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Report

Wnt/Wingless Pathway Activation and Chromosome 6 Loss Characterize a Distinct Molecular Sub-Group of Medulloblastomas Associated with a Favorable Prognosis

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KEY WORDS

medulloblastoma, β-catenin, Wnt/Wg, chromosome 6, prognosis

ABBREVIATIONS

FISH	fluorescence in situ hybridisation
IHC	immunohistochemistry
PCR	polymerase chain reaction
SHH	sonic hedgehog
UCSF	University of California San Francisco
Wnt/Wg	Wnt/Wingless

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ABSTRACT

The accurate assessment of disease risk remains a major goal in children with medulloblastoma. Activation of the canonical Wnt/Wingless (Wnt/Wg) signalling pathway occurs in up to 25% of cases and is associated with a favorable disease outcome. To explore the molecular pathogenesis of Wnt/Wg-active medulloblastomas, and to investigate any genetic basis for their observed clinical behavior, we assessed a series of primary medulloblastomas for evidence of Wnt/Wg pathway activation, alongside a genome-wide analysis of associated copy-number aberrations. Cases displaying evidence of Wnt/Wg activation (*CTNNB1* mutation and/or β-catenin nuclear stabilisation) were exclusively associated with a distinct genomic signature involving loss of an entire copy of chromosome 6 but few other aberrations ($p < 0.001$). In contrast, Wnt/Wg-negative tumors coclustered into an unrelated sub-group characterised by multiple established genomic defects common in medulloblastoma (losses of chromosomes 17p, 8, 10 and 16; gains of chromosomes 7 and 17q). Further investigation of specific genetic defects in a larger independent cohort demonstrated that loss of chromosome 6 was exclusively observed in Wnt/Wg-active tumors, but not in Wnt/Wg-negative cases (8/13 vs. 0/19; $p = 0.0001$), while pathway activation was independent of chromosome 17 aberrations, the most common chromosomal alterations detected in medulloblastoma ($p = 0.005$). Wnt/Wg-active tumors could not be distinguished on the basis of clinical or pathological disease features. Our data indicate that Wnt/Wg-active tumors represent an independent molecular sub-group of medulloblastomas characterised by a distinct pattern of genomic aberrations. These findings provide a strong biological basis to support (1) the idiosyncratic clinical behavior of Wnt/Wg-active medulloblastomas, and (2) the development of β-catenin status as an independent marker for therapeutic stratification in this disease.

INTRODUCTION

The accurate assessment of disease risk remains a major goal in the clinical management of patients with medulloblastoma, the most common malignant brain tumor of childhood. Despite recent advances in overall survival rates (to ~70% at five years), variability in outcome exists within current therapeutic risk groups defined by clinical and histopathological indices, and contemporary adjuvant therapies are associated with significant adverse effects.[1] The development of a robust schema for the accurate therapeutic stratification of medulloblastoma patients (i.e., intensive therapy for high-risk patients and reduced side-effects for low-risk cases), and the identification of biological markers to facilitate this, are therefore imperative.

Activation of the canonical Wnt/Wg signalling pathway is a feature of up to 25% of medulloblastomas. Pathway activation is mediated through the stabilisation and nuclear accumulation of β-catenin, a transcriptional activator, and is associated with activating mutations in its corresponding *CTNNB1* gene in the majority (~60%) of instances.[2-4] In a recent study of 109 patients enrolled in the SIOP PNET3 clinical trial, we showed that Wnt/Wg pathway activation is an independent marker of favorable outcome in medulloblastoma. Five-year overall survival rates were significantly higher for β-catenin nucleopositive than nucleonegative medulloblastomas (92.3% vs. 65.3%), and all children with β-catenin nucleopositive medulloblastomas displaying clinical or histopathological adverse-risk features (metastatic disease or large cell/anaplastic morphology) were alive at least five years post-diagnosis.[2]

To explore the molecular pathogenesis of Wnt/Wg-active medulloblastomas, and to investigate any genetic basis for their observed favorable prognosis, we assessed a series of

primary medulloblastomas for evidence of Wnt/Wg pathway activation, alongside a genome-wide analysis of associated genomic copy-number aberrations.

METHODS

Two independent cohorts of primary medulloblastomas were assessed, comprising 19 and 32 cases, respectively. Clinical details (age at diagnosis, sex, histopathological sub-type) are summarized in Figures 1 and 3. DNA was extracted using standard methods. Markers of Wnt/Wg pathway activation (β-catenin nuclear stabilisation; *CTNNB1* and *APC* mutation status) were assessed as previously described.[2]

Genomic aberrations were assessed by ar-CGH using arrays of 2464 genomic clones (BAC or P1), spaced across the human genome at an average interval of 1.2Mb, and printed in triplicate (HumArray2.0). ar-CGH analysis, imaging and data processing were carried out by the Microarray Core at the UCSF Comprehensive Cancer Center, as described previously.[5,6] Circular binary segmentation[7] was performed on the ar-CGH data by the Biostatistics Core at UCSF Comprehensive Cancer Center, as described previously, and mergeLevels[8] applied to translate experimental intensity measurements into regions of equal copy number, and to identify regions of copy number aberration. MAD values for all samples were <0.2, and minimum and maximum criteria used in mergeLevels were 0.05 and 2, respectively. Clones falling within regions of either copy number gain (green) or loss (red) were selected for unsupervised hierarchical clustering. Cluster analysis was performed using the Spotfire Decision Site 8.2.1 software (Somerville, MA) as described previously.[9]

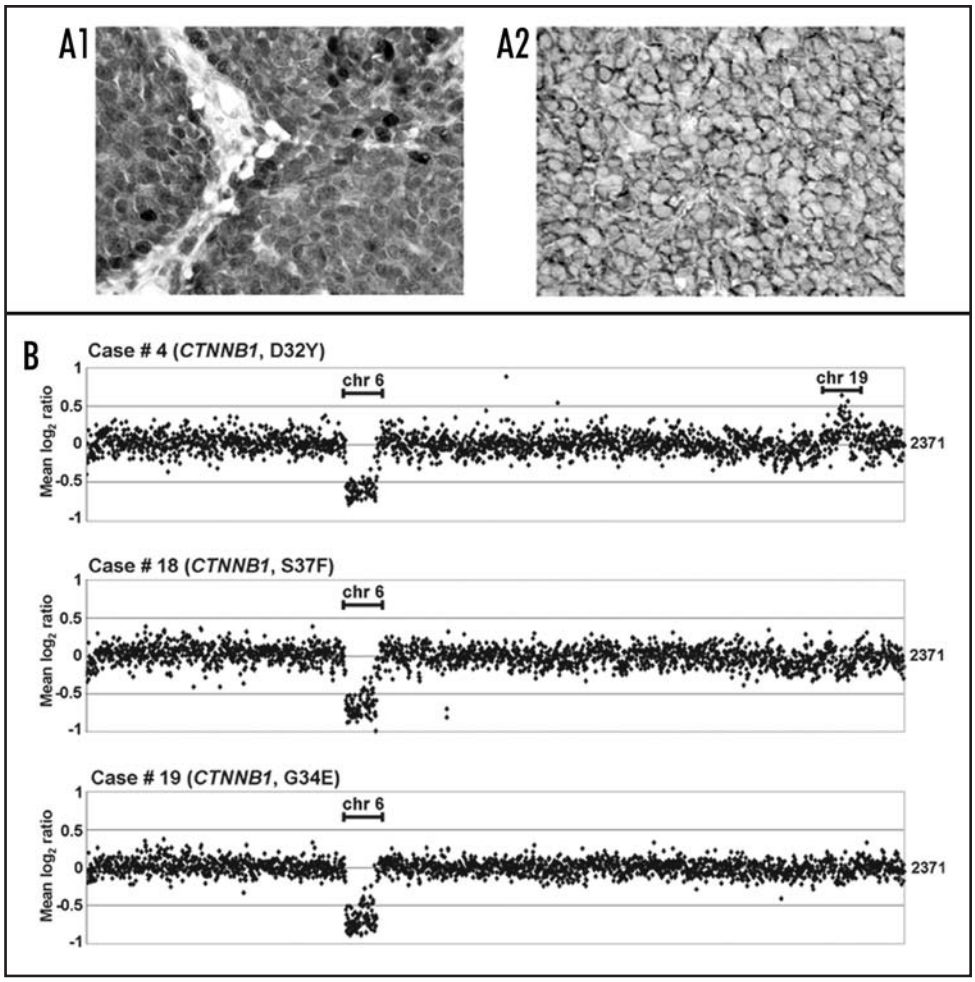

Figure 1. Assessment of Wnt/Wg pathway activation and associated genomic aberrations medulloblastomas. (A) β-catenin IHC, showing examples of strong combined cytoplasmic and nuclear reactivity (A1) and cytoplasmic, nonnuclear staining (A2), in two classic medulloblastomas. (B) ar-CGH data for three medulloblastoma samples which harbour *CTNNB1* mutations. Results are shown in genome order for 2371 probes, representing the human autosomes. Note clear evidence of loss of an entire copy of chromosome 6 in all 3 cases, with few additional chromosomal abnormalities.

Chromosome 6 status was determined by PCR and fragment analysis-based assessment of nine polymorphic microsatellite markers spanning the entire chromosome, using methods and criteria previously described by Langdon et al.[10] 17p status (encompassing 17p loss ocurring either in isolation or as a consequence of isochromosome(17q) was detected by fluorescence in situ hybridization (FISH) as described previously by Lamont et al.[11]

RESULTS

An initial cohort of 19 primary medulloblastomas was assessed for evidence of Wnt/Wg pathway activation (β-catenin nuclear stabilization; *CTNNB1* or *APC* mutation), alongside a genome-wide analysis of associated genomic copy-number aberrations by array-comparative genomic hybridisation (ar-CGH). Predicted activating mutations affecting the GSK-3β phosphorylation domain of *CTNNB1* were detected in 16% (3/19) of cases (D32Y (GAC>TAC), G34E (GGA>GAA) and S37F (TCT>TTT)), two of which were associated with strong combined cytoplasmic and nuclear immunoreactivity for β-catenin (Fig. 1; mutation data not shown). No further evidence of pathway activation was detected, with all remaining cases displaying cytoplasmic, nonnuclear β-catenin immunoreactivity.

ar-CGH analysis of this cohort revealed a spectrum of genomic aberrations consistent in number and nature with previous studies in medulloblastoma (reviewed in Ellison et al., 2003). The mean number of independent aberrations detected per sample was 5.5 ± 4.9 (± SD), with gains of chromosome 7 and 17q regions and losses of chromosme 8, 10p/q, 11p/q, 17p and 19q regions representing the most commonly affected regions (each in >20% of cases). A highly distinctive genomic profile was revealed for the three Wnt/Wg-active (i.e., *CTNNB1*-mutated) tumors; each case harbored a complete loss of one copy of chromosome 6, with few further detectable defects (no further alterations in two cases and two additional alterations in the remaining case) (Fig. 1).

To compare the genomic profiles of Wnt/Wg active medulloblastomas with our wider cohort, we performed an unsupervised hierarchical cluster analysis, which organised the full cohort into two major groups, A and B. A third cluster (C) comprised an individual sample with no detectable defects (Fig. 2). Tumors in clusters A (n = *5*) and B (n = *13*) were distinguished primarily by (1) the lower number of independent defects detected in group A cases

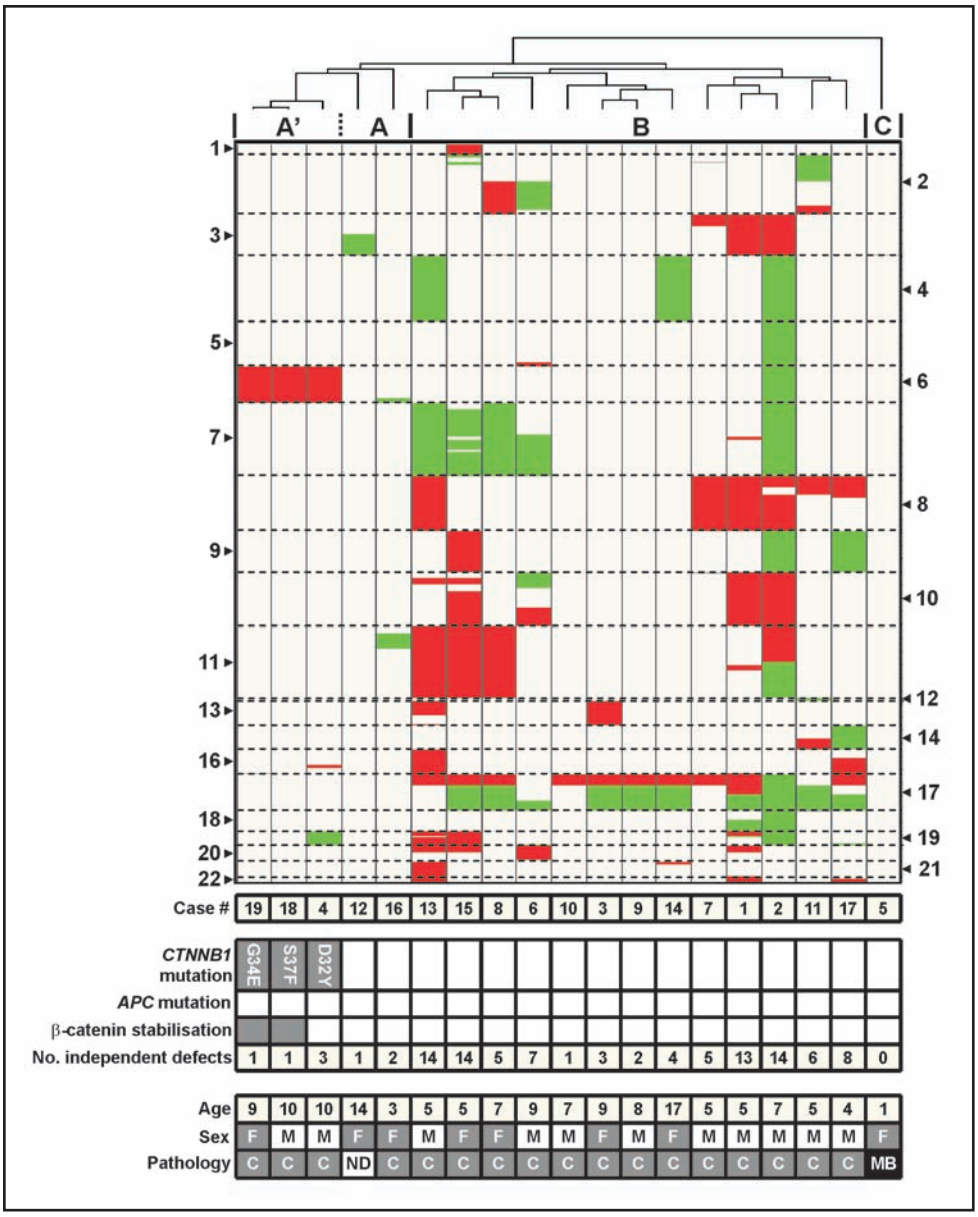

Figure 2. Cluster analysis of genomic aberrations detected in medulloblastomas, and their relationship to Wnt/Wg pathway activation status. ar-CGH clones falling within regions of either copy number gain (green) or loss (red) were selected for unsupervised hierarchical clustering as shown. Data are shown by chromosome, with major coclusters indicated (A, A', B and C). Wnt/Wg pathway status and the total number of independent regions of copy number aberration detected for each sample are also shown, alongside clinical descriptors (age in years; sex, male (M), female (F); histopathological sub-type, classic (C), nodular/desmoplastic (ND), medulloblastoma with extensive nodularity (MB).

[1.60 (±0.89) vs. 6.86 (±5.02); p = *0.02*, Mann-Whitney test], and (2) their distinct patterns of genomic aberrations. Wnt/Wg activation characterized the major specific sub-cluster (A'; n = *3*) within group A; alongside the significantly lower number of independent genomic defects detected in each of these cases, cluster A' cases were exclusively and significantly associated with *CTNNB1* mutation (p = *0.001*), β-catenin nuclear immunopositivity (p = *0.018*) and chromosome 6 loss (p = *0.001*; all Fisher's exact test), with none of these features observed in the remainder of the cohort. In contrast, cluster B represented the majority of tumors tested and was characterised by multiple genomic aberrations, including characteristic medulloblastoma genomic defects (see above). Notably, none of these defects was detected in the Wnt/Wg (A') cluster. Chomosome 17 defects, the most common genomic defects observed in medulloblastoma, were exclusively observed in cluster B cases, but occurred independently of the Wnt/Wg (A') cluster (p = *0.021*, Fisher's exact test). The remaining characteristic medulloblastoma defects (gain of chromosome 7, regional losses of chromosomes 8, 10 and 11) showed a trend towards exclusion from the Wnt/Wg cluster, although results did not reach statistical significance in this limited cohort.

To expand and validate these findings, we next assessed the relationship between Wnt/Wg pathway status and specific chromosmal aberrations in a second larger independent group, derived from our previous study of Wnt/Wg activation in medulloblastomas from the PNET3 cohort.[2,11] Briefly, this cohort comprised 13 Wnt/Wg-active tumors as determined by β-catenin nuclear immunoreactivity (of which 9 (69%) harbored *CTNNB1* mutations) and 19 Wnt/Wg-negative control cases. These cases were assessed for specific chromosomal defects (loss of chromosome 6 and 17p; Fig. 3), as their prior fixation in formalin precluded informative genome-wide ar-CGH analysis. In this wider cohort, loss of entire chromosome 6 was exclusively and significantly associated with Wnt/Wg-activation (p = 0.0001, Fisher's exact test). Chromosome 6 loss was a feature of 62% (8/13) of β-catenin nucleopositive cases, including cases with (6/9) and without (2/4) *CTNNB1* mutation. Again, Wnt/Wg activation occurred independently of chromosome 17p loss, which was only observed in Wnt/Wg-negative control cases (7/13 informative cases; p = 0.005, Fisher's exact test).

DISCUSSION

Our data indicate strongly that Wnt/Wg pathway activation defines a distinct molecular sub-group of medulloblastomas, which harbor a characteristic genomic signature involving chromosome 6 loss and few other detectable defects, and which appear to be independent of tumors containing common characteristic medulloblastoma defects, such as chromosome 17 aberrations. Notably, Wnt/Wg cases could not be distinguished within either cohort on the basis of basic clinical or histopathological information (age at diagnosis, sex, histopathological sub-type; all p > 0.05), futher supporting the utility of Wnt/Wg activation as an independent biological marker for medulloblastoma. However, the relevance of Wnt/Wg activation in nodular/desmoplastic medulloblastomas and medulloblastomas from children aged under three

years (ineligible for the PNET3 trial) remains undetermined. Our findings are corroborated by the recent study of Thompson et al.,[4] which reported characteristic gene expression patterns and genomic aberrations, including chromosome 6 loss, in five medulloblastomas harboring *CTNNB1* mutations. The unique molecular pathogenesis of Wnt/Wg medulloblastomas provides a strong biological basis to support (1) their idiosyncratic clinical behavior, and (2) the development of Wnt/Wg sub-group markers as independent indices for therapeutic stratification in this disease.

Whilst our data strongly support a unique genomic signature for Wnt/Wg medulloblastomas, more detailed analyses are now required to elucidate the molecular mechanisms underlying their pathogenesis and favorable response to therapy. In particular, further investigations involving larger cohorts and enhanced resolution mapping arrays are essential in the short-term, both to verify our findings and to identify further characteristic genomic events. An understanding of the contribution of chromosome 6 loss to Wnt/Wg tumor development may offer particular insights. Outstanding issues include (1) whether whole chromosome 6 loss is necessary, potentially involving a chromosome-wide reduction in "gene-dosage", or whether specific critical genes are targeted for inactivation by smaller genetic or epigenetic events not revealed at the ~1.4Mb mapping array resolution used in the present study, and (2) whether Wnt/Wg pathway activation and inactivation of genes on chromosome 6 cooperate directly in promoting medulloblastoma development. Moreover, the interplay between Wnt/Wg activation and additional genetic events could be complex; for instance, evidence of *MYC* amplification, a marker of poor prognosis in medulloblastoma, has been reported in a Wnt/Wg tumor.[2,11] Finally, the distinct genomic signature of Wnt/Wg tumors may reflect a unique ontogeny. While strong evidence exists to support the development of a subset of medulloblastomas (~25%), which display activation of the SHH signalling pathway, from the external granule layer of the cerebellum,[12] the developmental origins of other tumors are less clear. Gene expression data suggest that Wnt/Wg medulloblastomas and medulloblastomas displaying SHH activation are mutually exclusive,[4] and studies to investigate the cerebellar precursor cells from which they originate should be highly informative.

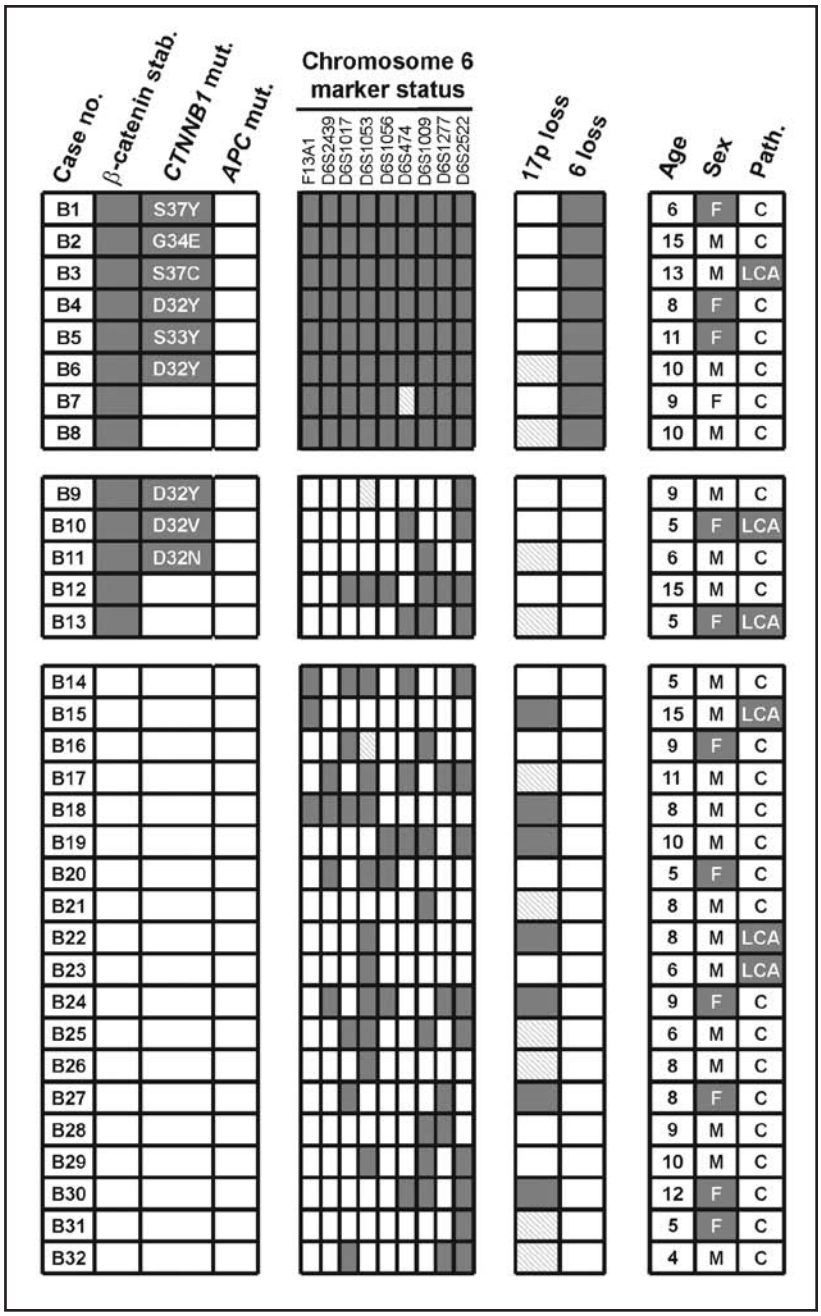

Figure 3. Wnt/Wg pathway activation, chromosome 6 loss and chromosome 17p status in an independent medulloblastoma cohort. Wnt/Wg pathway status and clinical features (age in years; sex, male (M), female (F); histopathological sub-type, classic (C), large cell/anaplastic (LCA)) are shown for 32 medulloblastomas. Polymorphic markers on chromosome 6 were classified as either homozygous (dark grey boxes) or heterozygous (white boxes). Using this approach, regions of contiguous homozygosity encompassing ≥ 5 markers are significantly associated with loss of heterozygosity (i.e., allelic loss). Noninformative markers are represented by cross-hatched boxes. A summary of chromosome 6 status determined for each sample is shown (dark grey boxes, allelic loss of entire chromosome 6; white boxes, no evidence of chromosome 6 loss). 17p status was determined for 22 cases (dark grey box, 17p loss; white box, no 17p loss; cross-hatched box, data not available).

In view of the favorable outcome observed for Wnt/Wg medulloblastomas,[2] Wnt/Wg status represents an attractive marker for the molecular stratification of favorable-risk medulloblastoma patients. Three alternative markers, which exclusively identify Wnt/Wg tumors, have now been identified; (1) β-catenin nuclear immunoreactivity, (2) *CTNNB1* mutation, and (3) chromosome 6 loss. All of these features were amenable to assessment using standard technologies (IHC, PCR and iFISH) in formalin-fixed material, in the present study. β-catenin nuclear stabilisation defined 15/16 of the Wnt/Wg sub-group cases reported here (Figs. 2 and 3) and, of these, the Wnt/Wg sub-type was further corroborated in 13/15 cases by the presence of either a *CTNNB1* mutation (11/15 cases) or chromosome 6 loss (10/15 cases). Thus, the application of β-catenin IHC as a primary marker, in conjunction with confirmatory *CTNNB1* mutation detection (PCR/sequencing) and assessment of chromosome 6 loss (by PCR or FISH-based methods), appears to offer a robust approach for the positive discrimination of Wnt/Wg sub-group medulloblastomas in diagnostic biopsies.

Molecular (17p loss, amplification of the *MYC* or *MYCN* oncogenes), clinical (metastatic and post-operative residual disease) and histopathological (large cell/anaplastic morphology) indices that predict a poor prognosis, have been identified in studies conducted in medulloblastoma clinical trials cohorts.[11,13] Stratification schemes involving the combined use of multiple indices may thus offer attractive systems for the robust positive discrimination of poor, average and favorable risk cases.[11] The interplay between Wnt/Wg activation and other biological, clinical and histopathological markers of prognosis in medulloblastoma should now therefore be considered in prospective group-wide biological studies involving uniformly treated cohorts, with a view to (1) validating present findings, (2) defining optimal stratification systems, and (3) assessing the feasibility of biological testing in multi-centre studies, including appropriate quality control procedures, prior to assimilation into routine clinical practice.

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