

UNIVERSITY OF CAPE TOWN, SOUTH AFRICA

DEPARTMENT OF ANATOMICAL PATHOLOGY

TITLE:

**MORPHOLOGICAL CLASSIFICATION OF CHILDHOOD MEDULLOBLASTOMAS WITH β -
CATENIN IMMUNOHISTOCHEMISTRY AND MYCN FLUORESCENT *IN SITU*
HYBRIDIZATION.**

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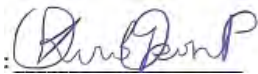
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Abstract

Introduction

Medulloblastoma is the most frequently occurring childhood malignant brain tumour, affecting 1 of 5 children presenting with a brain tumour, between the ages of 0 and 9 years. The basic prognostic stratification that relies on clinical and histological findings alone has proven unsatisfactory as an outcome predictor. Distinct molecular genetic profiles have been described, with four molecular variants of medulloblastoma with specific demographic and prognostic features. These are the WNT subgroup, SHH subgroup, Group 3 and Group 4 tumours.

The aim of this study was to describe the expression status of β -catenin, and MYCN, using IHC and FISH respectively, and to correlate these findings with clinico-pathological and demographic characteristics and clinical outcome.

Materials and Methods

This study was a nested retrospective analytical study, reviewing 54 cases of childhood medulloblastoma diagnosed between 1988 and 2014.

Results

Classic histology accounted for 40.7% of cases, LCA 37%, ND 16.7% and 5.6% MBEN). Based on B-catenin IHC, the WNT subgroup accounted for 16.7% of cases. This group had no mortalities or recurrences. Seven patients showed amplification of *MYCN* gene. The SHH group, defined by ND/MBEN histology and/or MYCN amplification, accounted for 27.7% of patients. Non-WNT/non-SHH tumours 30 patients (55.6%) showed a male predilection, and accounted for 37.5% recurrences and 50% mortalities also falling in this group.

Conclusions

Nuclear B-catenin identifies WNT tumours. Nodular desmoplastic morphology is useful in identifying some, but not all cases of SHH group medulloblastomas. MYCN positive tumours also showed classical, and LCA morphology.. Patients of all the beta-catenin positive cases were free of recurrence and alive at last follow up. Patients with *MYCN* amplification and non-ND histology (LC/A or classic) had poorer outcomes than patients with ND histology. One patient showed both MYCN amplification and nuclear B-catenin translocation, and had good clinical outcome. This finding requires validation with other molecular techniques.

List of Abbreviations

APC	Adenomatous polyposis coli
B-cat	β eta- catenin
CTNNB1	Cadherin-Associated protein beta-1
FAP	Familial Adenomatous Polyposis
FISH	Fluorescent in-situ hybridization
LC/A	Large Cell / Anaplastic
MBEN	Medulloblastoma with extensive nodularity
ND	Nodular Desmoplastic
IHC	Immunohistochemistry
PTCH	Patched
RCCH	Red Cross Children's Hospital
SHH	Sonic Hedge hog
Smo	Smoothened
Tcf	T-cell factor
WHO	World Health Organization
Wnt	Wingless-type

Dedication

To my Parents, Joseph and Damaris Okiro.
For your love, guidance and example.
Thank you for your constant prayers and support.

To my Husband and Best friend.
I cherish you.
Thank you for listening and being there for me through this journey.

To the Almighty God, thank you.

Acknowledgements

I am grateful to my supervisor Dr. Komala Pillay, whose scholarly advice, encouragement, commitment and patience have been invaluable to me throughout the course of this study.

I wish to thank Prof. Figaji and the other investigators in the parent study, for their support and advice during my study period.

I also wish to thank the Red Cross Children's Hospital, Groote Schuur Hospital Management, and the National Health laboratory Service (NHLS) staff, faculty and my colleagues for their input, and support.

To the patients, who every day afford us the opportunity to learn and understand more about the fascinating subject of pathology, thank you.

And finally, to my family, for their prayers, patience, and willingness to listen to my concerns, and making sure I kept my target and vision in sight. Mum and Dad, I have benefited greatly from your advice and wisdom.

Thank you all.

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1.0 Introduction

1.1 Background

Brain tumours are the second most common malignancy in children.^{1,2} Despite the importance of brain tumours, there is limited published data examining this group of patients, in South Africa and the African continent as a whole. There have been extensive and continual advances in paediatric cancer patient care over the last two decades; however brain tumour patient outcomes remain generally poor compared to outcomes of other childhood malignancies.

Medulloblastoma is the most common childhood malignant brain tumour. The prognostic stratification of medulloblastoma patients has been based on histological classification, as well as patient demographic and diagnostic findings. This basic classification has proved unsatisfactory as an outcome predictor, with survivors suffering serious long-term effects, particularly from over-treatment.

Recently, extensive work has been done to evaluate the molecular characteristics of medulloblastomas, in a bid to provide insights into the molecular biology of this potentially curable disease, and possibly facilitate improved prognostic and therapy planning process, and subsequently to facilitate the development of novel targeted therapies. These studies have culminated in the description of four distinct molecular variants of medulloblastoma, with specific demographic and prognostic features.³

1.2 Literature Review

Medulloblastoma is the most frequently occurring childhood malignant brain tumour, affecting one fifth of all children presenting with a brain tumour.⁴ It is an invasive malignant primitive neuroectodermal tumour of the cerebellum, seen most often in children between the ages of 0 and 9 years, but noted to occur at any age.⁵

The current incidence of medulloblastoma is reported as an estimated 0.5 per 100,000 children below the age of 15.³ Medulloblastomas affect nearly 2 people per million per year, affecting children 10 fold more frequently than adults.³ Medulloblastoma is the second most frequent brain tumor in children following Pilocytic astrocytoma, and is the most common malignant brain tumor in children, comprising 14.5% of newly diagnosed cases.⁶

The WHO defines medulloblastoma as a malignant embryonal tumour of the cerebellum with preferential manifestation in children, with predominantly neural differentiation, and an inherent tendency to metastasize via CSF pathways.⁷ Medulloblastomas most frequently occur in the posterior fossa, in the vicinity of the fourth ventricle.⁸

The WHO classification recognizes specific histological variants, associated with differing outcomes.⁹ These variants are the classic type of medulloblastoma, nodular desmoplastic variant, medulloblastoma with extensive nodularity (MBEN), and large cell/anaplastic variant.¹⁰

The classic type is characterized by sheets of undifferentiated cells with variable neuroblastic differentiation, indicated by the presence of Homer-Wright rosettes and palisades.¹⁰

The nodular desmoplastic variant, which on histology accounts for between 10% and 12% of medulloblastomas, exhibits biphasic architecture of nodules of

tumour cells surrounded by prominent amounts of connective tissue. The nodules are relatively hypocellular, composed of larger cells characterized by a neurocyte-like morphology, in a fine, neuropil-like background. The intervening internodular areas are hypercellular, and composed of primitive cells with high mitotic activity and proliferative indices. These areas, unlike the nodules, are reticulin rich. This variant of medulloblastoma is thought to be slightly more favourable prognostically than the classic medulloblastoma.^{4,10}

Medulloblastoma with extensive nodularity (MBEN) differs morphologically from the related nodular/desmoplastic variant by showing an expanded lobular architecture, characterized by enlarged reticulin free zones that are characterized by abundant neuropil-like material.¹¹

The large cell/anaplastic variant of medulloblastoma represents approximately 4% of these tumours, and are considered to be the most aggressive variant.¹² They show large, polygonal cells, nuclear pleomorphism and prominent nucleoli. Necrosis is a prominent feature.¹³

Histopathological subtyping has been shown to be a strong prognostic factor in certain patients, particularly the desmoplastic and anaplastic subtypes.¹⁴ However differences in risk stratification and outcomes despite the same histologic subtype, particularly for the classical and LC/A variants, have limited its value.¹⁵

Since 3 decades ago, medulloblastoma prognostic stratification has been based on this histological classification, as well as patient demographic and diagnostic findings, that include age at presentation, metastatic status at diagnosis, and extent of surgical resection.¹⁶

Based on these features, patients are stratified into two groups:

- a) Standard /Low risk: characterized by age of more than 3 years old, non-metastatic disease, total or near- total resection.¹⁷
- b) High risk: Leptomeningeal metastases at presentation and / or incomplete resection.¹⁸

This basic classification has proven unsatisfactory as an outcome predictor, with survivors suffering serious long-term effects, particularly from over-treatment.¹⁹ The current treatment modalities include surgical resection, radiotherapy and chemotherapy, with a cure rate of about 70%.²⁰ However, there is a continuing need to minimize overly aggressive therapy in patients with low-risk disease, to limit the occurrence of therapy associated complications such as cognitive impairment, stroke, neuropathies, seizures and secondary malignancies.²¹

Recent extensive study of the molecular characteristics of medulloblastomas using cytogenetic and genomic studies have resulted in the definition of distinct genetic profiles and pathway signatures that have been correlated with specific clinicopathological features.³ These studies have culminated in the description of four molecular variants of medulloblastoma, with specific demographic and prognostic features.

These variants are the Wingless-Type (WNT) subgroup, Sonic Hedgehog (SHH) subgroup, Group 3 and Group 4 tumours.¹⁶

Two of these molecular subgroups have lent themselves to extensive study, due to the identification of consistent distinct genetic profiles and molecular markers that are specific and recurrent, based on their similarities to hereditary syndromes with a proclivity for development of medulloblastomas. They are the WNT subgroup and the SHH subgroup.³

The Group 3 and 4 tumour subgroups are less well characterized, with multiple but non-recurrent molecular characteristics.

The Wingless-type (WNT) Subgroup.

This is the least common of the medulloblastoma subgroups, occurring in only 10% of cases.²² It is however the most curable and has the best prognosis of all the subtypes, with only rare metastasis to the neuraxis, and with more than 90% of patients surviving on current treatment modalities offered.²³ These tumours show a predominance of classic histology, with low incidences of metastatic disease. Rarely, the large cell anaplastic variants are seen.²² Males and females are equally affected, and the mean age at diagnosis is 10 years, with an age range extending to adulthood. ¹⁶ Genomic sequencing studies show that up to 90% of these tumours show activating mutations in *CTNNB1* (β -catenin, chromosome 3p21), encoding a pivotal molecule in the canonical WNT signaling pathway. ^{24,25.}

This group is eponymously designated after the predominant expression of components of the canonical WNT signaling pathway, a signal transduction pathway with a complex network of proteins best known for their roles in embryogenesis and cancer.²⁶ The major molecules within the *Wnt/APC* signal transduction pathway are the Adenomatous Polyposis coli (APC) and β -catenin proteins. *Wnt/Wg* genes, which are related to *Wingless* in *Drosophila*, encode several secreted proteins with key roles in development, cell fate and patterning.²⁷ In the absence of *Wnt* proteins, cells employ active measures to maintain low levels of the WNT signaling protein β -catenin.²⁸ At baseline, β -catenin is phosphorylated at its N-terminus by glycogen synthase kinase-3 β (GSK-3 β), targeting β -catenin for degradation by the ubiquitin-proteasome pathway.²⁹

The aberrant activation of the WNT signaling pathway represents a major oncogenic process that is an integral part of the evolution and development of many epithelial cancers, including colorectal cancer.³⁰ It has been demonstrated that a small percentage of sporadic medulloblastomas, and few inherited forms as in Turcot syndrome, are initiated by activating WNT pathway mutations, characterized by the stabilization of β -catenin and constitutive transcription by a β -catenin/T-cell factor (Tcf)-4 complex.³¹ Activation of this pathway prevents the

cells from either entering G1 arrest or undergoing terminal differentiation, and induces resistance to apoptosis. The end result is cellular proliferation.³²

Ellison et al,²³ in a study examining β -catenin status of childhood medulloblastomas, indicated that nuclear accumulation of this marker, which is in keeping with WNT group of tumours, showed significantly better overall survival and event free survival, indicating that it may be a marker of favourable outcomes in medulloblastoma patients.

The Sonic Hedgehog (SHH) Subgroup.

The SHH subgroup shows a bimodal age peak, arising in infants and young children on one end of the spectrum and adults on the other.³ There is no sex predilection, and metastatic involvement of the neuraxis is an uncommon finding.³³ The prognosis is favourable for infants and intermediate for other age groups.¹⁶

In the 1990s, Gorlin syndrome was attributed to inherited mutations of the *Patched 1 (PTCH1)* tumour suppressor gene on chromosome 9q22.32,³⁴ a finding that paved the way for studies that demonstrated recurrent somatic mutations of *PTCH1* in sporadic medulloblastomas.³⁵ The sonic hedgehog (SHH) pathway, a developmental signalling axis normally inhibited by PTCH1 protein, is aberrantly activated in about 30% of all medulloblastomas.³⁶ Activation of SHH signalling in these tumours is as a result of recurrent mutations and/or copy number aberrations that target multiple specific levels of the pathway.³⁷ In the absence of signal, the transmembrane protein Patched1 (Ptc1) keeps the pathway turned off by inhibiting the function of Smoothened (Smo), a second transmembrane protein.³⁸ Sonic Hedgehog (Shh) protein, a secreted protein, binds to and inactivates Ptc1, allowing activation of Smo. Smo then triggers target gene transcription through the Gli family of transcription factors.³⁷ The mechanism by which Shh inhibits Ptc1 and Ptc1 inhibits Smo is not understood in mammals.

The common genomic alterations in this subgroup include mutations of *PTCH-1* (chromosome 9q22.3), *SMO* (7q32.3), or *SUFU* (chromosome 10q24.32) that

disrupt sonic hedgehog signaling, *Gli2* amplification (chromosome 2q14), and MYCN amplification (chromosome 2p24.3) with MYCN overexpression.^{3, 8, 39}

The predominant histologic variants in this subtype include the nodular/desmoplastic type, with a small proportion of the classic and LCA types.⁸

Group 3 tumors, which account for 25% of medulloblastomas, arise in young children, and to a lesser extent in infants.³ A male predilection has been described. This subtype has the worst prognosis, and finding of metastases to the neuraxis is frequent.¹⁴ Several chromosomal aberrations have been described, including gains on chromosomes 1q, 7, 17q, and 18q, as well as losses on 5q, 8, 10q, 11p, and 16q. Isochromosome 17q is present.¹⁶ *MYCC* amplification (but not *MYCN*) and marked over expression are common.³

Histologically, this group shows LCA and classic morphology.

Group 4 tumours arise most frequently in children, and also show a male predilection.^{3,9} Infants and adults may also be affected. Metastasis to the neuraxis is frequent. The prognosis for this group of tumours is considered intermediate.¹⁶ The described chromosomal aberrations include *CDK-6* gene amplification, Isochromosome 17q, and involvement of the neuronal/glutamatergic pathway.⁴⁰ There is minimal MYC family expression.³ The histologic variant seen in group 4 tumours is most commonly the classic variant, but LCA histology is also noted.¹⁶

Statistical analysis of these 4 subgroups have revealed significant subgroup-specific clinical features, including patient age at diagnosis, sex, histology, metastatic status, and somatic variations, correlated with cytogenetic aberrations, providing an improved outcome prediction scheme.²⁷

β-catenin

Evidence of the involvement of the WNT cell signaling pathway in medulloblastoma first came to light from genetic studies of patients with Turcot

Syndrome, a syndrome characterized by germ-line mutations in the *Adenomatous Polyposis Coli (APC)* gene, who exhibit a 92-fold risk of developing medulloblastoma.

Sporadic medulloblastoma shows mutations in *CTNNB1* (β -catenin), *Axin-1*, and *APC*, whose phenotypic demonstration can be elucidated by immunohistochemical nuclear localization of β -catenin.⁴¹ This finding is fairly specific for WNT group of medulloblastomas, making this a fairly specific marker of this subgroup.^{3,43}

MYCN

Three MYC proteins have been described in genomic studies of medulloblastoma. They are MYC, MYCN and MYCL1.⁴² These proteins are important because they appear to demonstrate different roles in the different medulloblastoma subgroups.^{43,44} The expression and amplification of MYC and MYCN in medulloblastoma have been evaluated extensively, as they have previously been associated with poor outcomes and highly aggressive tumours.^{45, 46}

MYCN amplifications are seen to most commonly occur in the SHH subgroup, with a few occurring in group 4 tumours, and rarely in group 3.²⁷ Patients with MYCN amplification have a substantially worse prognosis than those without amplification, a finding that holds true for all patients.⁴⁷ Bearing in mind the fairly specific finding of nodular desmoplastic histology in SHH tumours, the finding of these two features is fairly specific for this subgroup, and would allow the evaluation of outcomes in this subgroup that are generally regarded as having favourable outcomes.³

This study is nested within a larger parent study that aims to collect data on childhood brain tumours at Red Cross Children's Hospital, a regional centre in South Africa, in a bid to develop capacity for infrastructure and future research.

1.3 Study Justification

The aim of this study was to describe the expression status of β -catenin, and MYCN, using immunohistochemistry and fluorescent in situ hybridization (FISH) respectively, and to correlate these findings with the various clinicopathological and demographic characteristics of the tumours including patient's age, sex, histopathological subtype and clinical outcome.

This study will set the background for further molecular characterization of medulloblastomas into the four molecular subtypes in the larger study that will include additional molecular investigations.

In order to diagnose and classify medulloblastomas into these genetic groups in practice, it is necessary to validate the genetic and IHC markers in formalin-fixed paraffin-embedded (FFPE) tumour tissue, comparing these findings with demographic and survival data in order to verify the usefulness of each marker for diagnostic practice.

1.4 Study Objectives

Main Objective:

To determine the β -catenin and MYCN expression status of medulloblastoma cases diagnosed at Red Cross Children's Hospital.

Specific Objectives:

1. To collect demographic and outcome data for childhood medulloblastomas diagnosed at RCCH.
2. To determine the β -catenin and MYCN expression status of these tumours by immunohistochemistry and fluorescent in situ hybridization (FISH) respectively, using standardized techniques.
3. To correlate this demographic data with the patterns of expression of β -catenin and MYCN.

2.0 Study Design and Methodology

2.1 Study Design

This study was a nested retrospective analytical study, reviewing cases of childhood medulloblastoma diagnosed between 1988 and 2014.

2.2 Study setting and population

This study was conducted at the Red Cross Children's' Hospital Cape Town, Pathology Department. This is a regional referral Centre in the Western Cape, South Africa.

The study was nested within a larger study examining Paediatric Brain tumours in South Africa, whose lead principle investigator is Prof. Anthony Figaji (Paediatric Neurosurgery).

This study sought and received ethical approval from the University of Cape Town Faculty of Health Sciences Ethics Committee (HREC/REF: 377/2014).

2.3 Sample Size

The sample size was based on convenience sampling, of cases with diagnostic material, clinical information available, and funding limitations.

2.4 Methods

2.4.1 Inclusion and exclusion criteria

All cases with histologically confirmed diagnosis of medulloblastoma, that also had clinical information available, were included. Any case in which tissue blocks and or adequate clinical information were not available, was excluded.

2.4.2 Recruitment and Enrollment

Cases were selected through a consecutive review of neurosurgical, oncology and pathology databases at the Red Cross Children's Hospital.

All medulloblastoma patients with a histological diagnosis managed at RCCH over the stated period, with tissue blocks and clinical information were included. Demographic and relevant clinical information of these cases were retrieved from patient files and recorded in data sheets.

2.4.3 Research Procedures and Data Collection Methods

Medulloblastoma patients were identified from neurosurgery and oncology databases, and searched for against pathology records. Archived slides and Formalin fixed, paraffin-embedded archival tissue blocks from these cases were retrieved and reviewed to confirm the diagnosis and histological sub-typing of medulloblastoma, according to the 2007 WHO classification for Central Nervous System tumours.

The patients were categorized into specific age groups: Infants (<3 years), children (3-10 years and over 10 years), as performed for similar studies²⁷, and as described for risk stratification.¹¹

Immunohistochemistry was conducted using antibodies against β -catenin.

Fluorescence in situ hybridization was conducted using probes to MYCN.

2.4.4 Laboratory Analysis

Microscopy

Haematoxylin and Eosin (H&E) stained slides were reviewed to confirm medulloblastoma diagnosis and histological sub-type.

Description of these characteristics was based on standard diagnostic protocols.

Data were entered into the data collection sheet (APPENDIX I)

Suitable blocks were then selected for immunohistochemistry and FISH.

Immunohistochemistry

(APPENDIX II)

The slide showing the best preserved tumour was identified. From the tissue block corresponding to the tumour slide identified, 3 micrometre thin tissue sections were cut. The sections were floated in a water bath at 37°C, and transferred to sialinised glass slides, which were dried in an oven at 60 degrees for 6 hours.

Automated immunohistochemical staining for β -catenin was performed. (See APPENDIX III) Heat induced epitope retrieval (HIER) was performed. The cases were incubated with the primary antibodies for durations defined by optimization done in the laboratory, and following the manufacturers' specifications (Novocastra TM Lyophilized Mouse Monoclonal Antibody Beta-Catenin, APPENDIX III). All slides were counterstained with Haematoxylin and cover slipped. Positive controls were run in parallel for each antibody.

β -catenin expression was evaluated, and cases segregated based on no or membrane staining alone, versus cytoplasmic and nuclear positivity in 10% or more of the tumour cells.

Fluorescent in situ Hybridization (FISH)

(APPENDIX III)

Formalin-fixed paraffin embedded blocks were analysed for *MYCN* gene amplifications via FISH using specific *MYCN* probes (Vysis LSI N-MYC SpectrumGreen/CEP2 SpectrumOrange probe).

Slide sections of tumour were treated with denaturing buffer, prewarmed to 73°C for 30 minutes. The slides were immersed in denaturation buffer at 73°C for 5 minutes. Slides were placed in 70%, 85% and 100% ethanol respectively at room temperature for 1 min each, and then warmed at 45 °C until ethanol evaporates.

Probes:

- Denatured at 73°C for 5 minutes and placed on ice for 2 minutes

- Briefly centrifuged
- Warmed at 37°C for 16hrs
- Washed in solution to remove surplus/free probe material
- 10µl of probe mixture was added to the slide (2µl probe + 8µl buffer)
- Slides were cover slipped
- Incubated at 37°C for 16hrs
- Washed in solution to remove surplus/free probe material
- 10µl of DAPI (complementary dye) was applied and the slide cover-slipped.

The slides were then visualized under a fluorescence microscope using appropriate filter sets. Under a Zeiss fluorescence microscope (Zeiss, Germany), the signals of the 2 probes in only intact nuclei were visualized through a triple bandpass filter (Vysis). Counting of hybridization signals was performed by examining a minimum of 50 distinct, non-overlapping nuclei were assessed per case.

2.4.5 Quality Assurance

1. Standard operating procedures were observed for routinely used stains
2. Suitable positive controls as defined by the manufacturer, were included for each slide for immunohistochemistry.

The slides were then visualized under a fluorescent microscope using appropriate filter sets. A minimum of 50 distinct, non-overlapping nuclei were assessed per case.

The findings of both FISH and immunohistochemistry were collated and statistical analysis was undertaken, along with patient demographic and outcome data. Slides were digitally photographed using the Olympus digital imaging software application available within the Division of Anatomical Pathology.

Privacy and Confidentiality

Patient anonymity was preserved throughout the study process. Records were de-identified and tracked via a designated study number.

Ethical Considerations.

Ethical approval was sought from the Human Ethics Board of the University of Cape Town before commencement of the study. The study does not impact on current patient care. All patient records were de-identified to maintain patient anonymity when analyzing the samples.

The parent study has received ethical approval. Funding was sourced from the parent study.

2.4.6 Data Management and analysis:

Data were recorded using a data sheet and then entered into an MS Excel spreadsheet (APPENDIX I). Statistical analysis was done using SPSS version 20.0 (SPSS Inc., Chicago). Values were represented as means and percentages. Chi square test (or Fisher's exact test when number was <5) was performed to determine the association between categorical variables.

3.0 Results

Demographic data.

54 cases met the inclusion criteria and were included in the study. 51.9% (n=28) of the patients were female and 48.1% were male. The mean age of the patients was 65.48 months (5years, 5 Months).

Based on age categories, 24,1% of patients were below the age of 3 years (See Table 1 below).

The tumours were predominantly midline (79.6%; n=43), with only 5.7% (2 right and one left) located laterally. There were 8 cases for which tumour location was unknown.

22.2% of the tumours were less than 4.1cms. 57.4% of tumours were greater than 4.1cms. Size was not known for 11 cases (20.4%).

A summary of the findings is represented in Table 1.

		Number (n=54)	%
Sex	Male	26	48.1%
	Female	28	51.9%
Age	< 3 years	13	24.1%
	3-10 years	37	68.5%
	> 10 years	4	7.4%
Site of Tumour	Midline	43	79.6%
	Right	2	3.7%
	Left	1	1.9%
	Unknown	8	14.8%
Tumour size	<4.0cm	12	22.2%
	>4.0cm	31	57.4%
	Unknown	11	20.4%
Risk stratification	Low risk	19	35.2%
	High risk	34	63.0%
	Unknown	1	1.9%
Extent of Resection	Biopsy	3	5.6%
	Subtotal	4	7.4%
	Near-total	22	40.7%
	Total	25	46.3%
Radiotherapy	Yes	44	81.5%
	No	10	18.5%
Chemotherapy	Yes	40	74.1%
	No	14	25.9%
Tumour Recurrence	None	46	85.2%
	Recurrence	8	14.8%

Mortality Status	Alive	41	75.9%
	Deceased/palliation	12	22.2%
β -catenin status	Membrane only	45	83.3%
	Nuclear/cytoplasmic	9	16.7%
MYCN Status (N=30)	Negative	23	76.7%
	Amplified	7	23.3%

Table 1: Data Summary (N=54; MYCN N=30)

Histological subtypes.

Classic histology accounted for 40.7% (n=22) of cases, being the most frequent histological subtype. Large cell / Anaplastic subtype was the second most common, accounting for 37% (n=20) of cases of medulloblastoma (See Figure 1)). Only 3 cases (5.6%) of medulloblastoma with extensive nodularity (MBEN) were seen (Figure 2 to 8).

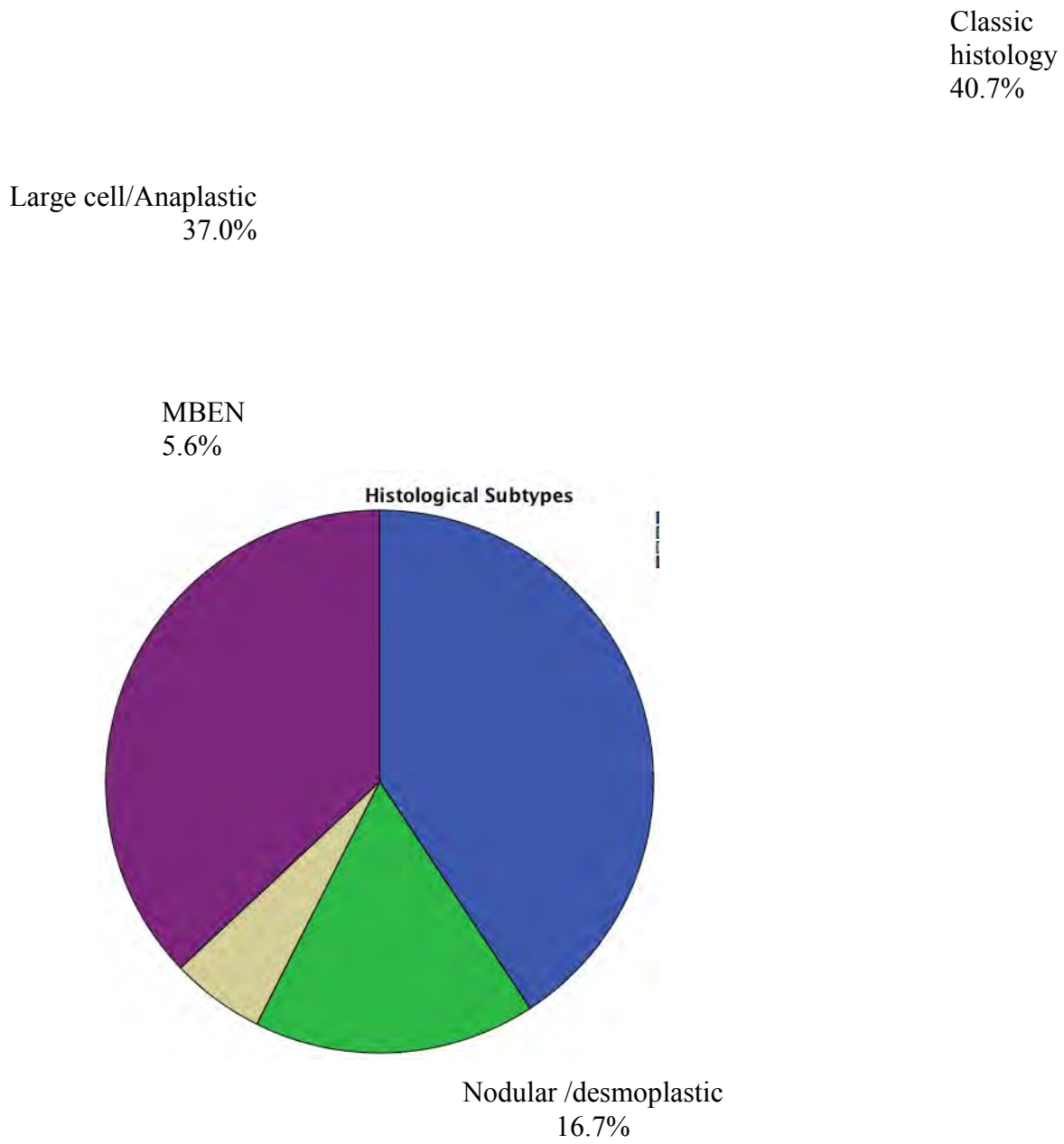


Figure 1: Histological subtypes (n=54).

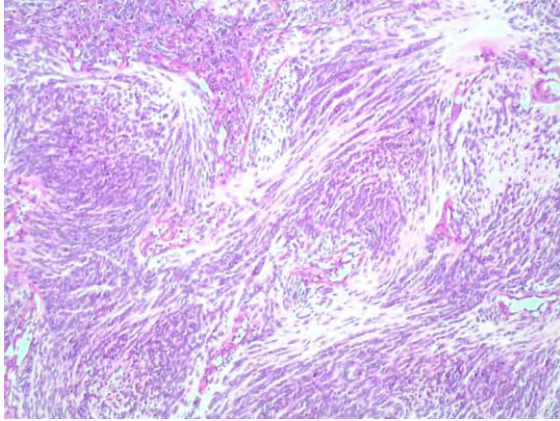


Figure 2: Medulloblastoma with extensive Nodularity (H&E, x4 magnification)

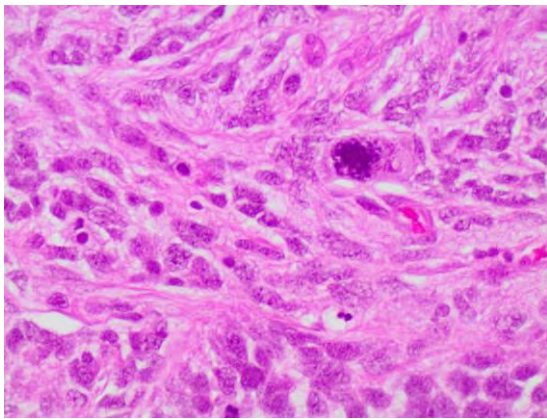


Figure 3: Large cell / anaplastic subtype of medulloblastoma with large atypical mitotic figure (H&E, x40 magnification)

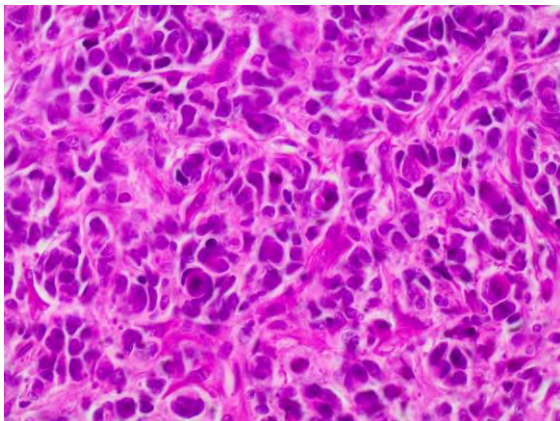


Figure 4: Large cell / anaplastic subtype of medulloblastoma with cell wrapping (H&E, x40 magnification).

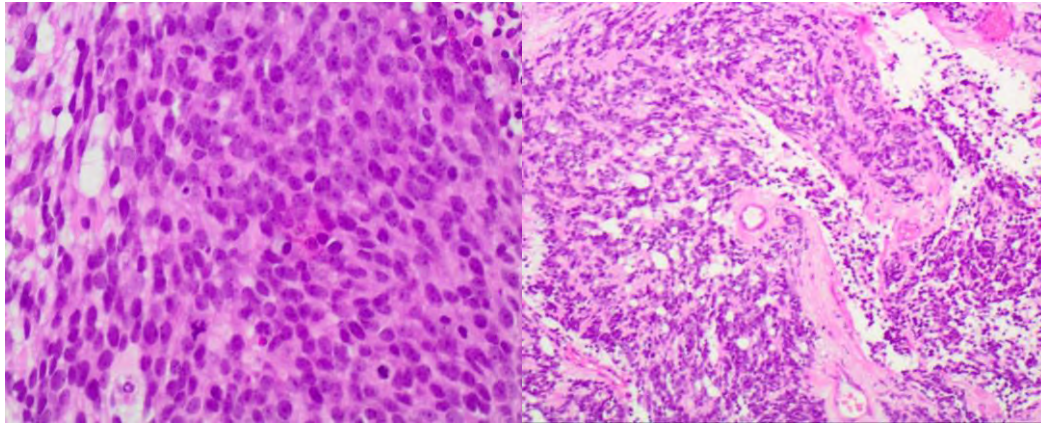


Figure 5 (A x40 and B x4 magnification): Classical medulloblastoma, with Homer-Wright rosettes.

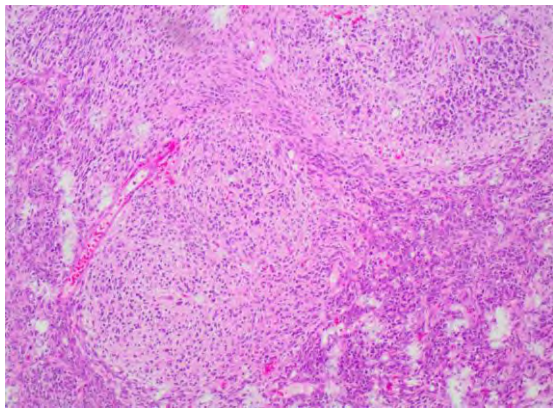


Figure 6: Nodular desmoplastic subtype of medulloblastoma (H&E, x4 magnification)

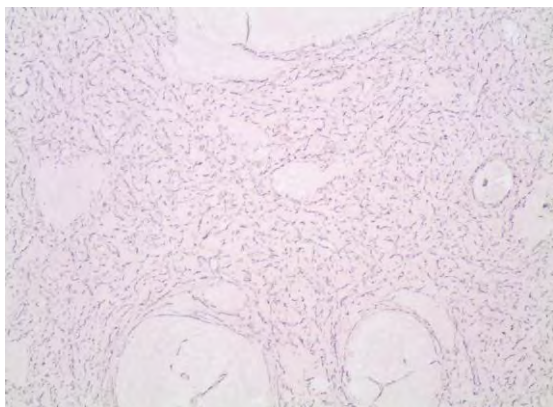


Figure 7: Reticulin stain showing no reticulin fibres within the nodules of the desmoplastic subtype of medulloblastoma (Reticulin stain, x10 magnification)

Based on clinical criteria described, 63% (n=34) of cases were classified as high risk. Risk data was unavailable for one case.

12 cases showed mixed histology, with 7 cases of the classic or large cell anaplastic tumours showing nodular desmoplastic areas, and 4 cases with focal anaplasia (figure 8).

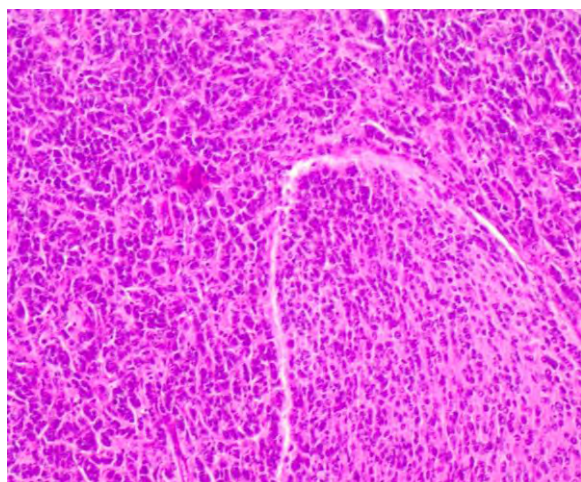


Figure 8: Biphasic tumour pattern showing large cell /Anaplastic and classic patterns (H&E, x10 magnification)

Histological Subtype and outcome.

Classic histological subtype accounted for the largest percentage of mortality /palliation outcomes (41.7%; n=5), followed by large cell/anaplastic (33.3%). Neither Nodular Desmoplastic nor MBEN had any mortality outcomes (Table 2).

Histological subtype	Mortality Status		
	Alive (n=41)	Deceased/palliation (n=12)	Unknown (n=1)
Classic	16 (39.0%)	5 (41.7%)	1 (%)
ND	6 (14.6%)	3 (25.0%)	0 (0%)
MBEN	3 (7.3%)	0 (0.0%)	0(0%)
LC/A	16 (39.0%)	4 (33.3%)	0(0%)

Table 2: Histological Subtypes and Mortality status Cross tabulation

One patient died of conditions not directly related to the tumour.

β -catenin staining (WNT Group)

With regards to β -catenin status, 16.7% (n=9) showed nuclear-cytoplasmic staining. 26% (5/19) of these patients were in the low risk group, and 11% (4/34) were in the high-risk group. (Figure 9 to 12).

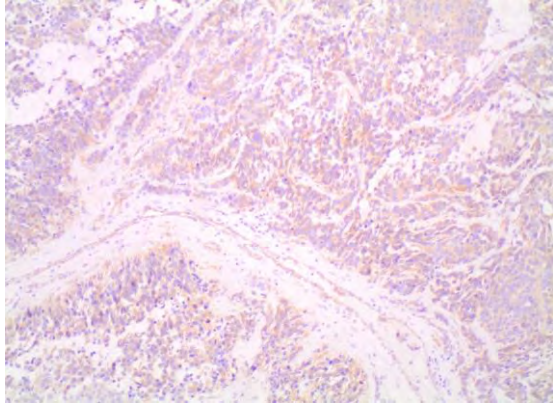


Figure 9: β -catenin Immunohistochemical stain showing membrane staining only (x4 magnification)

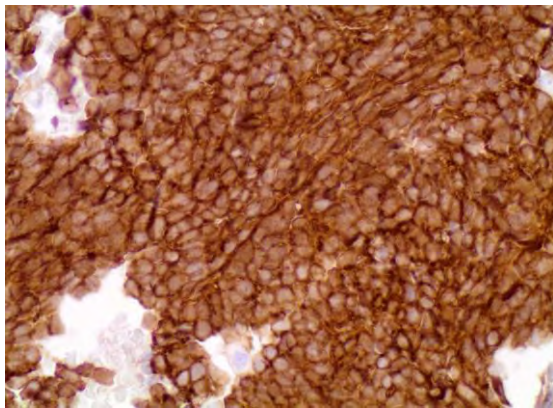


Figure 10: Strong membrane and nuclear β -catenin staining (x 40 magnification)

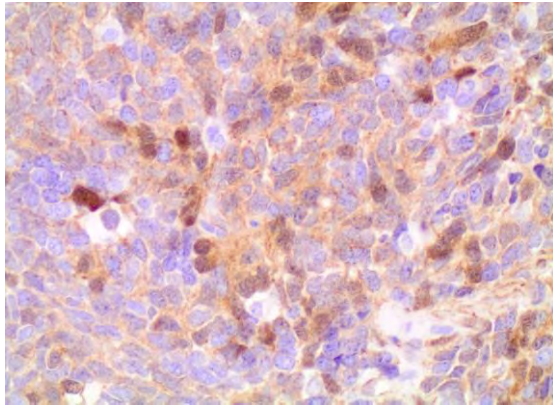


Figure 11: Variable β -catenin nuclear staining (x 40 magnification)

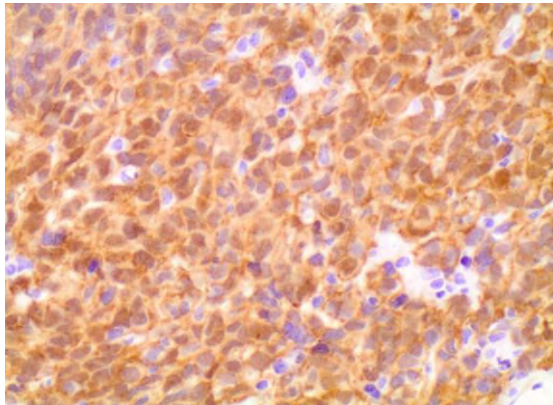


Figure 12: Moderate cytoplasmic and nuclear β -catenin staining (x 40 magnification)

β -catenin and histological subtypes.

Of the cases that showed aberrant nuclear-cytoplasmic β -catenin staining, 4 showed classic histology, and 4 LC/A, with one showing ND morphology. Two of the LCA cases and one classic case showed >50% staining. The others showed 10-50% staining. If staining was less than 10% of the tumour cells, it was considered negative.

β -catenin and outcome.

All patients with aberrant β -catenin expression were alive at last follow-up (Table 3).

	Mortality Status
--	------------------

β -catenin status	Alive (n=41)	Deceased (n=12)
Membrane only	32 (78.0%)	12 (100.0%)
Nuclear/cytoplasmic ('positive')	9 (22.0%)	0 (0.0%)

Table 3: β -catenin - Mortality status Cross-tabulation

This finding approached, but did not however attain statistical significance ($p=0.066$, Fisher's exact test, one sided).

β -catenin status had a trend towards association with tumour recurrence ($p=0.065$, Fisher's exact test, one sided), in that none of the tumours with aberrant β -catenin staining recurred, but this did not reach statistical significance. A cross tabulation of recurrence and β -catenin status is represented in Table 4.

There was no evidence of an association between β -catenin status and clinical risk status ($p=0.357$).

			Tumour Recurrence		Total
			No recurrence	Recurrence	
β-catenin	membrane	No.	37	8	45
		% with Tumour Recurrence	80.4%	100.0%	83.3%
	Nuclear-cytoplasmic	No.	9	0	9
		% with Tumour Recurrence	19.6%	0.0%	16.7%
Total			46	8	54

Table 4: β -catenin- Tumour Recurrence Cross tabulation.

MYCN and outcome

Thirty cases were stained for MYCN using FISH probes (Figures 13 and 14 below). 7 cases (23.3%) were positive for MYCN amplification.

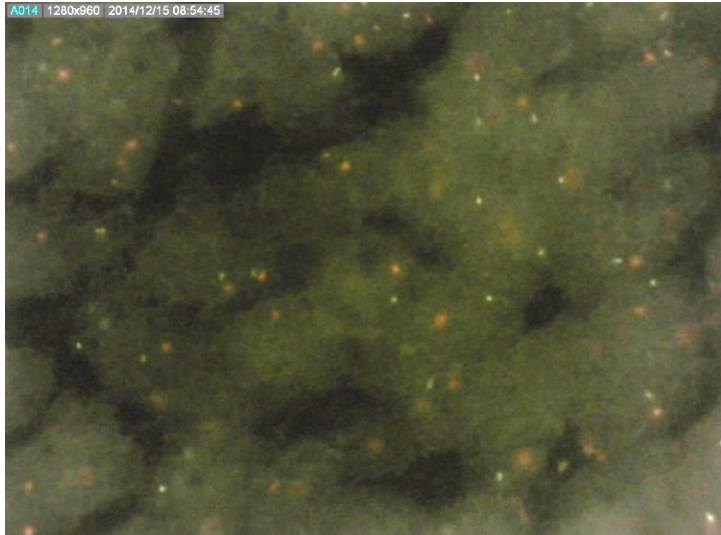
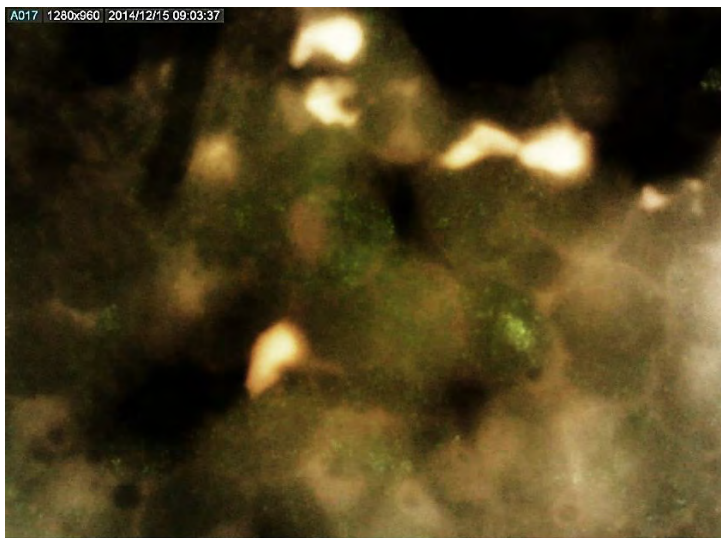
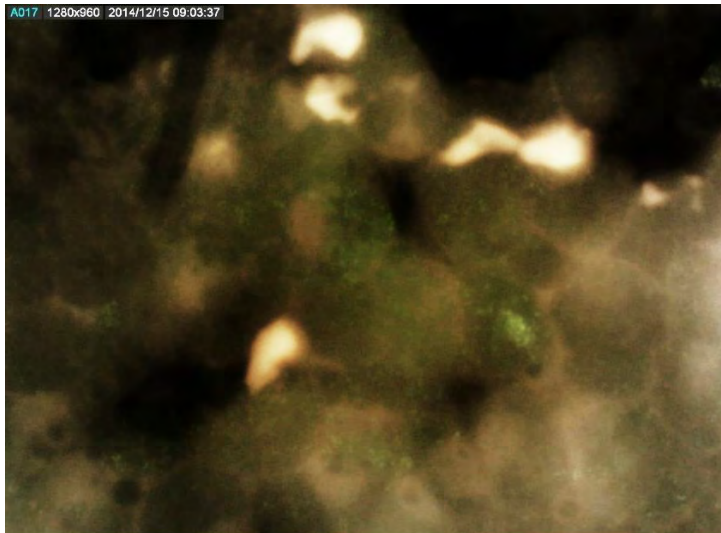


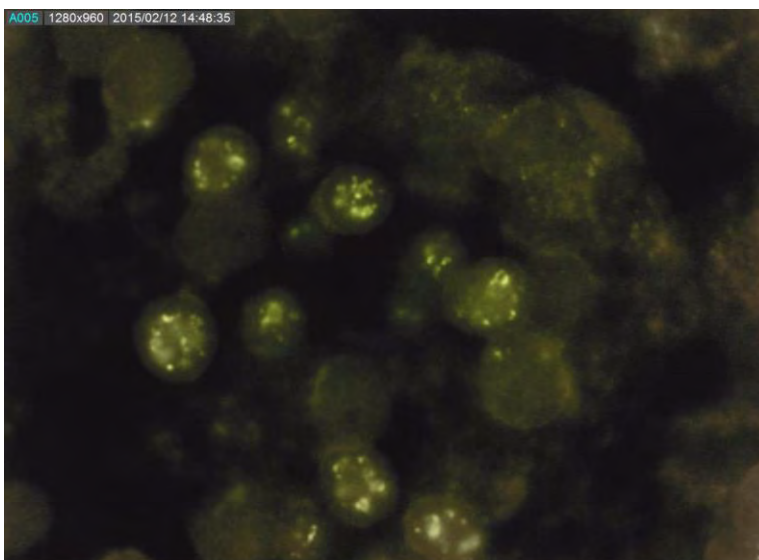
Figure 13: MYCN FISH not amplified showing two green and red signals within each nucleus, with a ratio of <math><6:1</math> (x40 magnification)



1



2



3

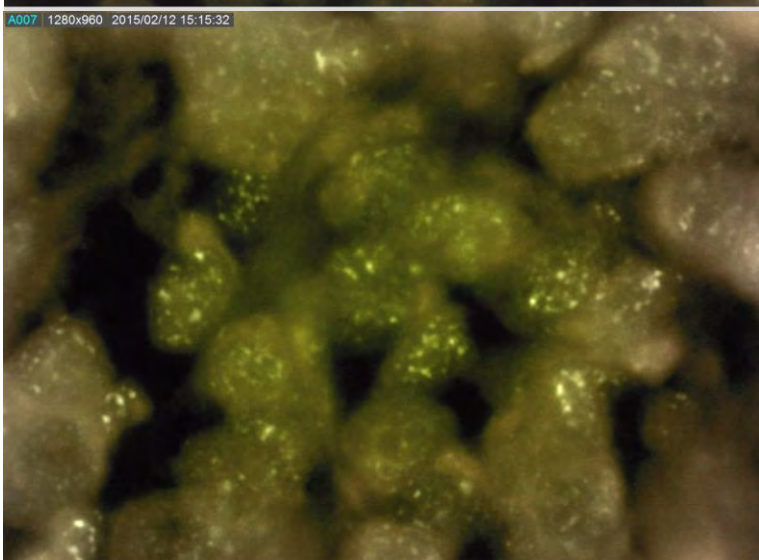


Figure 14: MYCN FISH amplification (Cases 1,2- triple filter; Cases 3, 4-green filter) >6:1 green signal. (x40 magnification).

The histological subtypes of these cases were 2 classic, 3 nodular/desmoplastic and 2 Large cell /anaplastic.

Comparison with tumour recurrence and mortality/palliation are tabulated in tables 5 and 6 below.

		Tumour Recurrence		Total
		No recurrence	Recurrence	
MYCN status	Negative	21(91.3%)	2(8.7%)	23 (76.7%)
	Positive	5(71.4%)	2(28.6%)	7(23.3%)
Total		26	4	30

Table 5: MYCN Status – Tumour Recurrence Cross tabulation

		Mortality status		Total
		Alive	Deceased/palliation	
MYCN status	Negative	19(82.6%)	4 (17.4%)	23
	Positive	4(57.1%)	3(42.9%)	7
Total		14	6	30

Table 6: MYCN status - Mortality status Cross tabulation

These findings however, did not achieve statistical significance (p=0.225 and 0.185 respectively, Fisher exact test).

SHH Group

15 cases were classified as belonging to the SHH group, classified based on Nodular desmoplastic/MBEN morphology, and/or MYCN over-expression.

Nine of these patients were below the age of 3 years, with 18 aged 3 to 10 and 3 over the age of 10. Seven patients were male (46.7%). Seven cases each were classified as high or low risk respectively, with risk status unavailable for one.

This group accounted for 27.7% of patients.

Nine patients in this group were alive at last follow-up. These cases showed MBEN histology (3 cases), Nodular desmoplastic histology (5 cases) and classic histology (1 case). Three patients showed MYCN amplification (2 ND, 1 Classic). MYCN status was unknown for 6 of these patients.

Six of the patients were deceased or on palliation (40%), with 3 showing ND histology, two LC/A and one classic. Of these, 5 had recurrences. One was not operated on due to the size of the tumour, and its increased growth despite chemotherapy. For 3 of these patients, MYCN status was unknown.

	Frequency (n=15)	Percent
No recurrence	10/15	66.7%
Recurrence	5/15	33.3%
Alive	9	60%
Deceased/palliation	6	40%

Table 7: SHH group and recurrence/outcome cross tabulation.

Non Wnt/Non SHH" group

This group was composed of cases that did not fit the above descriptive criteria, which is no nuclear β -catenin, non-ND or MBEN histology and no MYCN amplification.

A summary of the findings is represented in Table 8 below.

		Number (n=30)	%
Sex	Male	17	56.7%
	Female	13	43.3%
Age	< 3 years	9	30.0%
	3-10 years	18	60.0%
	> 10 years	3	10.0%
Risk stratification	Low risk	7	23.3%
	High risk	23	76.7%
Tumour Recurrence	None	27	90.0%
	Recurrence	3	10.0%
Mortality Status	Alive	23	76.0%
	Deceased	6	20.0%
	Unknown	1	4.0%

Table 8: Data summary of cases under the “non-WNT/non-SHH” group. (n=30)

Over 10 (30%)

Under 3 (10%)

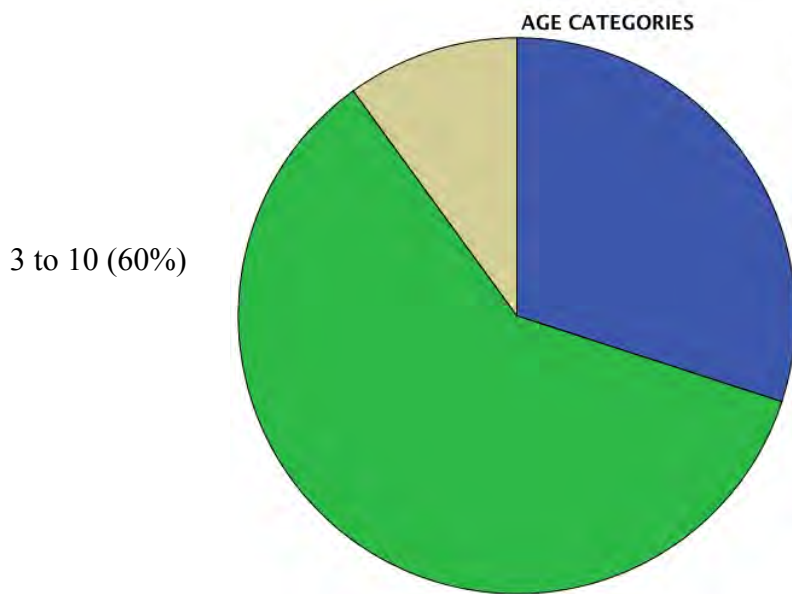


Figure 15: “Non WNT/non-SHH” group. Distribution of patients by age ranges (n=30)

Table 9 summarizes the tumour size, therapy and outcomes of the MYCN and aberrant β -catenin tumours.

MYCN					
Tumour size	Resection	RXT	CXT	Recurrence	Deceased/palliation
Bigger	4	Y	Y	No	No
Bigger	4	No	Y	Yes	Yes
Unknown	3	Y	Y	No	No
Bigger	4	Y	Y	No	No
Bigger	3	Y	Y	Yes	Yes
Bigger	4	Y	Y	No	No
Bigger	1	No	Y	No	Yes
Aberrant β-catenin					
Smaller	3	Y	Y	No	No
Bigger	2	Y	No	No	No

Unknown	3	Y	Y	No	No
Unknown	3	Y	Y	No	No
Bigger	4	Y	Y	No	No
Bigger	4	Y	Y	No	No
Smaller	4	Y	Y	No	No
Smaller	4	Y	Y	No	No
Bigger	4	Y	Y	No	No

Table 9: Outcomes and therapy for MYCN and B-catenin positive tumours

4.0 DISCUSSION

Medulloblastoma is a complex disease, characterized by described genetic changes that have facilitated a molecular classification of these tumours. The molecular classification of medulloblastomas is aimed at enabling the prediction of the pathogenesis and biological behavior.

Historically, tumour classification has been based on histological classification alone, and management based on clinical characteristics that stratify patients into low or standard risk groups. However, studies have shown varied responses to treatment, and varied outcomes have been described for the same histologic subtype, with patients who are potentially curable with less aggressive management strategies suffering significant treatment related morbidity, thus highlighting the need a molecular classification. Patient management thus far has been based on standard therapy protocols (APPENDIX IV)

There is evidence of unique molecular pathogenetic pathways for medulloblastoma. However, there is a need for accessible techniques that can identify surrogate markers for these molecular pathways that are potentially usable in routine diagnostic pathology, specifically immunohistochemistry and FISH.

The aim of this study was to determine the expression status of β -catenin by immunohistochemistry and MYCN over-expression by FISH, and to correlate these findings with patient and demographic data.

Demographic findings

Our patient ages ranged from 3 months to 13 years, with a mean age of 5 years and 5 months. There was a slight female predominance (51.9% versus 48.1%), compared to other demographics that indicate a male preponderance.⁵

Overall, post-treatment outcomes were comparable to the findings of Gajjar *et al*,²⁰ at 76%, to their recorded 70%.

Using the molecular groups that have been defined for medulloblastoma, and using described immunohistochemical, FISH and morphological features as surrogate markers, the cases can be divided into the following groups:

	WNT group (Aberrant β - catenin- nuclear- cytoplasmic)	SHH group (ND/MBEN and MYCN over- expression)	Non- WNT /Non SHH
Number of cases	9	15	30
Percentages	16.7%	27.7%	55.6%

β -catenin is a specific marker for the WNT pathway, which has been shown to be a category with a more favourable outcome than any of the other groups.²³ In a study by Fattet *et al*,⁴⁸ who looked at the correlation between immunohistochemistry findings for nuclear β -catenin staining and compared this to genetic mutational status, found that presence of more than 5% nuclear staining correlated with *CTNNB1* mutational status.

In this study, the WNT subgroup accounted for 16.7% of cases, compared to the 10% described by Northcott *et al*.¹⁶ Other independent studies have reported rates of 12.5%,⁴⁸ 16%,⁴⁹ and 25%.²³

This group had 2 males and 7 females. The sex ratio for WNT tumour subgroup has been reported as 1:1,¹⁶ with better outcomes reported in females overall due

to their more common representation in the WNT group, as was the case in our study population, compared to the nearly 1:1 ratio overall.

Another noted distinction is the incidence of Large cell / Anaplastic morphology, which accounted for 33% (n=3) of cases in this group. Generally, this histological subtype is considered only a rare occurrence in the WNT group.³ da Silva *et al*⁴¹ reported an incidence of 18% in their analysis of medulloblastoma. One case showed nodular desmoplastic histology and will be discussed in greater detail.

All patients found to have aberrant β -catenin staining were over the age of 3 years, in keeping with reports of this tumour expression occurring in older children.^{3,16}

Clinical risk stratification has all along been a major factor in determination of patient management strategies.

Of patients with nuclear/cytoplasmic β -catenin staining (WNT), 5 were classified as low risk by clinical criteria, and 4 were classified as high risk. Clinical risk status did not show a statistically significant association with either β -catenin or MYCN.

All recurrences in our patient population showed nuclear negativity for β -catenin. Although the p-value for the association between β -catenin expression and recurrence and mortality outcome tended toward but did not achieve statistical significance, it is of note that all the patients with nuclear β -catenin positive tumours are alive at last follow-up (Tables 3 and 4).

Seven patients showed amplification of *MYCN* gene. The histological subtypes of these cases were 2 classic, 3 nodular/desmoplastic and 2 Large cell /anaplastic. Two (28.3%) of these patients had tumour recurrence, and 3 (42.9%) are deceased. Of the two who had recurrences, their tumours were LCA, both at initial diagnosis and recurrence. The third patient had classic histology but tumour resection was not possible, and the patient was referred for palliation.

Because FISH for MYCN was performed on only 30 of the 54 cases, it may be an under-estimation of the occurrence of this genetic amplification.

Of the 7 cases that showed MYCN amplification, they showed Nodular desmoplastic, Large cell /Anaplastic and Classical histology, consistent with the heterogeneity described for this group of tumours.

Three of the MYCN amplified patients are deceased or on palliation (2 deceased, one on palliation, with four months follow-up). The two deceased patients had LC/A histology and recurrence of tumour; the other had classic histology, and tumour was noted to have increased in size despite chemotherapy. Three of the 4 living patients showed ND histology and the last was classic.

Of cases in the SHH group, classified based on Nodular desmoplastic/MBEN morphology, and/or MYCN over-expression,³ this group accounted for 27.7% of our patients, a figure in keeping with the 30% described by Northcott *et al.*¹⁶ This group has an intermediate prognosis, and can be represented by any of the histologic subtypes.

The 15 patients in this group had varied outcomes. Nine were alive at last follow-up, and these cases showed MBEN histology (3 cases), Nodular desmoplastic histology (5 cases) and classic histology (1 case). Three patients showed MYCN amplification (2 ND, 1 Classic). MYCN status was unknown for 6 of these patients.

Six of the patients were deceased or on palliation, with 3 showing ND histology, two LC/A and one classic. Of these, 5 had recurrences. One was not operated on due to the size of the tumour, and its increased growth despite chemotherapy. For 3 of these patients, MYCN status was unknown.

Korshunov *et al.*⁴⁷ in a study examining MYCN-MB histology in 67 tumours, demonstrated heterogeneity of outcomes, clinical biology and tumour genetics.

All other tumours that did not fit into the two described groups were together considered to be part of the Group 3 and Group 4 tumours (Non-WNT/Non-SHH). This group consisted of 30 patients (55.6%). Ellison *et al*³⁸ described this group as representing 55% of their study population. This group represents the assumed group 3 and 4 tumours, which account for 25% and 35% of medulloblastomas respectively (aggregate of 60%).¹⁶

These tumours have a male predilection, and can occur from infancy to adulthood.³ Nine of these patients were below the age of 3 years, with 18 aged 3 to 10 and 3 over the age of 10.

Of the 8 recurrences overall, 3 (37.5%) are in this group, with half of the 12 mortalities also falling in this group. Group 3 tumours have the worst prognosis, with Group 4 tumours showing intermediate prognosis.¹⁶

This is however an unsatisfactory categorization, as Group 3 tumours have by far the worst prognosis, and should be considered independently.

The patient with nodular desmoplastic histology within the WNT group also showed MYCN amplification. Based on the B-catenin amplification, it was classified in the WNT group. This patient is alive post-treatment.

MYCN moderate overall expression has been described in WNT tumours,⁴⁴ but is an high expression is an unusual finding. Further molecular studies would be needed to further characterise this case.

An observation of note is the finding of mixed subtypes in 12 of our cases. The most common finding was of a nodular /desmoplastic component within a predominantly classic or large cell anaplastic tumour (7 cases), followed by focal anaplasia, not fulfilling criteria for LC/A, seen in other subtypes (4 cases). Classification was based on the predominant component.

5.0 Study Limitations

The major limitation to this study is the limited funding that did not allow MYCN characterization of all the tumours.

Another limitation is the absence of markers for group 3 and group 4 tumours. The parent study aims to address this issue by exploring four antibodies that have been described for each sub-type, along with other molecular studies, and this data will be collated along with their findings.

The short duration of follow-up for some of the patients included also limits the prognostic value gleaned from the findings.

The retrospective nature of the study limited some aspects of clinical information, particularly data on radiological staging. Some of the clinical folders could not be retrieved.

Finally, for patients who are alive post therapy, the data does not reflect the functional outcomes post therapy, an important factor that has informed the search for molecular characterization of these tumours.

6.0 CONCLUSIONS

As described by other authors, β -catenin nuclear translocation serves as a robust marker for the WNT group of tumours, which can present with varied histology, and generally have good clinical outcome.

Nodular desmoplastic morphology is useful in identifying some, but not all cases of SHH group medulloblastomas. MYCN positive tumours also showed classical, and LCA morphology. Although for the purposes of this study, cases showing MYCN amplification were considered to belong to SHH group, one case also showed aberrant nuclear β -catenin staining. This finding requires validation with other molecular techniques.

Patients of all the aberrant β -catenin cases were free of recurrence and alive at last follow up and this finding approached statistical significance compared to a 42.9% mortality in the MYCN positive group and 40 % mortality in the SHH group as a whole. Patients with *MYCN* amplification and non-ND histology (LC/A or classic) had poorer outcomes than patients with ND histology. One patient showed both MYCN amplification and nuclear β -catenin translocation, and had good clinical outcome.

APPENDICES

APPENDIX I: DATA COLLECTION SHEET

Data number	Age Months)	Age ranges	Sex	Histological subtype	MYCN Status	B-catenin status	Tumour location	Tumour size	Risk	Extent of resection	Radiotherapy	Chemotherapy	Recurrence	Outcome
1	32	1	1	1	N/A	1	1	2	2	2	1	1	1	1
2	37	2	2	2	N/A	1	1	1	1	4	1	2	1	1
3	29	1	2	2	2	1	1	2	2	4	1	1	1	1
4	88	2	2	4	N/A	1	1	?	2	2	1	1	1	1
5	122	3	1	4	N/A	2	1	1	2	3	1	1	1	1
6	62	2	2	2	N/A	1	?	?	1	4	1	2	1	1
7	16	1	2	4	N/A	1	1	2	2	3	2	2	1	1
8	155	3	1	1	N/A	1	1	1	2	3	1	1	1	1
9	39	2	2	4	N/A	2	1	2	2	2	1	1	1	1
10	118	2	2	4	N/A	1	1	?	1	4	1	2	1	1
11	27	1	1	2	N/A	1	1	2	2	4	1	1	2	2
12	113	2	1	1	N/A	1	1	1	1	4	1	2	1	1
13	88	2	2	4	N/A	1	1	2	1	4	2	2	1	1
14	41	2	1	2	N/A	1	1	1	1	4	2	2	2	2
15	136	3	1	1	N/A	1	1	1	2	3	1	1	1	1
16	45	2	2	2	N/A	1	1	2	2	3	1	1	2	2
17	12	1	1	4	N/A	1	1	2	2	3	2	1	1	2
18	39	2	1	4	N/A	2	1	?	2	3	1	2	1	1
19	108	2	2	4	N/A	1	1	2	2	3	1	1	1	1
20	156	3	2	4	N/A	1	1	?	2	3	1	1	1	1

21	72	2	2	4	1	1	1	2	2	3	1	1	1	1
22	72	2	1	4	2	1	1	2	1	4	2	1	2	2
23	64	2	1	1	1	1	1	2	2	3	1	1	1	1
24	88	2	1	1	1	1	1	1	2	3	1	1	1	1
25	12	1	2	1	1	1	1	2	2	1	2	2	1	1
26	13	1	1	3	1	1	1	2	2	4	2	1	1	1
27	108	2	1	1	N/A	2	?	?	2	3	1	1	1	1
28	64	2	2	1	1	1	2	2	2	3	1	1	1	1
29	51	2	1	1	2	1	?	?	0	3	1	1	1	1
30	44	2	1	1	N/A	1	?	?	2	3	1	1	2	2
31	96	2	2	2	2	1	1	2	1	4	1	1	1	1
32	84	2	2	1	N/A	1	?	?	1	4	1	1	1	3
33	67	2	1	4	N/A	1	1	2	2	3	1	?	1	1
34	35	1	1	4	1	1	1	2	1	4	1	2	1	1
35	72	2	1	4	N/A	1	?	?	1	4	1	1	1	1
36	72	2	1	3	1	1	1	1	2	4	1	1	1	1
37	66	2	2	4	2	1	1	2	2	3	1	1	2	2
38	69	2	1	1	1	1	1	2	1	4	1	1	1	1
38	3	1	2	1	1	1	?	?	2	1	2	2	1	2
40	96	2	1	4	1	2	1	2	1	4	1	1	1	1
41	72	2	2	4	1	1	1	2	2	3	1	2	1	1
42	72	2	2	1	1	2	3	2	1	4	1	1	1	1
43	108	2	1	2	2	2	2	1	1	4	1	1	1	1
44	96	2	1	1	1	2	1	1	1	4	1	1	1	1
45	30	1	2	1	1	1	1	1	2	3	1	1	2	2
46	95	2	2	4	1	1	1	2	2	3	1	1	2	2
47	48	2	1	2	1	1	1	2	1	4	1	1	1	1
48	59	2	1	1	1	2	1	1	1	4	1	1	1	1
46	96	2	1	3	1	1	1	2	1	4	1	1	1	1

50	18	1	1	1	1	1	1	2	2	2	2	2	1	2
51	72	2	2	1	1	1	1	2	2	4	1	1	1	1
52	11	1	2	1	2	1	1	2	2	1	2	1	1	2
53	10	1	2	1	1	1	?	2	2	4	1	1	1	1
54	38	2	2	4	1	1	1	2	2	3	1	1	1	1

Codes provided below.

DATA SHEET CODING.

1. Data number: _____
2. Age(yrs and months): _____
3. Age ranges

<3	1
3-10	2
>10	3
4. Sex:

Female-	1
Male-	2
5. Histological Subtype

Classic	1
Nodular/Desmoplastic	2
Medulloblastoma with extensive nodularity	3
Large cell/Anaplastic	4
6. MYCN Status

Negative	1
Positive (amplified)	2
Not done	-
7. Beta catenin staining

Membrane	1 (Membrane staining only, or nuclear <10%)
nuclear-cytoplasmic	2 (Cytoplasmic and / nuclear staining; >10%; 10-50%; >50%)
8. Tumour location

Midline	1
Right	2
Left	3
Unknown	-
9. Tumour size

Smaller (<4.1cm)	1
Bigger (>4.1cms)	2
Unknown	-
10. Risk

Low risk	1
High risk	2
11. Extent of Resection

Biopsy	1
Subtotal resection	2

Near total resection 3
Total resection 4

12. Radiotherapy

Yes 1
No 2

13. Chemotherapy

Yes 1
No 2

14. Tumour recurrence

No 1
Yes 2

15. Mortality

Alive 1
Deceased/Palliation 2

APPENDIX II

Immunohistochemistry:

High temperature Antigen Unmasking Technique for Immunohistochemical Demonstration on Paraffin Sections.

Leica Biosystems Newcastle Ltd. 95 8147 Rev A

1. Cut and mount section on slides coated with suitable tissue adhesive.
2. Deparaffinize sections and rehydrate to distilled water.
3. Place sections in 0.5% hydrogen peroxide/methanol for 10 minutes (or use other appropriate endogenous peroxidase blocking procedure). Wash sections in tap water .
4. Heat 1500mls of the recommended unmasking solution (0.01M buffer, pH 6.0 (or Epitope retrieval solution, RE 7113)unless otherwise indicated overleaf. Until boiling in a stainless steel pressure cooker. Cover but do not lock the lid.
5. Position slides into metal staining racks (do not place slides close together as uneven may occur)and lower into pressure cooker ensuring slides are completely immersed in unmasking solution. Lock lid.
6. When the pressure cooker reaches operating temperature and pressure, (after about 5 minutes) start a timer for 1 minute (unless otherwise indicated on the data sheet).
7. When the timer rings, remove pressure cooker from heat and run under cold water with lid on. DO NOT OPEN LID UNTIL THE INDICATORS SHOW THAT PRESSURE HAS BEEN RELEASED> Open lid, remove slides, and place immediately in a bath of tap water .
8. Wash sections in TBS buffer (pH 7.6) for 1x5 minutes
9. Place sections in diluted normal serum (or RTU Normal Horse Serum) for 10 minutes.

10. Incubate sections with primary antibody. Use antibody diluent RE7133 (where available).
11. Wash in TBS buffer for 2x5 minutes.
12. Incubate sections in an appropriate biotinylated secondary antibody.
13. Wash in TBS buffer for 2x5 minutes.
14. Incubate slides in ABC reagent (or RTU streptavidin/peroxidase complex).
15. Wash in TBS buffer for 2x5 minutes.
16. Incubate slide in DAB or other suitable peroxidase substrate
17. Wash thoroughly in running tap water.
18. Counterstain with Haematoxylin, dehydrate and mount.

SOLUTIONS

0.01M CITRATE BUFFER (PH6.0) OR RE7133 (where available).

Add 3.8 g of citric acid (anhydrous) to 1.8L of distilled water. Adjust to pH 6.0 using concentrated NaOH. Make up to 2L with distilled water.

1mM EDTA 9pH 8.0) or RE 7116

Add 0.37g of EDTA (SIGMA product coded E-5134) to 1L of distilled water. Adjust pH to 8.0 using 1.0M NaOH.

20mM TRIS/0.65mM EDTA/ 0.65% TWEEN (pH 9.0) or RE 7119 (where available)

Dissolve 14.4g of Tris (BDH product code 271197K) and 1.44g EDTA (SIGMA product code E-5134) to 0.55L of distilled water. Adjust pH to 0.9 with 1 M HCL and add 0.3ml Tween 20 (SIGMA product code P-1379) Make up to 0.6L with distilled water. This is a 10x concentrate which should be diluted with distilled water as required (eg 15ml diluted with 1350mL of distilled water).

**in most applications, 10mM phosphate, 0.15M NaCl, pH 7.6 (PBS) can be used instead of 50mM Tris, 0.15M NaCl, pH 7.6 (TBS).*

Novocastra TM

Lyophilized Mouse Monoclonal Antibody

Beta-Catenin

Product Code:	NCL-B-CAT
Intended Use	FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
Specificity	Human beta-catenin
Clone	17C2
Ig Class	IgG2a
Antigen Used for Immunizations	Prokaryotic recombinant protein corresponding to a 160 amino acid region of the C-terminus of the beta-catenin molecule.
Hybridoma Partner	Mouse myeloma (p3-NS1-Ag4-1).
Preparation	Lyophilized tissue culture supernatant containing sodium azide. Reconstitute with the volume of sterile distilled water indicated on the vial label.
Effective on Frozen Tissue	Yes
Effective on Paraffin Wax Embedded Tissue	Yes.
Recommendations on Use	Immunohistochemistry: typical working dilution 1:100-200. High temperature antigen unmasking technique. 60 min primary antibody incubation at 250C. Standard ABC technique on paraffin sections.
Positive controls	IHC: Tonsil, squamous epithelium
Staining pattern	Membrane

The slides were reviewed on an Olympus multi-header microscope.

APPENDIX III

Fluorescent in situ Hybridization (FISH) Protocol

The procedure will take place as follows:

Formalin-fixed paraffin embedded blocks will be analysed for MYCN gene amplifications via FISH using specific MYCN probes.

Sections of paraffin embedded blocks:

- Denaturing buffer will be pre-warmed to 73°C for 30 mins.
- Slides immersed in denaturation buffer at 73°C for 5 mins.
- Slides placed in 70%, 85% and 100% ethanol respectively at room temperature for 1 min each.
- Slides warmed at 45 °C until ethanol evaporates

Probes:

- Denatured at 73°C for 5 mins and placed on ice for 2 mins
- Briefly centrifuged
- Warmed at 37°C for 16hrs
- Washed in solution to remove surplus/free probe material
- 10µl of probe mixture will be added to the slide (2µl probe + 8µl buffer)
- Slides will be cover slipped
- Incubated at 37°C for 16hrs
- Washed in solution to remove surplus/free probe material
- 10µl of DAPI (complementary dye) will be applied and the slide cover-slipped.

The slide will then be visualized under a fluorescent microscope using appropriate filter sets. A minimum of 50 distinct, non-overlapping nuclei will be assessed per case.

The fluorescence in situ hybridization (FISH) technique was performed on tissue slides. The Vysis LSI N-MYC SpectrumGreen/CEP2 SpectrumOrange probe, designed specifically to detect amplification of the MYCN gene on chromosome 2p24 using CEP2 as an internal control, was used.

Interpretation:

A normal nucleus will show two green signals (MYCN) and two orange signals (CEP2) while an abnormal case, positive for amplification of MYCN, will have 6 or more green signals.

APPENDIX IV

CHEMOTHERAPY OF BRAIN TUMOURS RX 9941

Commences after the postoperative CT or MR scan and myelogram and within 4 weeks after surgery.

Week 0: Day 1: Carboplatinum 500mg/m² IV

Vincristine 1.5mg/m²

Etoposide 100mg/m² IV

Day 2-5 Etoposide 200mg/m² po daily

Week 1-6 Vincristine 1.5mg/m² IV Weekly

(Dosage for infants: Carboplatinum 16.5mg/kg, Etoposide 3.3mg/kg, Vincristine 1.5mg/kg)

- a) Patients under 3 years of age: repeat Carboplatinum + Etoposide (with dosage modification) every 3 weeks unless delayed by cytopenia) for a total of 8 courses.
- b) Patients over 3 years of age: repeat above (with appropriate dose modification) at week 3, unless delayed by cytopenia.

Radiotherapy is given after course 2 of chemotherapy. On completion, chemotherapy is recommenced immediately (if blood counts permits) to a total of 8 courses. Given according to standard procedure.

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