Neuroblastoma tumour genetics: clinical and biological aspects

N Bown

Abstract
Neuroblastoma tumour cells show complex combinations of acquired genetic aberrations, including ploidy changes, deletions of chromosome arms 1p and 11q, amplification of the MYCN oncogene, and—most frequently—gains of chromosome arm 17q. Despite intensive investigation, the fundamental role of these features in neuroblastoma initiation and progression remains to be understood. Nonetheless, great progress has been made in relating tumour genetic abnormalities to tumour behaviour and to clinical outcome; indeed, neuroblastoma provides a paradigm for the clinical importance of tumour genetic abnormalities. Knowledge of MYCN status is increasingly being used in treatment decisions for individual children, and the clinical value of 1p and 17q data as adjuncts or refinements in risk stratification is under active investigation. Reliable detection of these molecular cytogenetic features should be regarded as mandatory for all new cases at presentation.

Keywords: neuroblastoma genetics; 17q gain in neuroblastoma; neuroblastoma: 1p and MYCN; tumour genetics and prognosis

Neuroblastoma is an embryonal tumour of neuroectodermal cells derived from the neural crest and destined for the adrenal medulla and sympathetic nervous system. The disease accounts for approximately 6% of childhood cancers, with an annual incidence of 8/million children under the age of 15. The median age at diagnosis is 22 months, and more than 95% of cases present by 10 years of age. There are approximately 90 new cases each year in the UK. In contrast, infants with neuroblastoma tend to present with lower stage disease (stages 1, 2, and 4s), and the clinical behaviour of these tumours differs greatly from the aggressive forms; they are generally chemosensitive and high cure rates are obtained. Moreover, a proportion of lower stage tumours show spontaneous regression, even those showing widespread dissemination in stage 4s disease. This extreme clinical heterogeneity has led some workers to question whether neuroblastoma may consist of two distinctly different diseases.

Hence, the well established clinical indicators of adverse prognosis are age > 1 year at diagnosis and advanced tumour stage. Clinical risk systems based on tumour histology—degree of ganglionic differentiation, extent of Schwannian stroma, etc—have also been developed.

The biological hallmark of neuroblastoma is the complexity of the genetic abnormalities acquired by the tumour cells, and some of these abnormalities are powerful prognostic markers independent of the clinical features. This fact helps in risk stratification of patients at presentation, with the most intensive treatments being reserved for high risk cases, so that children with relatively benign tumours can be spared the deleterious effects of unnecessary chemotherapy. Clinical trials in neuroblastoma are increasingly basing treatment selection on tumour genetic variables. Furthermore, it is envisaged that research directed at sites of genomic imbalance will provide insights into the fundamental biology of neuroblastoma initiation and progression.

This review will describe and illustrate the salient genetic changes associated with neuroblastoma tumours, and will outline the links between tumour genetics and tumour behaviour. Reflecting the author’s background (diagnostic cytogenetics), the focus will be on genomic imbalances rather than abnormalities of gene expression.

Ploidy
Using flow cytometry, Look et al distinguished between diploid and hyperdiploid tumour cell DNA content in a series of neuroblastoma tumours from infants. Hyperdiploid tumours showed DNA indices ranging between 1.07 and 2.42. In this study, DNA content was significantly linked to tumour stage, with diploidy...
much more frequent in higher stage tumours. DNA content was also shown to correlate with response to treatment: 17 of 17 hyperdiploid tumours had complete or partial responses, whereas 0 of 6 diploid tumours responded to treatment. Several subsequent flow cytometric studies⁴⁻¹¹ have confirmed and extended these findings, namely: that a hyperdiploid DNA content of tumour cells characterises low stage disease in younger patients with a favourable clinical course, whereas a normal cellular DNA content is associated with unfavourable clinical features and significantly reduced survival probability.

The series of Look et al was extended in 1991 to include 298 patients.¹² Tumour DNA content was diploid in 34%, hyperdiploid in 65%, and hypodiploid in 1%. MYCN gene amplification—seen in 25% of the cases overall—was significantly more frequent in diploid than in hyperdiploid tumours. For advanced disease, hyperdiploidy was closely associated with long term disease free survival in infants and younger children (ploidy was not significant for patients with stage 4 tumours over the age of 2 years because their survival rate was already dismal).

In parallel with flow cytometric analyses, direct chromosome counting by classic cytogenetics has also provided data on the role of ploidy changes in neuroblastoma. Two studies¹³,¹⁴ involving a total of 81 karyotypes provided very consistent results. Both distinguished three ploidy levels; near diploidy, near triploidy, and near tetraploidy, and found that 1p abnormalities, double minute chromosomes (dmin), homogenously staining regions (hsrs), and other structural chromosome aberrations were much more prevalent in diploid and tetraploid tumours. MYCN amplification detected by Southern blot was also largely restricted to the diploid group. Ploidy correlated strongly with age, stage, and survival, near triploidy being associated with a significantly more favourable outcome.

Taken together, the consensus on the results from both flow cytometric and cytogenetic investigations indicates that patients with near triploid tumours are distinctly different from those with diploid or tetraploid tumours, and that ploidy is a significant prognostic factor.

At a practical level, what is the optimum technique for determining tumour cell ploidy? Direct chromosome counting by conventional cytogenetic analysis is only sporadically successful because of the low success rate for cytogenetic analysis of neuroblastoma material. Flow cytometry is in routine use in a large number of centres—and can readily be applied to paraffin wax embedded archival material—but requires substantial quantities of tumour material. An alternative, requiring less material and applicable to needle biopsies, fine needle aspirates, and touch imprint slides, is static DNA cytometry. Finally, as demonstrated in other malignancies,¹⁵ in situ hybridisation (FISH) with combinations of centromeric probes enables approximations of the ploidy level to be derived.

**Deletions/allelic losses from 1p**

**GENETIC: CLINICAL CORRELATIONS**

The importance of the short arm of chromosome 1 in neuroblastoma genetics was first identified in 1977 when Brodeur et al noted recurrent deletions of 1p in cytogenetic analyses of primary tumours and cell lines.¹⁶ Subsequent cytogenetic studies¹⁷ confirmed the high frequency of deletions and other rearrangements of 1p (fig 1A), and—until very recently—this feature was held to be the most common genetic aberration of neuroblastoma tumour cells. The inference that distal 1p contains a gene or genes important in the development of neuroblastoma quickly gained ground. For both simple terminal deletions and unbalanced rearrangements, where material from another chromosome has replaced the distal segment of 1p, cytogenetic studies revealed a very wide range of breakpoints on 1p, from 1p22 to 1p36.

It is important to appreciate that rearrangements of 1p and losses of 1p material are not specific to neuroblastoma, but characterise a wide range of human malignancies, including both solid tumours and haematological cancers (reviewed by Atkin¹⁸ and Schwab and colleagues¹⁹).

It was subsequently established that the presence of 1p abnormalities in neuroblastoma tumours correlated with unresectable and metastatic disease, whereas localised and clinically favourable tumours showed an intact chromosome 1.²⁰ Furthermore, it was shown that 1p deletion was associated with adverse clinical and genetic indicators such as MYCN gene amplification—for example, Fong et al found that 62% of tumours showing 1p LOH were MYCN amplified compared with only 3% of 1p intact tumours.²¹ Finally, several cytogenetic studies²²⁻²⁴ showed 1p deletion to be a significant predictor of adverse outcome.

There has been ongoing controversy about the precise prognostic power of 1p deletion in relation to other variables, particularly MYCN. For example, in the series of Gehring et al the negative survival impact of 1p loss was restricted to cases with MYCN amplification²⁵; 1p loss of heterozygosity (LOH) had no independent predictive significance. In other studies, however, LOH 1p has been identified as the most powerful predictor of adverse outcome in multivariate survival analyses.²⁶⁻²⁸ In considering these discrepancies between study findings, Maris et al have drawn attention to the different outcome criteria used by different groups, some of which used event free survival (EFS), whereas others used overall survival (OS).²⁹ These authors presented results of a large series (238 cases) analysed under both criteria. Overall, 35% of tumours showed 1p LOH. In multivariate analysis, 1p36 loss was a significant independent predictor of decreased EFS, but had no significant effect on OS probability, implying that knowledge of 1p status may be useful in predicting salvageable relapses in otherwise low risk patients. The study demonstrated no prognostic impact of 1p loss in high risk groups (particularly, MYCN amplified tumours).
With the appreciation of the high incidence of this feature and its association with adverse outcome, other methodologies more reliable than classic cytogenetics were co-opted to detect 1p deletions in tumour cells. Molecular analyses of LOH using Southern blotting have in turn been largely superseded by genomic polymerase chain reaction (PCR), which requires only very small quantities of tumour DNA (fig 1D). For diagnostic purposes, two colour interphase FISH is also commonly used; the most common FISH approach involves simultaneous visualisation of differentially labelled probes for chromosome 1 pericentromeric region (for example, puc177) and for 1p36 (for example, D1Z2) (fig 1B).

Deletions of distal 1p are not restricted to diploid tumours, but occur also in triploid neuroblastomas showing three copies of chromosome 1. Thus, interphase FISH analysis may reveal three pericentromeric signals, but only two signals from the 1p36 probe (FISH imbalance). In this situation, it is not immediately clear whether a reduction to hemizygosity has occurred, or whether both maternal and paternal alleles have been retained in the intact.

Figure 1 (A) G-banded metaphase chromosomes 1 showing a large deletion including the p36 band. (B) Two colour interphase fluorescent in situ hybridisation (FISH) detection of 1p deletion; tumour nuclei showing two centromeres for chromosome 1 but only a single signal for 1p36. (C) Comparative genomic hybridisation (CGH) profile; the shift in the fluorescence ratio profile indicates that a large segment of 1p (indicated by red bar) is absent from the tumour genome. (D) Genomic PCR analysis of two 1p36 alleles in a neuroblastoma tumour (t) and the patients constitutional DNA (c); deletion at marker D1S76, marker D1S80 was not informative (constitutionally homozygous). (E) Double minute chromosomes in a tumour metaphase. (F) Interphase FISH showing a MYCN amplified nucleus. (G) Appearance of MYCN amplification by CGH; excess tumour DNA is present binding to the 2p24 locus of MYCN. (H) Southern blot detection of MYCN status (image courtesy of J Board); lanes 1–3 non-amplified tumours, lane 4 showing MYCN amplification. (I) Two examples of unbalanced translocations resulting in gain of distal 17q segments. (J) Interphase FISH analysis of chromosome 17; tumour nuclei showing two signals for chromosome 17p and three signals for distal 17q. (K) CGH profile showing gain of 17q.
1p segments. Molecular analysis by Southern blot or genomic PCR may then clarify the allelic status of 1p, showing either a biallelic pattern with attenuation of one band (allelic imbalance) or reduction to a single band. Very few studies have investigated LOH of 1p in the context of chromosome 1 copy number, but Kaneko et al established that heterozygosity for 1p alleles was retained for 11 of 12 trisomy 1 tumours showing 1p loss by FISH analysis.\(^m\) In the same study, it was demonstrated that the negative prognostic impact of 1p loss was restricted to diploid tumours, with loss of 1p from trisomy 1 tumours having no impact on survival.

THE SEARCH FOR 1p TUMOUR SUPPRESSOR GENES

The extensive basis for the involvement of 1p in neuroblastoma established by classic cytogenetic studies has been reinforced by reports of a child with a constitutional deletion of this region who developed neuroblastoma at age 5 months,\(^m\) and a patient with neuroblastoma who was found to have a balanced translocation t(1;17)(p36;q11–12) disrupting the 1p36 region.\(^m\) Further evidence for the biological importance of 1p came from chromosome transfer experiments in which the introduction of 1p material into neuroblastoma cells in vitro resulted in morphological differentiation and suppression of tumorigenicity.\(^m\) These findings provided a major spur for the study of 1p LOH, specifically attempts to find the putative tumour suppressor gene (TSG). To this end, great efforts have been made in several centres to delineate the smallest region of overlap (SRO) for the 1p deleted region. Early Southern blot restriction fragment length polymorphism (RFLP) analyses identified predominantly terminal deletions with proximal SRO boundaries in 1p36.1.\(^m\) White et al analysed 122 tumours that were clinically and biologically representative of the disease as a whole, and found allelic loss in 32 (26%).\(^m\) The proximal deletion border varied considerably, but a single consensus deleted region of approximately 8 Mb was identified in 1p36.2–3, distal to marker D1S228.

The analysis of 1p deletions and the search for the 1p TSG entered a new level of complexity with the publication of two reports in 1994, each identifying two distinct regions of allelic loss. Using Southern blot analysis, Takeda et al analysed the extent of 1p deletions in 21 tumours showing LOH and found evidence for two groups.\(^m\) The first showed small interstitial deletions in chromosome bands 1p35–36, with retention of the most telomeric marker (D1S80); these deletions were found in tumours showing favourable clinical and biological features—lower stage disease, triploidy, single copy MYCN—and significantly longer EFS and OS. The second group showed larger deletions including the distal marker D1S80 and extending proximally into chromosome band 1p21. These cases were of higher clinical stage, were much more likely to have MYCN amplification, and showed poor survival. These workers proposed that two TSGs must be present in 1p, one in 1p36 (NB1) and one in 1p32 (NB2).

A PCR study reported at the same time\(^m\) reached very similar conclusions. The patterns of allelic loss in 22 tumours allowed two consensus regions of loss to be delineated, one very distal in 1p36, and one more proximally in 1p32–35. Correlation with other features was restricted to MYCN status; amplification was found to be restricted to the group of tumours showing deletion of the more proximal consensus region.

This model of two TSGs with distinct biological properties received further support from a cell line study\(^m\) in which the extent of the 1p deletions was determined by a combination of FISH and Southern blotting. Neuroblastoma cell lines lacking MYCN amplification showed a small SRO in 1p36.23–33, whereas MYCN amplified lines showed a much larger SRO, extending from 1p35–36.1 to the telomere.

A further complication emerged from studies of the parental origins of deleted regions, which produced evidence for genomic imprinting, implying functional differences between maternal and paternal copies of TSGs. Genomic imprinting refers to the differential expression of paternally and maternally inherited alleles (reviewed by Hall\(^m\) and Yun\(^m\)). In one model of the role of imprinting in oncogenesis, it is assumed that epigenetic modification (for example, by DNA methylation) of a TSG during spermatogenesis results in constitutively reduced or absent expression in offspring. Somatic loss of the active maternal copy would then contribute to malignant initiation.

For neuroblastoma, initial studies of 1p LOH gave conflicting results concerning the parental origin of allelic loss. Thus, whereas Caron and colleagues\(^m\) identified a significant non-random loss of maternally derived alleles in 1p deleted neuroblastoma tumours (13 of 15 cases), Cheng and co-workers\(^m\) found a random pattern of loss with regard to parental origin. This discrepancy was subsequently resolved when the MYCN status of 1p deleted tumours was taken into account; Caron and colleagues\(^m\) found that MYCN normal tumours showed preferential maternal LOH (16 of 17 cases maternal), whereas 1p LOH in MYCN amplified tumours was of random parental origin (18 maternal, 12 paternal)—the earlier study of Cheng et al had focused exclusively on MYCN amplified cases.

These studies, in conjunction with other series,\(^m\) suggest the presence of at least two TSGs on 1p, one located distally in 1p36.2–3, subject to genomic imprinting and being lost in MYCN-non-amplified tumours, and one more proximal in 1p35–36.1, showing random origin of allelic loss and an association with MYCN amplified disease.

1p LOSS AND 17q GAIN

With the increasing recognition of the frequency and clinical importance of 17q gain in neuroblastoma (see below), the importance of
unbalanced translocation with 17q as a mechanism for 1p loss must be highlighted. Cytogenetic and metaphase FISH studies have shown that the unbalanced translocation der(1)t(1p;17q)—with loss of distal 1p and gain of distal 17q—is a recurring feature of neuroblastoma cell lines and primary tumours (fig 2A). In a recent audit, 1p was the most common site for 17q translocation (33 of 75 identified translocations), and this mechanism accounted for approximately 42% of 1p losses (30 of 72 rearrangements of 1p), with simple deletion comprising 32% and unbalanced translocations between 1p and other chromosomes comprising 26% of visible 1p segment loss. Hence, cytogenetic evidence suggests that unbalanced translocation with 17q is the most frequent mechanism for 1p loss. Because 17q gain is itself a powerful independent predictor of poor survival (see below), the failure to recognise concurrent loss of 1p and gain of 17q—caused by this common translocation mechanism—may be a significant shortcoming of the molecular assays of 1p LOH.

Given the frequency of unbalanced translocation der(1)t(1p;17q), it is interesting to ask whether this rearrangement has prognostic consequences distinct from its constituent elements of 1p loss and 17q gain; preliminary evidence suggests not; that is, for a given tumour with both 1p deletion and 17q gain, clinical outlook appears to be equally bleak whether these two features occur independently or are physically interlinked.

**MYCN**

THE CYTOGENETICS OF GENE AMPLIFICATION
dmin and hsrs were identified in the earliest phase of neuroblastoma cytogenetics (fig 1E). Studies of drug resistance in human cell lines in vitro had previously implicated these structures as cytological evidence of gene amplification, and in 1983 Schwab and colleagues showed that the amplified domain in neuroblastoma cell lines and a primary tumour showed sequence homology to the human cellular oncogene c-myc. From Southern blot results, the sequences were calculated to be amplified up to 130 fold, and the authors were able to show by in situ hybridisation (radio-labelled) that hsrs in neuroblastoma cell lines were the site of the amplified myc related material.

At around the same time, the amplified gene was termed N-myc and its origin identified as the short arm of chromosome 2. MYCN amplification was found to be relatively specific for tumours of neurogenic origin. It was quickly appreciated that MYCN amplification is never manifest at the 2p23–24 resident site of the gene itself, but is found in hsrs on other chromosomes, or in extrachromosomal dmin. In neuroblastoma cell lines, FISH studies have shown retention of a single copy of the MYCN gene at 2p23–24 concurrent with multiple copies in transposed hsrs—these observations have prompted a model of the amplification process involving unscheduled replication and recombination to produce circular extrachromosomal structures; these integrate at other chromosomal sites and a process of secondary in situ amplification then results in an hsr. The reason that hsrs are rare in primary tumour cells compared to dmin remains obscure.

Until recently, no pattern had been described for the integration sites of MYCN hsrs; however, FISH techniques now show that these structures are frequently flanked by segments of 17q material, suggesting that 17q may be a preferential recombination site for MYCN.

In rare instances in cell lines, the amplified domains of hsrs have been shown to be complex constructs including both MYCN and sequences derived from other sites—for example, MYCN plus MDM2 in the 12q hsr of the LS cell line, or MYCN plus the MEIS1 homeobox gene from 2p15 in the 1p hsr of the IMR32 cell line.

**CLINICAL IMPORTANCE OF MYCN AMPLIFICATION**

When the focus of MYCN studies was shifted from cell lines to primary tumours, amplification was confirmed as a frequent feature of...
neuroblastoma in vivo. In general, MYCN status appears to be a fixed and stable feature of the neuroblastoma genome and the MYCN gene copy number—whether amplified or not—does not alter in the course of tumour development. For example, Brodeur et al found that MYCN copy numbers were consistent within different areas of individual tumour masses, between primary tumours and their corresponding metastases, and between matched presentation and relapse samples. Amplification of MYCN is well known to correlate with advanced stage disease; for example, in the study of Brodeur et al, amplification was found in 24 of 48 stage 3/4 tumours but in none of 15 stage 1 or 2 tumours. Multiple studies subsequently confirmed this association with progressive disease, and showed that MYCN amplification correlates with a greatly increased risk of fatal outcome. In the survival analysis of Seeger et al, patients whose tumours had a normal MYCN status had an 18 month progression free survival of 70%, compared with 30% and 5% for tumours showing 3–10 or > 10 copies of the oncogene, respectively. The adverse associations of MYCN amplification appeared to be independent of clinical stage: amplification was linked to treatment failure in low stage (2) disease, whereas normal MYCN status was associated with continuing remission in stage 3 and 4 tumours. MYCN amplification was thus firmly implicated in the malignant aggressiveness of neuroblastoma tumours, and established as a powerful clinical marker of high risk disease. Currently, MYCN is the only tumour genetic feature used as a basis for treatment stratification in neuroblastoma clinical trials.

Two techniques predominate in the routine detection of MYCN status for clinical purposes. The molecular analysis of tumour DNA by Southern blotting has been used extensively (fig 1H), but genomic PCR is gaining ground and has the advantage of requiring only 100 ng of tumour DNA, compared with 5–10 μg for Southern blotting. Both molecular techniques purport to provide quantitative information; that is, assessments of MYCN gene copy number.

An alternative approach is interphase FISH using cosmid or yeast artificial chromosome (YAC) probes (fig 1F). The results are usually highly concordant with those from molecular analyses, but the FISH approach has several advantages. There is only a very minimal requirement for tumour material because FISH is readily applicable to tumour touch imprint slides, fine needle aspirates, and paraffin wax embedded tissue sections. More importantly, FISH results are seen at an individual cell level and are hence more sensitive than Southern analysis. For example, high level gene amplification present in only a minority of cells—perhaps because of non-malignant cells in the tumour sample—may well lead to misinterpretation as low level amplification or non-amplification by molecular analysis, but will be readily apparent by FISH.

INSIGHTS FROM MYCN FISH
The advent of MYCN FISH has led to several advances in understanding the nature of MYCN amplification. First, it has allowed the great heterogeneity of MYCN gene copy number within individual amplified tumours to be recognised. It is now well known that individual cells from MYCN amplified tumours typically diverge widely from the mean copy numbers suggested by molecular assays. Usually, a mixture of cells is seen, with copy numbers ranging from < 10 to many hundreds. Two processes may account for this extreme heterogeneity. Most amplification in primary tumours takes the form of dmin and, because these are acentric structures, they tend to be lost during mitosis, or to be unequally divided between daughter cells. Recently, a more active process of expulsion of amplified MYCN has been reported from FISH studies of both neuroblastoma cell lines and primary tumours. The intriguing possibility that this process could modulate the aggressiveness of MYCN amplified tumours remains to be investigated.

A final insight from MYCN FISH has been the observation of MYCN duplications. These were first reported at 2p24 (the resident site of MYCN) in several MYCN non-amplified cell lines, and were proposed to represent a prelude to amplification or an alternative pathway for MYCN activation. Duplications of 2p segments including the MYCN locus have been observed in cytogenetic studies (N Bown, 1993, unpublished) and in comparative genomic hybridisation (CGH) analyses, and gain of 2p/MYCN by unbalanced translocations has also been reported as a frequent finding in neuroblastoma cell lines. The potential biological relevance of these duplications/low copy number gains is underlined by reports of neuroblastomas arising in children with constitutional duplications of 2p.

MYCN EXPRESSION
MYCN amplification leads to high concentrations of the gene product, a nuclear phosphoprotein (Myc). The oncogenic properties of MYCN overexpression have been demonstrated both by malignant transformation of normal cells in vitro after transfection of a MYCN expression vector, and also by the reliable development of neuroblastoma tumours in mice with constitutive overexpression of MYCN in neuroectodermal cells. However, the ultimate mechanism by which Myc protein contributes to malignant development remains obscure, but it is thought to involve binding of the Myc protein to the transcriptional regulator Max, leading to inappropriate activation of growth promoting genes (reviewed by Maris and Matthay).

Concentrations of the Myc protein are generally high in tumours showing amplification of the gene, but may also be increased in MYCN silenced copy disease, and the relation between MYCN expression, clinical parameters, and outcome in the latter context is controversial (reviewed by Bordow).
Neuroblastoma tumour genetics

The same phenomenon was observed in other families. The occurrence of translocations involving this chromosome was noted in virtually all neuroblastoma cell lines. The phenomenon came to focus on 1p deletion and MYCN amplification. However, it was noted that near triploid tumours frequently showed gains of whole chromosome 17 over and above their diploid level (four to five copies of chromosome 17). The advent of FISH techniques in the mid-1990s brought 17q to the forefront of neuroblastoma research. The extent and structure of the amplified domains that include the MYCN gene have been investigated extensively. The amplicon size is heterogenous between cells derived from different tumours, and can vary between 100 kb and 1 Mb. The amplicon is randomly located, and includes a consistently amplified core domain of 100–200 kb containing the MYCN gene. The very large size of the amplicon in relation to the size of the MYCN gene has prompted several investigations into the nature of the coamplified sequences and their possible role in modulating the clinical behaviour of MYCN amplified tumours. The most frequently coamplified gene is DDX1, encoding an RNA helicase, which is located within 400 kb of MYCN (reviewed by George and Squire). However, DDX1 amplification has not been observed in the absence of MYCN amplification, implying that it has a secondary, passenger status to MYCN. The possibility remains that the malignant behaviour of MYCN amplified tumours may be influenced by coamplification of DDX1 or other 2p24 genes, such as the recently discovered NAG (neuroblastoma amplified gene).

17q gain

CYTOGENETICS AND CGH STUDIES

Chromosome 17 abnormalities were first implicated in neuroblastoma in the early 1980s. G-banded cytogenetic analyses of cell lines and primary tumours recorded gains of chromosome segment 17q21–qter in tumour karyotypes, but these observations were not developed further and genetic research interest focused on 1p deletion and MYCN amplification. However, it was noted that near triploid tumours frequently showed gains of whole chromosome 17 over and above their diploid level (four to five copies of chromosome 17). The advent of FISH techniques in the mid-1990s brought 17q to the forefront of neuroblastoma research. The extent and structure of the amplified domains that include the MYCN gene have been investigated extensively. The amplicon size is heterogenous between cells derived from different tumours, and can vary between 100 kb and 1 Mb. The amplicon is randomly located, and includes a consistently amplified core domain of 100–200 kb containing the MYCN gene.

COAMPLIFIED GENES

The extent and structure of the amplified domains that include the MYCN gene have been investigated extensively. The amplicon size is heterogenous between cells derived from different tumours, and can vary between 100 kb and 1 Mb. The amplicon is randomly located, and includes a consistently amplified core domain of 100–200 kb containing the MYCN gene. The very large size of the amplicon in relation to the size of the MYCN gene has prompted several investigations into the nature of the coamplified sequences and their possible role in modulating the clinical behaviour of MYCN amplified tumours. The most frequently coamplified gene is DDX1, encoding an RNA helicase, which is located within 400 kb of MYCN (reviewed by George and Squire). However, DDX1 amplification has not been observed in the absence of MYCN amplification, implying that it has a secondary, passenger status to MYCN. The possibility remains that the malignant behaviour of MYCN amplified tumours may be influenced by coamplification of DDX1 or other 2p24 genes, such as the recently discovered NAG (neuroblastoma amplified gene).

17q gain

CYTOGENETICS AND CGH STUDIES

Chromosome 17 abnormalities were first implicated in neuroblastoma in the early 1980s. G-banded cytogenetic analyses of cell lines and primary tumours recorded gains of chromosome segment 17q21–qter in tumour karyotypes, but these observations were not developed further and genetic research interest focused on 1p deletion and MYCN amplification. However, it was noted that near triploid tumours frequently showed gains of whole chromosome 17 over and above their diploid level (four to five copies of chromosome 17).

The extent and structure of the amplified domains that include the MYCN gene have been investigated extensively. The amplicon size is heterogenous between cells derived from different tumours, and can vary between 100 kb and 1 Mb. The amplicon is randomly located, and includes a consistently amplified core domain of 100–200 kb containing the MYCN gene. The very large size of the amplicon in relation to the size of the MYCN gene has prompted several investigations into the nature of the coamplified sequences and their possible role in modulating the clinical behaviour of MYCN amplified tumours. The most frequently coamplified gene is DDX1, encoding an RNA helicase, which is located within 400 kb of MYCN (reviewed by George and Squire). However, DDX1 amplification has not been observed in the absence of MYCN amplification, implying that it has a secondary, passenger status to MYCN. The possibility remains that the malignant behaviour of MYCN amplified tumours may be influenced by coamplification of DDX1 or other 2p24 genes, such as the recently discovered NAG (neuroblastoma amplified gene).
the chromosome 17 status of 160 neuroblastoma tumours.\textsuperscript{116} The most significant predictive factors were serum ferritin ($p = 0.0128$), stage ($p = 0.0477$), 1p status ($p = 0.008$), and 17q status ($p = 0.0001$). A collaborative compilation of data from six European centres\textsuperscript{53} provided 313 cases with known 17q status of tumour cells. OS at five years was 30.6% for the 168 cases with 17q gain, compared with 86.0% for the 145 without this feature ($p < 0.0001$). 17q status provided significant prognostic information within both the 1p non-deleted group and the MYCN non-amplified group (both $p < 0.001$). In stepwise multivariate analysis, significant independent predictors of lethal outcome were 1p deletion ($p = 0.02$), stage 4 disease ($p = 0.004$), and 17q gain ($p < 0.001$).

Most recently, a Swedish series\textsuperscript{113} based on FISH detection found 17q gain in 31 of 48 neuroblastomas and highlighted its strong correlation with reduced survival probability. Therefore, from preliminary studies it is clear that unbalanced gain of distal 17q is a feature of significant independent prognostic importance and may, in fact, identify a larger proportion of high risk tumours with greater predictive power than any other clinical or tumour genetic factor.

In view of the emerging clinical importance of 17q gain, its reliable detection in new cases of neuroblastoma is likely to be of increasing concern. CGH, outlined above, is effective but not widely available, whereas metaphase chromosome analysis and metaphase FISH are highly informative but unreliable (owing to the difficulty of producing chromosome spreads from neuroblastoma material). Interphase FISH techniques have been performed using differentially coloured markers on either side of the 17q breakpoint\textsuperscript{53,113} (fig 1J). Molecular assays of 17q gain are problematic because it is often necessary to detect a single gained 17q segment against a background of two normal chromosomes 17; however, detection by both Southern blot\textsuperscript{112} and genomic PCR\textsuperscript{117,118} have been described.

**THE BIOLOGY OF 17q GAIN**

Although the prognostic significance of 17q gain has been powerfully demonstrated—albeit in a very limited number of studies—the investigation of the fundamental biology of this feature is at a very early stage. In principle, the underlying molecular genetic event could be either fusion of a specific gene flanking the 17q translocation breakpoint, or a gene dosage effect arising from the unbalanced nature of the rearrangements. The delineation of the 17q translocation breakpoints is a prerequisite for testing these alternative hypotheses.

At a cytogenetic level of resolution ($>1$ Mb/visible chromosome band), the breakpoints are most often located in 17q11–21.\textsuperscript{10,11} FISH analysis of 12 translocations in primary tumours showed heterogeneous breakpoints, with the segment 17q23.1–qter being consistently gained.\textsuperscript{100} Extensive analysis of breakpoint locations has been carried out in 10 neuroblastoma cell lines and 21 primary tumours using 13 YAC or cosm id DNA probes.\textsuperscript{119–122} At least seven different breakpoints were identified, ranging over the proximal half of 17q between the centromere and marker 17p11.2. The findings strongly implicate a gene dosage effect, rather than rearrangement of a specific 17q gene, as the important consequence of the unbalanced translocations. However, the possibility remains that the partial trisomy might be merely a gigantic marker for a single gene abnormality, as with trisomy 11 and internal duplications of the MLL gene in acute myeloid leukaemia,\textsuperscript{122} or duplication of mutant MET genes by trisomy 7 in hereditary papillary renal carcinoma.\textsuperscript{123}

Given that whole chromosomal 17 gain and partial 17q gain have such divergent associations and prognostic effects, it may be postulated that the important event is imbalance between two or more genes on either side of the translocation breakpoint, rather than simple gain of a single gene. The search for the relevant genes is a daunting challenge, but may be facilitated by recent technical developments and genome sequencing efforts. In particular, genome-wide studies for differential gene expression—for example, by using cDNA microarray analysis or the SAGE (serial analysis of gene expression) technique (see Going and Gusterson\textsuperscript{124} for review) may reveal candidate genes. Currently, genes implicated in apoptosis, cell cycle control, and neuronal differentiation are of particular interest. These include the nm23 gene located at 17q21–22, for which a metastasis suppressor role has been implicated because reduced expression of the gene has been reported in a wide range of highly malignant rodent and human tumours. In neuroblastoma, by contrast, nm23 expression is increased in aggressive tumours compared with localised disease,\textsuperscript{125} and sequence mutations have been demonstrated in nm23 in a high proportion of advanced neuroblastomas.\textsuperscript{126} Other studies have linked the increased expression to genomic copy number increases, and to a significant adverse impact on clinical outcome.\textsuperscript{127,128} In this last study, 1p deletion and MYCN amplification were found in only a subset of the tumours showing nm23 copy number gains, suggesting that genomic gain of this gene may be a prognostic predictor independent of other tumour genetic factors.

Another 17q gene attracting increasing interest is survivin, which is normally consequent on retinoic acid exposure. Thus, survivin is clearly a candidate gene for the 17q effect, but no study has yet directly related protein concentrations to genomic 17q copy number in neuroblastomas.\textsuperscript{129} The same authors also demonstrated that transfection of survivin into a neuroblastoma cell line (CHP134) resulted in potent inhibition of the apoptosis normal consequent on retinoic acid exposure. Thus, survivin is clearly a candidate gene for the 17q effect, but no study has yet directly related protein concentrations to genomic 17q copy number in neuroblastomas.

How specific to neuroblastoma is the phenomenon of 17q gain? 17q gain has also been seen in a variety of other neoplasms including...
Neuroblastoma tumour genetics

Table 1 Reported regions of genomic loss in neuroblastoma tumours

<table>
<thead>
<tr>
<th>Region</th>
<th>Frequency of segment loss in CGH studies</th>
<th>Frequency of LOH in molecular studies</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>2q</td>
<td>0–6%</td>
<td>32%130</td>
<td>SRO in 2q33 includes locus of caspase 8 gene</td>
</tr>
<tr>
<td>3p</td>
<td>21–32%</td>
<td>10–15%131,132</td>
<td>Outcome difference between LOH over whole chromosome 3 (favourable) and LOH restricted to 3p (unfavourable). SRO 3p25.3–p14.3</td>
</tr>
<tr>
<td>4p</td>
<td>15–33%</td>
<td>12–20%133</td>
<td>No correlation with 1p or MYCN status141</td>
</tr>
<tr>
<td>9p</td>
<td>6–21%</td>
<td>17–30%134</td>
<td>Associated with advanced stage and poor outcome132</td>
</tr>
<tr>
<td>11q</td>
<td>11–44%</td>
<td>19–41%135,136,137</td>
<td>Evidence for two regions of deletion in 9p21</td>
</tr>
<tr>
<td>14q</td>
<td>8–48%</td>
<td>16–50%138,139,140,141</td>
<td>Associated with 1p LOH but no correlation with age or stage142</td>
</tr>
<tr>
<td>16p</td>
<td>0–6%</td>
<td>9–24%143,144</td>
<td>SRO in 11q23.3. Inverse correlation with MYCN amplification. Marker of poor prognosis in MYCN non-amplified cases144,145</td>
</tr>
<tr>
<td>18q</td>
<td>0–16%</td>
<td>21%146</td>
<td>No correlation with clinicopathological features</td>
</tr>
</tbody>
</table>

CGH, comparative genomic hybridisation; LOH, loss of heterozygosity; SRO, smallest region of overlap.

In conclusion, the advent of molecular cytogenetic analyses has firmly established the high incidence of chromosome 17 abnormalities in neuroblastoma. In localised tumours with near triploid DNA content without structural changes, whole chromosome 17 gains are observed, whereas in advanced near diploid or tetraploid neuroblastoma, extra copies of 17q are present. 17q gain is strongly associated with other known prognostic factors, but is in itself a very powerful independent predictor of adverse outcome. Finally, the role at the cellular level of chromosome 17 or 17q gain is currently unknown and more intensive research in this area is warranted.

OTHER REGIONS OF GENETIC IMBALANCE

In addition to ploidy changes, 1p rearrangements, MYCN gene involvement, and gain of 17q, several other sites of recurrent genomic imbalance are well established in neuroblastoma. Alloclic losses have been reported for a large number of regions; table 1 summarises the principal findings to date. In the LOH studies listed, allelic losses were established by molecular techniques (most commonly genomic PCR, less frequently Southern blot analysis), in which the pattern of DNA polymorphisms for locus specific markers is compared between the constitutional and the tumour genomes. Chromosome segment loss is also readily detectable by CGH analysis, and results from the four largest CGH series are given in table 1. However, it is important to appreciate that losses detected by LOH analysis and by cytogenetics/CGH are not necessarily concordant. Thus, although clonal monosomy or partial monosomy of a chromosome or segment mandates LOH, the reverse does not necessarily hold because mechanisms can be envisaged whereby LOH could occur without visible loss of chromosome material—for example, submicroscopic deletion below the level of cytogenetic/CGH resolution, or loss of an entire chromosome followed by duplication of the remaining homologue.

Sequence losses from the long arm of chromosome 11 have been the subject of a large recent survey, which merits further consideration. Guo et al found loss of 11q alleles in 41% of 349 primary tumours, indicating that this is the most frequently deleted region in the neuroblastoma genome.136,137 A 2.1 cM consensus region of deletion was identified in band 11q23.3. Loss of 11q was significantly associated with adverse clinical parameters (age > 1 year, stage 4 disease, unfavourable histology), but strongly inversely correlated with MYCN amplification. Although there was a non-significant trend for worse survival for 11q LOH tumours overall, this was more pronounced among the MYCN non-amplified subset (three year overall survival of 75% for 98 children with 11q LOH tumours compared with 91% for 82 children with 11q intact tumours). The authors concluded that a tumour suppressor gene located within 11q23.3 is inactivated during malignant progression in a large proportion of neuroblastoma tumours. CGH analyses have also identified recurrent losses of 11q segments in MYCN normal tumours and have found a significant association between 11q loss and 3p loss, suggesting a distinct pathway of genetic evolution.

The importance of 11q loss in neuroblastoma is further underscored by rare cases of constitutional abnormalities of this region in children who develop the disease, including deletions,147 inversions,148,149 and balanced 11q translocations.150,151

Although regions of recurrent genomic loss are thought to represent sites of potential tumour suppressor genes; as yet, few strong candidate genes have been identified for any of the reported regions of LOH. One exception concerns the involvement of the caspase 8 gene in allelic losses from 2q. This gene is normally
involved in induction of cell death pathways, and its inactivation by methylation—a frequent event in MYCN amplified tumours—results in the suppression of apoptosis and in the survival of tumours.

The caspase 8 locus is in 2q33, which is the smallest common region of LOH in the 32% of tumours showing allelic loss for 2q, strongly implicating caspase 8 as a TSG in neuroblastoma.

However, it is not widely appreciated that many of the other reported sites of LOH are also sites at which 17q segment gain occurs through unbalanced translocation. For example, chromosome arm 11q is the second most common site for 17q translocations (after 1p), and such translocations—resulting in concurrent loss of distal 11q and gain of 17q—account for approximately 50% of 11q “deletion” events. Similar 17q translocations have been repeatedly observed on 3p, 4p, 9p, and 14q. The biological relevance of 17q involvement as a predominant mechanism for LOH at these multiple sites remains to be explored. As noted above and in table 1, several LOH studies have assessed the possible prognostic impact of particular losses; in view of the frequent involvement of 17q translocation and the powerful independent clinical impact of 17q gain, it is reasonable to regard these assessments with caution because none of these studies took 17q status into account.

With the exception of 17q, gain of chromosome segments appears to be a less important process than loss, and few studies have focused on such changes. However, in one, gain of 1q21–25 was identified by CGH in eight of 16 stage 4 tumours but in only one of 11 tumours of lower stage. 1q gain was found in all cases of progressive and chemoresistant disease and was associated with fatal outcome, whereas it was not seen at all in cases with a favourable clinical course. Cosmid FISH analysis was used to narrow the consistent region of gain to 1q23. These intriguing results await corroboration by other workers.

Abnormalities of gene expression

In addition to genomic imbalances, which are the main subject of this review, alterations in the degree of expression of specific genes have been studied extensively in neuroblastoma. In addition to nm23 and survivin (mentioned above), interest has been directed particularly at four other genes.

TELOMERASE

Progressive telomere shortening is implicated in cell senescence and apoptosis. High activity of the telomerase enzyme has been shown to override this process in many human malignancies, stabilising the telomere, and “immortalising” the malignant cell population. Expression of the telomerase gene has been studied in several neuroblastoma series; high concentrations of telomerase are associated with unfavourable clinical and tumour genetic features and with reduced survival probability. In stage 4 disease, telomerase values distinguished strongly between those tumours showing spontaneous regression and those that progressed with lethal outcome.

TRK

Trk is a transmembrane tyrosine kinase that acts as a receptor for the neurotrophin nerve growth factor. High expression of this gene is strongly associated with low stage disease and has been shown to be a significant predictor of favourable outcome.

MULTIDRUG RESISTANCE GENES

Overexpression of the gene for multidrug resistance associated protein (MRP) may confer resistance to chemotherapeutic agents, and has been shown to correlate closely with MYCN amplification. In multivariate analysis, high level MRP expression was also shown to be a significant prognostic indicator independent of MYCN status.

CD44

CD44 is a cell surface glycoprotein involved in cell adhesion. In neuroblastoma, its expression...
Neuroblastoma tumour genetics

is reportedly low or absent in higher stage disease. Christiansen et al found no correlation between CD44 expression and disease stage, but significantly better survival associated with CD44 expression was evident across all stages. This was powerfully confirmed by the study of Comberet et al, in which CD44 expression was identified as a significant independent factor in multivariate analysis of 140 patients.

Recently developed techniques such as suppression subtractive hybridisation (SSH), SAGE, and cDNA microarrays are powerful approaches to expression profiling, which promise to identify large numbers of overexpressed sequences in the neuroblastoma genome; this may expedite the screening of candidate genes of biological importance.

Summary

Neuroblastoma has been more extensively studied than any other childhood solid cancer and a perplexing variety of tumour genetic and other biological factors have been shown to play important roles in tumour development. Constellations of genetic abnormalities show close associations with tumour stage and aggressiveness (fig 3A,B). Despite intensive research efforts, as yet no single tumour genetic aberration has been found to be present in all cases, and none has been identified as the causative event in disease initiation. The fundamental biology of the three most important abnormalities—1p loss, 17q gain, and MYCN amplification—remains obscure. In contrast, a great deal of progress has been made in inter-relating tumour biological factors with clinical behaviour; the most widely accepted model is that of Brodeur, who identified three genetically distinct disease subgroups, namely: (1) infants with low stage disease and excellent prognosis whose tumours are triploid with intact 1p and normal MYCN copy number; (2) children aged > 1 year with stage 3 or 4 disease whose tumours are diploid or tetraploid with normal MYCN but who may have 1p allelic loss—these cases showing intermediate survival; and (3) older children with clinically advanced disease, diploid, or tetraploid tumours, 1p loss, and MYCN amplification conferring a dismal clinical outlook.

Most recently, Lastowska and colleagues refined this model by incorporating the effect of 17q gain and the new International neuroblastoma pathology system, which seeks to relate tumour morphology to prognosis. Applying this approach to a series of 80 cases revealed two distinct types of poor prognosis tumour; the first with 17q gain but no MYCN amplification (these showing frequent deletions of 1p, 3p, and 4p and CD44 expression, differentiating morphology, and tumour calcification), and the second with both 17q gain and MYCN amplification (correlating with 1p loss but few other chromosome changes, lack of CD44 expression, undifferentiated morphology, and absence of calcification). On a practical clinical level, there remains a need to define more precise prognostic factors to assist in treatment stratification. Only a relatively small number of studies have reported multivariate survival analyses of multiple clinical and tumour genetic/biological factors and fewer still have included histopathological parameters. The non-overlapping subsets of features investigated hamper any overall synthesis. In various studies, features identified as being of primary significance include 1p deletion, MYCN amplification, 17q gain, MRP expression, and CD44 expression. To resolve these conflicting claims will require rigorous, large scale biological studies linked to clinical trials with patients receiving standardised treatments; this is the approach currently being adopted in Europe by the collaborating biologists and clinicians of the International Paediatric Oncology Society (SIOP).


Neuroblastoma tumour genetics


910

Bown


