Comparing histopathological classification with MYCN, 1p36 and 17q status detected by fluorescence *in situ* hybridisation from 14 untreated primary neuroblastomas in Singapore

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ABSTRACT

Introduction: Neuroblastoma is the most common extracranial solid tumour in children, accounting for about 5.3 percent of all childhood cancers in Singapore. Several genetic abnormalities have been reported as prognostic markers, including amplification of the MYCN gene, deletion of the short arm of chromosome 1 (1p) and gain of the long arm of chromosome 17 (17q). However, the correlation between tumour histology and these genetic parameters remains to be established in our local population.

Methods: 14 untreated primary neuroblastoma tumours, diagnosed consecutively in our hospital between 2003 and 2007, were included for this study. Tumour tissues were classified histologically as favourable or unfavourable, according to the modification of World Heath Organization Classification of Tumours, by associating the degree of differentiation and mitotic-karyorrhectic index of the neuroblastoma to the age of the patient. Fluorescence *in situ* hybridisation analysis for MYCN, 1p status and 17q status were subsequently performed on tumour touch imprints.

Results: Five tumours with favourable histology were all negative for the three genetic parameters being investigated. The other nine tumours showing unfavourable histology exhibited one or more of the three genetic parameters. All MYCN amplified tumours either had additional 1p deletion and/or 17q gain.

Conclusion: Our limited data suggests that 1p deletion and 17q gain are reliable independent parameters correlating with an unfavourable histology and poor clinical outcome. The use of 1p deletion and 17q gain studies, in addition to MYCN amplification studies, should be considered routinely in predicting prognosis in neuroblastomas.

Keywords: 1p deletion, 17q gain, fluorescence *in situ* hybridisation, genetic markers, MYCN amplification, neuroblastoma

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INTRODUCTION

Neuroblastoma is an embryonal tumour of neuroectodermal cells arising from the neural crest. The disease accounts for approximately 5.3% of childhood cancers in Singapore (based on the Singapore Childhood Cancer Registry 1997–2005). Neuroblastoma is a heterogeneous disease, making it difficult to achieve a reproducible and uniform morphological classification system with prognostic significance. Based on parameters such as the amount of Schwannian stroma, degree of differentiation, mitotic-karyorrhectic index (MKI) and age at diagnosis, Shimada et al proposed to differentiate tumours with favourable histology (FH) from those with unfavourable histopathology (UH).[1,2]

Several genetic abnormalities have also been reported as prognostic markers, including amplification of the MYCN gene, deletion of the short arm of chromosome 1 (1p) and gain of the long arm of chromosome 17 (17q).[3] MYCN amplification is found in 25%–30% of neuroblastomas, and is strongly correlated with accelerated tumour progression and poor prognosis.[4] Deletion of 1p is present in 25%–35% of neuroblastoma primary tumours, and is often associated with MYCN amplification and commonly found in patients with advanced stages of the disease.[3] High-level amplification, besides MYCN, is rare in primary neuroblastomas. However, an unbalanced gain of 17q, usually with

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Table I. Prognostic categories based on age, degree of differentiation and mitotic-karyorrhectic index of neuroblastoma.

Age (years)	Differentiation	Mitotic-karyorrhectic index	Prognostic category
< 1.5	Undifferentiated	Any	Unfavourable
< 1.5	Poorly differentiated Differentiating	Low to intermediate	Favourable
< 1.5	Any	High	Unfavourable
1.5–5	Undifferentiated Poorly differentiated	Any	Unfavourable
1.5–5	Differentiating	Low	Favourable
1.5–5	Differentiating	Intermediate to high	Unfavourable
> 5	Any	Any	Unfavourable

only an additional 1–3 copies, is found in about half of primary tumours and is associated with a more aggressive disease.[5]

Although there were few reports on the association of tumour histology and genetic parameters like amplification of MYCN[4,6] and 1p36 status,[7] correlation between tumour histology and genetic parameters, other than these two genetic markers, remains to be established, especially in the local Southeast Asian population. Therefore, we aimed to investigate the correlation between MYCN, 1p and 17q status and the prognostic morphological parameters to address the biology underlying the clinical complexity of neuroblastomas.

METHODS

A total of 14 consecutive untreated primary neuroblastoma tumours, diagnosed between 2003 and 2007 in KK Women's and Children's Hospital, Singapore, were included in this study. The patients, comprising six males and eight females, were aged between < one month and six years. This institution sees about 55% of all newly-diagnosed neuroblastoma cases in Singapore.

Pathology classification of tumour tissues were performed according to the World Heath Organization Classification of Tumours.[8] Representative sections were reviewed by pathologists, who were blind to the molecular genetic data. Three morphological features, viz. degree of differentiation of neuroblasts, proportion of mitotic/karyorrhectic cells and percentage of nuclear positive cells by MiB-1 immunostain, were considered for prognostic categorisation. Touch imprints representative of the tumour were prepared by the pathologists and sent for MYCN, 1p status and 17q status fluorescence *in situ* hybridisation (FISH) analysis.

Neuroblastomas are schwannian stroma-poor neuroblastic tumours which can be divided into three subtypes. Undifferentiated neuroblastoma consists of cellular neuroblasts with no differentiating features in

routine sections and requires supplementary techniques such as immunohistochemistry for the diagnosis. Poorly-differentiated neuroblastoma is a tumour with a background of readily-recognisable neurophils. Most tumour cells in this subtype are undifferentiated and only ≤ 5% of the cell population has cytomorphological features of differentiation. Differentiating neuroblastoma is a tumour with usually abundant neurophils, and ≥ 5% of the tumour are cells showing differentiation toward ganglion cells.

The proportion of tumour cells showing mitosis and karyorrhexis determined whether the neuroblastoma has low (< 2% mitotic-karyorrhectic cells [MKC]), intermediate (2%–4% MKC) or high (> 4% MKC) MKI. Altungoz et al linked the degree of differentiation and MKI of the neuroblastoma to the age of the patient to two prognostic categories of FH and UH.[7] The prognostic categories are defined in Table I.

SpectrumOrange labelled LSI-N-MYC (2p24.1) DNA probe (Vysis, Downer Grove, IL, USA; now Abbott Molecular) was used to enumerate the number of MYCN copies per nucleus. Amplification of MYCN was considered in cases where the number of signals clearly exceeded ten copies per nucleus.[9] For 1p36 status analysis, fluorescein isothiocyanate (FITC) labelled Chromosome 1p36 Midi-Satellite (D1Z2) DNA probe (MP Biomedicals, Irvine, CA, USA) were mixed with SpectrumOrange labelled chromosome 1 specific centromeric region CEP 1 (D1Z5) DNA probe (Vysis, Downer Grove, IL, USA; now Abbott Molecular). Interpretations for 1p alterations were done according to the guidelines of the European Neuroblastoma Quality Group.[10] Briefly, the ratio of D1Z2 to CEP 1 signals below 0.67 was considered as an unbalanced loss of 1p. The presence of 17q gain were detected using a mixture of digoxigenin labelled Myeloperoxidase (MPO, 17q21.3–q23) DNA Probe (MP Biomedicals, Irvine, CA, USA) and SpectrumOrange labelled chromosome 17

Table II.The comparison of FISH data for MYCN amplification, 1p and 17q status with histological parameters.

Patient	Age (months)	MKI	MiB-1 mitosis	Histological diagnosis of neuroblastoma	Prognostic category	MYCN amplification	1p loss	17q gain	Clinical outcome
1	24	H	H	Undifferentiated	Unfavourable	Yes	Yes	Yes	Died
2	1	L	1	Poorly-differentiated	Favourable	No	No	No	Died of infection after chemotherapy
3	60	1	1	Differentiating	Unfavourable	No	Yes	Yes	Died
4	60	H	H	Undifferentiated	Unfavourable	Yes	Yes	No	Not followed up
5	< 1	L	1	Poorly-differentiated	Favourable	No	No	No	Alive
6	60	H	H	Undifferentiated	Unfavourable	Yes	Yes	Yes	Alive but on palliative care
7	30	H	H	Poorly-differentiated	Unfavorable	No	Yes	No	Died
8	24	H	H	Poorly-differentiating	Unfavourable	No	No	Yes	Died
9	60	L	1	Differentiating	Unfavourable	No	No	Yes	Died
10	11	1	1	Poorly-differentiating	Favourable	No	ND	ND	Alive
11	4	1	1	Poorly-differentiating	Favourable	No	No	No	Alive
12	44	L	L	Differentiating	Favourable	No	No	No	Alive
13	20	1	1	Poorly-differentiating	Unfavourable	No	Yes	No	Died
14	39	H	H	Undifferentiated	Unfavourable	No	No	Yes	Died

H: high; L: low; I: intermediate; ND: not done

specific centromeric region CEP 17 (D17Z1) DNA probe (Vysis, Downer Grove, IL, USA; now Abbott Molecular). The MPO DNA probe was detected via digoxigenin by FITC, using Anti-Digoxigenin FITC (Roche Diagnostics GmbH, Mannheim, Germany) diluted 1:100 in *In Situ* Hybridisation Blocking Solution (Vector Laboratories, Burlingame, CA, USA). A ratio of MPO to CEP 17 signals of 1.5:2.0 was considered an unbalanced gain of chromosome 17q.[5]

FISH was performed on touch imprints from fresh tumour samples and fixed immediately in modified Carnoy's fixative (3:1 methanol/glacial acetic acid). Hybridisation and wash protocols were performed as described elsewhere.[7] The slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) in antifade solution (Vectorshield, Vector Laboratories, Burlingame, CA, USA). The FISH preparations were analysed under an Olympus BX 60 Fluorescence Microscope equipped with filter sets for DAPI, FITC, Rhodamine, dual band-pass for FITC/ Rhodamine and triple band-pass for FITC/Rhodamine/ DAPI. Images were acquired via a CCD camera (COHU), and digitised and processed with Powergene MacProbe Imaging software (Applied Imaging, Newcastle-upon-Tyne, UK; now Genetix Ltd). At least 100 interphase cells were scored for MYCN status and 50–200 cells were scored for 1p and 17q statuses. For one case (Patient 10), 1p deletion and 17q gain studies by FISH were not performed because of insufficient cells and a severely crushed tumour.

RESULTS

A total of 14 primary neuroblastoma tumours were classified according to the modified Shimada classification and analysed by FISH for MYCN, 1p and 17q status. Of these 14 tumours, five showed FH while the other nine had UH. All nine tumours showing UH exhibited one or more abnormalities of the three genetic parameters being investigated, i.e. MYCN amplification, 1p deletion and 17q gain. One (Patient 4) of these nine patients went overseas for treatment and was not followed up in our institution. Of the other eight patients, only one (Patient 6) was alive and on palliative care at the time of writing. The five tumours that showed a FH did not have MYCN amplification, 1p deletion or 17q gain. Four of these five patients (Patients 5, 10, 11 and 12) were alive at the time of writing. One patient (Patient 2) died from infection after chemotherapy.

All the MYCN-amplified tumours either had additional 1p deletion and/or 17q gain. 1p deletion and 17q gain were found as the sole genetic abnormality in two and three primary tumours, respectively. Overall, three out of 14 tumours (21.4%) showed MYCN amplification, six (42.9%) tumours showed 1p deletion and six (42.9%) tumours showed 17q gain. The results are tabulated in Table II. In spite of our small sample size, these frequencies did not differ significantly from larger studies.[3-5]

DISCUSSION

Neuroblastoma is characterised by its clinical heterogeneity

and the complexity of its genetic abnormalities. Some of these abnormalities are powerful independent prognostic markers and will help in the risk stratification of patients at presentation. It is envisaged that research directed at sites of genetic imbalances will provide insights into the fundamental biology of its initiation and progression.

MYCN locus amplification at chromosome band 2p24 is the most well-known recurrent genetic abnormality of neuroblastomas. In this study, three out of 14 (21.4%) of the tumours had MYCN amplification. MYCN amplification is associated with rapid tumour progression and a poor prognosis, and its prognotic influence is more significant in the otherwise favourable group of infant patients with stage 4S disease.[11] MYCN amplification can be manifested as double minutes (dmin) and/or homogeneously staining regions (HSR). However, it was found that there was no difference in the event-free survival or overall survival in patients with MYCN amplification manifesting as either dmins or HSRs.[12] Therefore, in this study, we did not differentiate between MYCN amplifications that were due to HSRs or dmins. This study showed that all tumours having amplification for MYCN were also in the category for UH (Patients 1, 4 and 6). Moreover, it was found that all our MYCN-amplified tumours coexisted with either 1p deletion (Patient 4) or 17q gain, or both (Patients 1 and 6). This data supports the hypothesis that neuroblastomas exhibit a high degree of clonal heterogeneity and evolution at the genetic level during the course of the clinical progression.[13] It is likely that 1p deletion and/or 17q gain may occur prior to MYCN amplification as the disease progresses.

1p deletion was identified in six out of 14 (42.9%) of primary tumours in this study. The tumour suppressor gene(s) involved in neuroblastomas have not been identified, but the most common deleted region has been narrowed to a 1Mbp region within 1p36.3.[14-17] Hence, we used a DNA probe for the locus D1Z2 at 1p36.3 to detect deletion of this region. In this study, all tumours with 1p deletion were found in patients with UH and were highly associated with MYCN amplification (three out of six cases). A similar study using the same DNA probe for 1p, also found a significant correlation between both 1p36 deletion and MYCN amplification with a lack of differentiation of the tumour.[7] Although the independent prognostic influence of 1p loss is controversial,[3] previous studies have shown, by univariate analyses, that allelic loss at 1p36 is associated with a poor outcome and is predictive of a worse progression-free survival.[18-21]

In our study, an unbalanced gain of one to three additional copies in 17q was present in six out of 14 (42.9%) primary neuroblastomas, and all these tumours

with 17q gain had UH. 17q gain is associated with more aggressive tumours and has been recommended to be the most powerful genetic predictor of adverse outcome for patients.[5,16] The preferential gain in the region from 17q22-qter suggested that an overexpression of one or more genes in that region provided a selective advantage for these tumour cells.[22] The candidate genes likely to be involved include BIRC5,[23] NME1[24,25] and PPM1D.[26] Therefore, like others,[27] we chose a DNA probe spanning MPO (localised between NME1 and PPM1D) at 17q23.1-q23.2 to investigate 17q gain.

With the exception of a single patient (Patient 4) whom we could not contact, all the other patients with UH and at least one of the three genetic abnormalities, either died or had a poor clinical prognosis (Patient 6). On the contrary, those patients who had a FH with none of the three genetic abnormalities were alive and well, with the exception of Patient 2 who died from infection following chemotherapy and not directly from the disease. The clinical outcome of the patients in this study correlated well with their histological and genetic parameters.

The ability to detect risk factors at diagnosis and tailor the therapy accordingly can make the treatment of cancer more effective. Although MYCN amplification is a hallmark of an adverse outcome, there exists a subset of patients who will eventually develop aggressive disease despite the absence of MYCN amplification in their tumour. European studies suggest that the inclusion of chromosome 1p status of neuroblastoma cells may improve risk grouping,[28] and the clinical significance of additional tumour genetic characteristics, including 17q gain, 1p deletion and 11q deletion, are under study. In our study, all the patients (Patients 3, 7–9, 13 and 14) with either 1p deletion or 17q gain died even when their tumours did not show MYCN amplification. Our limited data suggests that the 1p and 17q status of neuroblastoma cells should be considered for risk grouping, as these parameters correlate with an UH and a poor clinical outcome.

Although FISH was proven to be useful for the detection of 1p deletion, MYCN amplification and 17q gain in neuroblastomas, the development of more recent technology, like multiplex ligation-dependent probe amplification[29] and array-CGH, offers the potential to detect a wider genome-wide copy number imbalance in a single reaction. We are not aware of similar studies done locally and hope that our limited data can contribute to motivate further studies.

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