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MYB upregulation and genetic aberrations in a subset of pediatric low-grade gliomas

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Abstract

Recent studies of genetic abnormalities in pediatric low-grade gliomas (LGGs) have focused on activation of the ERK/MAPK pathway by *KIAA1549-BRAF* gene fusions in the majority of pilocytic astrocytomas (PAs) and by rare mutations in elements of the pathway across histopathologically diverse LGGs. This study reports that *MYB*, an oncogene not previously implicated in gliomagenesis, is activated in a diverse subset of pediatric LGGs. The study cohort comprised 57 pediatric LGGs and a comparative cohort of 59 pediatric high-grade gliomas (HGGs). The LGG cohort included 34 PAs and 23 diffuse gliomas; fibrillary astrocytomas (n=14), oligodendroglial tumors (n=7), and angiocentric gliomas (n=2). *MYB* copy number abnormalities were disclosed using Affymetrix 6.0 SNP arrays and confirmed using interphase fluorescence *in situ* hybridization. Novel *MYB* amplifications that upregulate *MYB* RNA and protein expression were demonstrated in 2/14 diffuse astrocytomas. In addition, focal deletion of the terminal region of *MYB* was seen in 1 of 2 angiocentric gliomas (AGs). Increased expression of *MYB* was demonstrated by quantitative RT-PCR and immunohistochemistry. *MYB* upregulation at the protein level was demonstrated in a proportion of diffuse LGGs (60%), pilocytic astrocytomas (41%), and HGGs (19%), but abnormalities at the genomic level were only a feature of diffuse gliomas. Our data suggest that *MYB* may have a role in a subset of pediatric gliomas, through a variety of mechanisms in addition to *MYB* amplification and deletion.

Keywords

MYB; glioma; pediatric; amplification

Introduction

Central nervous system (CNS) tumors constitute approximately 25% of neoplastic disease in childhood [17,39]. They are the major cause of cancer-related death in children and young adults [16]. Primary CNS neoplasms in adults and children are classified into four grades by histopathological features and broadly subdivided into low-grade (WHO grades I-II) and high-grade (WHO grades III-IV) tumors [30]. High-grade gliomas (HGG) predominate in adults, and low-grade gliomas (LGGs) form the majority of tumors in children. Excluding (low-grade) ependymomas, which are regarded as a distinct disease, pediatric LGGs are a heterogeneous group, comprising tumors thought to be derived from astrocytic or oligodendroglial lineages. They include pilocytic astrocytoma (PA, WHO grade I) and a range of WHO grade II tumors: diffuse astrocytoma (DA), oligodendroglioma (O), oligoastrocytoma (OA) and rarer entities, such as pilomyxoid astrocytoma, pleomorphic xanthoastrocytoma (PXA), and the recently described angiocentric glioma (AG) [30].

Adult and pediatric gliomas share similar histological appearances, but display different genetic and biological characteristics. In adults, mutations affecting the two isocitrate dehydrogenase (IDH) enzymes characterize the majority of WHO grade II-III astrocytomas [2,38,59]. *IDH1* mutations have been reported in pediatric DAs, but at a much lower frequency than in adults [11,60]. *TP53* mutations are also common in adult diffuse (fibrillary) astrocytomas (WHO grade II), and the majority of these tumors will undergo

further transformation to anaplastic astrocytoma or glioblastoma [37]. In contrast, *TP53* mutations are rarely seen in pediatric DAs, and less than 10% of these tumors undergo progression to a HGG [7,35,43]. Deletions of chromosome arms 1p and 19q are seen in approximately 80% of adult oligodendrogliomas and are associated with a favorable outcome [23,49,53]. Oligodendrogliomas in childhood are rare; few contain 1p/19q loss, and no survival advantages are associated with 1p/19q deletions in this age group [15,25,42].

Pediatric LGGs are generally regarded as a single group for therapeutic purposes, despite their histopathological heterogeneity. For many types, such as PA, surgical resection is the treatment of choice, and completeness of excision is the main determinant of survival [46]. However, complete resection is often not possible when tumors are situated deep within midline structures at the base of the brain or brain stem, or when tumors are diffusely infiltrative. In these situations, adjuvant chemotherapy and radiotherapy are effective [14], but overall survival beyond fifteen years for unresectable tumors is poor. Pediatric LGGs may cause significant long-term morbidity, despite their indolent clinical course [45]. Such challenges can only be met through an improved understanding of the biology and genetics of pediatric LGG. Increased understanding of the disease will facilitate more effective treatment strategies, particularly those using targeted small molecule drugs.

In this study, a genome-wide analysis of copy number abnormalities (CNAs) was undertaken in pediatric LGGs (WHO grades I and II). Novel CNAs at 6q23 involving gain of *MYB* were revealed in two DAs and subsequently confirmed as amplifications by interphase fluorescence *in situ* hybridization (iFISH). *MYB* was overexpressed, and nuclear accumulation of MYB protein was demonstrated by immunohistochemistry in both cases. Focal deletion of the 3' region of *MYB* was also found in 1 of 2 angiocentric gliomas (AGs). Both AGs displayed increased *MYB* expression and nuclear immunoreactivity for the protein. *MYB* is an oncogene, which has not previously been implicated in gliomagenesis. Our data are presented in the context of examining *MYB* status and defining other genetic abnormalities across a large series of pediatric LGGs and in a comparative cohort of pediatric HGGs.

Materials and methods

Tumor cohort

LGGs were surgical specimens from 57 patients (Tables 1a and 1b) and included 14 diffuse (fibrillary) astrocytomas (DAs), 5 oligoastrocytomas (OAs), 2 oligodendrogliomas (Os) and 2 angiocentric gliomas (AGs), all from cerebral cortex, 25 cerebellar PAs, and 9 supratentorial PAs from the cerebral cortex (n=6) and thalamus (n=3). Age at diagnosis ranged from 1-19 years, with a median age of 6 years. There were 29 males and 28 females within the cohort. Tissue samples were snap-frozen at the time of surgical resection and were obtained prior to adjuvant therapy. Matching formalin-fixed, paraffin-embedded (FFPE) samples of these tumors were used for immunohistochemical and molecular cytogenetic studies. A series of archived FFPE pediatric HGGs (n=59) represented a comparative series for immunohistochemical and cytogenetic studies and consisted of 42 glioblastomas (GBs), 11 anaplastic astrocytomas, 3 anaplastic OAs, and 3 (high-grade) astroblastomas. None appeared to have progressed from a low-grade astrocytoma. All patients were negative for NF1, and optic pathway gliomas were not included. A detailed histopathological review of all tumors was undertaken (DWE). DNA and RNA were extracted from frozen tissue using previously described methods [13].

The principles of the study and access to tumors and clinical data were in accordance with Institutional Review Board and MREC regulations: XPD07-107/IRB and Tissue Resource

Request No. 07-007 (St Jude Children's Research Hospital); REC No. 2002/112 (University of Newcastle) and ICMS/PR/09/77 (University of London).

Affymetrix 6.0 SNP array analysis

Tumor DNA from 54 LGGs was analyzed for CNAs using the Affymetrix Genome-Wide Human 6.0 SNP array according to standard Affymetrix protocols. Processed data were normalized and subjected to circular binary segmentation using previously described methods [13]. Genomic Identification of Significant Targets in Cancer (GISTIC) was used to identify significant CNAs [4]. Data were visualized using the Integrative Genomics Viewer (IGV; 1.4.2), <http://www.broadinstitute.org/igv>.

Dual-color interphase fluorescence *in situ* hybridization (iFISH)

Dual-color iFISH was performed on 5µm FFPE tissue array sections using standard methods [13]. Probes were derived from BAC clones (BACPAC Resources, Oakland, CA), labeled with either AlexaFluor-488 or Rhodamine fluorochromes and validated on normal control metaphase spreads to confirm chromosomal location. BAC clone RP11-104D9 was used to target *MYB* at 6q23.3 in combination with a control probe to 6p (RP11-945O22 and RP11-186N7). Clones RP11-702H8 and RP11-1025G11 were used to probe the co-amplified regions of gain at 6q23.3 in DA1 and DA2, respectively. Clones RP11-366H19 and RP11-170P19 were used to probe the regions of possible copy number loss in DA1 and DA2. RP1-233K16, RP1-120G22 and RP1-202O8 were used to probe 1p36.3, in combination with control probes RP11-54H19 and RP11-336K24 to 1q22. Clones RP11-927F22 and RP11-105H7 were used to probe 19q13.32, in combination with control probes CTD-2538G9 and CTD-2528A14 to 19p13.11.

Immunohistochemistry

Immunohistochemistry was undertaken on FFPE material using a monoclonal antibody to the N-terminal region of *MYB* (Abcam EP769Y; Cambridge, MA). Staining was performed on the BondMax automated staining system using standard methodology (Leica Microsystems, Bannockburn, IL). Slides were deparaffinized, pretreated with ER2 (EDTA pH8) and incubated with antibody (1:50) dilution for 15 minutes at room temperature. Detection was performed using the Refine Polymer from Leica Microsystems and slides were counterstained with hematoxylin.

DNA qPCR

The human genomic *MYB* gene sequence was retrieved from Ensembl (Transcript ID: ENST00000341911) and used as a template for designing all real-time PCR oligonucleotide primers (Table 2). Individual real-time PCR reactions (20µl) contained 1 × SYBR Green I master buffer, different concentrations of sense and antisense primers (Table 2) and 50ng of genomic DNA. Real-time PCR was performed in a LightCycler480® (Roche), using the default thermocycler program for all genes: 5 minutes of pre-incubation at 95°C followed by 45 cycles for 10 seconds at 95°C, 15 seconds at 60°C and 30 seconds at 72°C. At the end of each reaction, melting curves were acquired and analyzed. For real time PCR data analysis, an advanced relative quantification method was adapted to estimate copy numbers in *MYB* using Lightcycler 480® software (Roche). Human genomic DNA extracted from blood (Roche) contained 2 normal copies of *MYB* and was used as the copy number calibrator. Two genes (*GPR15* and *ZNF80*) contained constant copy number in all LGG samples and were used as reference genes to normalize the quantitative data. Results for each tumor sample were expressed as the N-fold copy number of *MYB* relative to the calibrator. Assays were repeated if amplification curves did not reflect exponential kinetic parameters or if the N-fold copy number was lower than 0.7 or higher than 1.3. When N-fold was between these

values ($0.7 < N\text{-fold} < 1.3$), tumor samples were judged to contain 2 normal copies of the target gene.

Quantitative reverse-transcription PCR (RT-PCR)

First strand cDNA was synthesized using Superscript II reverse transcriptase and random hexamers according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Primers were designed from published *MYB* cDNA sequences using Primer 6 software (Table 3). RT-PCR amplification mixtures (25 μ l) contained 25ng template cDNA, 1 $\times$ SYBR Green I Master mix buffer (Roche, Indianapolis, IN) and 300 nM forward and reverse primers. Reactions were run on a Lightcycler 480® Real-Time PCR machine (Roche, Indianapolis, IN). The cycling conditions comprised 10 minutes at 95°C and 45 cycles at 95°C for 10 seconds, 60°C for 15 seconds and 72°C for 20 seconds. *MYB* gene expression was analyzed by an Advanced Relative Quantification method using Lightcycler 480® software (Roche, Indianapolis, IN). In brief, target quantities were normalized to *GAPDH* and *B2M* (references) and calibrated using qPCR Human Reference cDNA (Clontech, Mountain View, CA) prepared from a mixture of total RNAs collected from adult normal human tissues. All quantities were expressed as N-fold related to the calibrator.

5' and 3' rapid amplification of cDNA ends (RACE)

5' and 3' RACE was performed using the SMARTer RACE cDNA amplification kit (Clontech, Mountain View, CA), following the manufacturer's instructions. Reactions were run on a Lightcycler 480® Real-Time PCR machine (Roche, Indianapolis, IN).

Gene sequence analysis

The mutational hotspots of *BRAF* (exon 15) and *KRAS* (exons 2 and 3) were sequenced using previously published primers [13]. The mutational hotspots for *IDH1* (exon 4) and *IDH2* (exons 4-5) were also sequenced using the following primers; *IDH1* exon 4 5'-GAGCTCTATATGCCATCACTGC-3' (sense) and 5'-CAAGTTGGAAATTTCTGGGC-3' (antisense), *IDH2* exons 4-5 5'-ATTCTGGTTGAAAGATGGCG-3' (sense) and 5'-CAGAAGAAAGGAAAGCCACG-3' (antisense). Direct sequencing of PCR products was performed using BigDye version 3.1 chemistry and a 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). Results were screened using MacVector version 8.3 sequence analysis software (Cary, NC).

Statistical Analysis

Comparisons of *MYB* immunoreactivity between different tumor groups were performed using Pearson's χ^2 test. The Wilcoxon rank sum test was used to compare the cumulative genomic burden between *MYB*-amplified and non-amplified samples.

Results

MYB copy number abnormalities in LGGs

Analysis of 54 LGGs using the Affymetrix 6.0 SNP array revealed discrete copy number gains at chromosome band 6q23 in 2 (14%) of 14 DAs (Fig. 1a, Table 1a). DA1 showed a 90Kb region of copy number gain at 6q23. This included *MYB* exons 1-8 and terminated within *MYB* exon 9 (Fig. 1b). DA2 contained a 43.6Kb region of gain encompassing the majority of the *MYB* locus, ending within the 3' untranslated region (UTR) of *MYB* exon 16. Distinct regions centromeric or telomeric from *MYB* were also co-amplified in each case. DA1 showed a large 370Kb region of copy number gain at 6q23 centromeric to *MYB*, including the non-coding gene *AJ606331*. The two gained regions in DA1 were separated by approximately 309Kb of possible copy number loss. DA2 showed a 136Kb region of gain

telomeric to *MYB*, including the first exon of *MAP7* and the terminal exon of *MAP3K5*. In this tumor, the two amplified regions were separated by approximately 1200Kb of apparent copy number loss. Regions of copy number gain within *MYB* were subsequently confirmed by DNA qPCR. DNA copy number levels, expressed as a normalized ratio, were elevated at *MYB* exon 8 in DA1 and DA2 (equal to 9 and 8, respectively). The DNA copy number ratio at *MYB* exon 16 was also elevated (equal to 7) in DA2, and was normal in DA1, matching the copy number aberrations identified by SNP array analysis. Sixteen representative LGGs were investigated in this way, and no other samples showed DNA copy number gains within *MYB*.

Dual-color interphase FISH (iFISH) confirmed amplification of the *MYB* locus at 6q23 in both DAs with copy number gains. Multiple copies of the *MYB* signal were seen in the form of double minutes in more than 80% of tumor cell nuclei, in the presence of two homologues of chromosome 6p (Fig. 2a-b). Fifty-two further LGGs were probed in this way, but did not demonstrate *MYB* amplification (Tables 1a and 1b). Three LGGs (DA13, OA3, PA12) showed polysomy 6, with 3-8 copies per cell.

Further iFISH was performed to investigate the co-amplified regions that included *AJ606331* or *MAP7/MAP3K5* in DA1 and DA2, respectively. Multiple copies of these co-amplified regions were found as double minutes, and these were confirmed to co-localize with *MYB* (Fig. 2c-f). Regions of apparent copy number loss identified by array analysis were also probed using iFISH. Hemizygous deletion was confirmed between the amplified region of *MYB* and *MAP7/MAP3K5* in DA2; however, no deletion was found in the region between *AJ606331* and *MYB* in DA1, where two copies of the target probe were present (data not shown).

Affymetrix SNP array analysis also revealed a discrete region of DNA copy number loss at 6q23 in one angiocentric glioma (AG1) (Figs. 1a, b). AG1 showed a region of loss of approximately 59Kb, including *MYB* exon 16. The coding exons for the 5' promoter and central transactivation region remained intact in this case. This region of copy number loss was subsequently confirmed by DNA qPCR. A normalized ratio of 0.5 was shown at *MYB* exon 16, with normal DNA copy number at *MYB* exon 8, matching the copy number change shown by SNP array analysis. No other LGG showed DNA copy number loss within *MYB* by DNA qPCR.

MYB expression by RT-PCR and immunohistochemistry in LGGs

RT-PCR was undertaken to measure *MYB* expression levels in all 57 LGGs, using primers situated within *MYB* exons 8-9. The central transactivating domain in *MYB* is encoded by exons 6-8 (amino acids 223-313). Increased *MYB* expression was seen in 8 LGGs. These comprised 5 DAs, 2 AGs, and 1 oligodendroglioma, and included all cases with *MYB* copy number aberrations (Table 1a). No PAs showed increased expression of *MYB* by RT-PCR.

MYB immunoreactivity was investigated in 49/57 LGGs (Tables 1a, 1b). Two patterns were demonstrated; strong nuclear immunoreactivity was a feature of nearly all tumor cells in 6 LGGs, all diffuse gliomas; 4 of 11 DAs, including one *MYB*-amplified case (DA1), 1 of 5 oligoastrocytomas, and 1 of 2 angiocentric gliomas (Fig. 3). Weak to moderately strong immunoreactivity was found in scattered tumor cells in 18 LGGs. These comprised 4 of 11 DAs, including the second *MYB*-amplified case (DA2), 1 of 2 oligodendrogliomas, 12 of 29 PAs, and the second angiocentric glioma. No immunoreactivity was found in the remaining 25 LGGs. The frequency of *MYB* immunoreactivity was higher among DAs (8/11; 73%) when compared to PAs (12/29; 41%). Five diffuse gliomas with increased *MYB* RNA and protein expression showed no corresponding copy number changes within *MYB* by DNA

qPCR, array or FISH analysis (Table 1a). These data indicate that *MYB* upregulation may be modulated by further mechanisms, in addition to DNA CNAs.

Analysis of *MYB* abnormalities in high-grade gliomas

Pediatric HGGs (n=54) were evaluated by iFISH, and none demonstrated *MYB* amplification. Many tumors (n=21; 38%) had a balanced (2:2) profile, but a high proportion of assessable tumors (23/55; 42%) showed polysomy (3N or 4N) of chromosome 6. Relative loss of *MYB* against a background of hyperploidy was detected in 6 tumors, and monosomy of chromosome 6 was detected in 4 HGGs. The remaining tumor, a giant cell GB, contained between one and four extra copies of *MYB* in its cells.

MYB immunoreactivity was assessed in 59 HGGs. A minority (19%) contained scattered tumor cells with weak to moderately strong nuclear immunoreactivity (Fig. 3). None contained widespread strong immunoreactivity, as seen in some low-grade diffuse gliomas. Anaplastic astrocytomas (5/11) and GBs (5/42) were equally represented among immunopositive tumors, and there was one immunopositive astroblastoma. However, the frequency of *MYB* immunoreactivity among anaplastic astrocytomas (45%) was significantly higher than among GBs (12%; $P=0.011$).

No gene fusions involving *MYB*

Potential gene fusions were sought between the co-amplified genomic regions in DA1 and DA2 using several methods. DA1 had limited material available for study. PCR for rapid amplification of cDNA ends (RACE) was performed in 5' and 3' directions for both samples. No novel sequences were identified to suggest that gene fusions with *MYB* had occurred. The 5' region was intact in both DAs; however, the sequence from DA1 was found to terminate within *MYB* exon 9 as indicated by array analysis. Oligonucleotide primers were designed within the candidate fusion partner genes *MYB*, *MAP7* and *MAP3K5*, and PCR was then performed using cDNA from DA2. No PCR products could be amplified. Finally, genomic DNA from DA2 was investigated for the presence of DNA insertions flanking the 5' and 3' ends of *MYB* using standard methods [19]. No additional genetic material was detected.

No chromosomal instability in *MYB*-amplified DAs

Chromosomal instability was investigated as a possible cause for the genetic abnormalities in the two DAs with *MYB* amplification. The number of genetic lesions (sum of gains and losses) and the cumulative genetic burden (sum of cumulative gains and losses in Kb) were calculated. Segmentation data were filtered to exclude known germline copy number variants, using ≥ 8 markers within a segment. Data were compared between the two DAs with *MYB* amplification and 38 representative LGGs, to identify whether more genetic rearrangements were present in the two DAs, indicating chromosomal instability. No differences were found in the number of genetic lesions ($P=0.73$) or in the cumulative genetic burden ($P=0.43$), respectively.

Further genetic aberrations in LGGs

Twenty-one LGGs contained a discrete copy number gain of 1.9Mb at chromosome band 7q34 by Affymetrix 6.0 SNP array analysis; all were PAs (Table 1b). The cohort was screened for the presence of *KIAA1549-BRAF* fusions as previously described [13]. Twenty-seven of 34 PAs (79%) were subsequently confirmed to contain *KIAA1549-BRAF* fusions and five different *KIAA1549-BRAF* fusion variants were identified. Seven PAs did not contain *KIAA1549-BRAF* fusions. Of these, one cerebellar PA contained an *SRGAP3-RAF1* fusion. One oligodendroglioma (O2) and one oligoastrocytoma (OA5) contained

hemizygous deletions of chromosomes 1p and 19q by array analysis and iFISH. Fifty-five LGGs were probed in this way, and no other cases contained 1p/19q deletions.

Mutation screening

Fifty-seven pediatric LGGs were screened for activating mutations at known mutation hotspots in *BRAF* (exon 15), *KRAS* (exons 2 and 3) and for somatic mutations known to perturb enzymatic function in *IDH1* (exon 4) and *IDH2* (exons 4 and 5). Two samples were found to contain the *BRAF* p.V600E activating mutation. One was a cerebral DA which also showed homozygous *CDKN2A* loss by copy number analysis and iFISH. The other was a thalamic PA. This sample had not been screened by SNP array, but showed polysomy at the *CDKN2A* locus by iFISH. A *KRAS* p.G12A activating mutation was identified in one fusion-negative cerebellar PA. No mutations were identified within *KRAS* exon 3, *IDH1* exon 4 or *IDH2* exons 4-5 within the cohort.

Discussion

Pediatric LGGs are dominated by cerebellar PAs (WHO grade I), which are generally cured by surgery [1,46]. However, diffusely infiltrating gliomas (WHO grade II) and PAs that seed the neuraxis or occur at sites where resection is impossible cause significant morbidity and mortality and represent a considerable therapeutic challenge. This challenge will only be met by an increased understanding of the biology of these tumors. Recent recognition that pediatric PAs demonstrate activation of the ERK/MAPK pathway, many as a result of a *KIAA1549-BRAF* gene fusion, has helped to direct functional studies and drug discovery for this disease [13,20,21,52,55]. Grade II pediatric gliomas remain enigmatic entities. Extrapolation from data on adult grade II LGGs to childhood disease clearly has limitations; while the histological features of adult and pediatric LGGs show considerable overlap, their clinical and molecular characteristics do not. The rate of anaplastic progression in pediatric grade II LGGs is much lower than in their adult counterparts, and the frequency of specific molecular genetic abnormalities, such as *TP53* or *IDH1* mutations in diffuse astrocytomas [2,35,43,51] and 1p/19q loss in oligodendrogliomas [42,47], is much lower in pediatric tumors. The present study of 57 well characterized pediatric LGGs reports novel data on *MYB*, a gene not previously implicated in gliomagenesis. Amplifications of *MYB*, apparently driving upregulation of the gene product, occurred at low frequency in DAs, but not in PAs.

The oncogene *MYB* encodes a nuclear DNA-binding leucine zipper transcription factor, which can act as both a transcriptional activator and repressor [3,48]. *MYB* is the cellular homologue of *v-Myb*, which is found in the avian retroviruses E26 and AMV and induces leukemic transformation in birds [5,29]. *MYB* is one of a family of closely related transcriptional regulators that modulate cell maturation and proliferation and determine lineage fate. Others include *MYBL1* (*AMYB*) and *MYBL2* (*BMYP*). All three proteins contain a highly conserved DNA binding site (the *MYB* binding site, *MBS*), which binds to the sequence t/cAACT/gG [6]. Three key domains are found within *MYB*; the N-terminal DNA binding domain, where three *MBS*s are situated as tandem repeats, the centrally located transactivating domain, and the C-terminal negative regulatory domain [36]. *MYB* has a short half-life of approximately 30 minutes *in vivo*, and undergoes various post-translational modifications, including ubiquitylation, phosphorylation, acetylation and sumoylation, which modify levels of DNA-binding or transcriptional activation. In general, *MYB* induces gene activation, although repression has been documented in a few situations. Over 80 genes have been confirmed as *MYB* targets, including *MYB* itself, but these vary considerably between different cell types [28,48]. Most *MYB* studies have been conducted on the hematopoietic system, and little is known about the actions of *MYB* within the CNS. *MYB* expression has recently been shown to be negatively regulated by microRNAs,

including miR-15a, miR-16-1, and miR-150. These bind within the 3'UTR and inhibit mRNA translation of *MYB* [8,58,61].

MYB is critically important for hematopoiesis and is highly expressed within immature multi-potent stem cells. Levels are successively down-regulated as differentiation proceeds along myeloid and lymphoid lineages [3,56]. Complete loss of *Myb* is lethal in mouse embryos, which appear to develop normally until day 14, but then die as myeloid and erythroid development is severely disrupted [34]. Further murine studies have demonstrated functions for *cMyb* in colonic stem cells and in regions of postnatal neurogenesis [48,54]. Normal mice express high levels of *cMyb* mRNA at E15 within brain, colon and liver. Levels fall in brain and liver by P7, and are almost undetectable in adult mice [50]. Relative to cortex, high levels of *cMyb* mRNA and protein are found in the subventricular zone (SVZ) of adult mice [31], and *cMyb* immunoreactivity has been identified in the majority of SVZ cell types.

Various human neoplasms harbor genomic rearrangements and CNAs within *MYB*. Balanced reciprocal t(6;7)(q23;q34) translocations have been found in a few patients with T-ALL [9]. These create a derivative chromosome 6 with a novel juxtaposition involving *MYB* and the regulatory sequence for the T-cell receptor-beta gene. *MYB* duplications characterize disease in an additional group of T-ALL patients. Focal copy number gains were found to encompass the whole *MYB* locus, and tandem duplication was demonstrated using FISH [9,26]. *MYB* amplifications have also been identified in several solid tumors. High-level amplifications occurred in 29% of hereditary *BRCA1* breast cancers, which compares with 2% in sporadic breast cancers [24]. Furthermore, over-expression of *MYB* mRNA was seen by Northern blot analysis in 2 of 5 *BRCA1* tumors with no *MYB* amplification, suggesting the presence of an alternate mechanism of up-regulation. *MYB* DNA copy number gains have been demonstrated in approximately 10% of pancreatic carcinomas and FISH using the P2 pancreatic cancer cell line confirmed amplification of *MYB* as homogeneously staining regions [57]. Studies of CNS tumors using array-based comparative genomic hybridization (CGH) have identified *MYB* copy number gain in one supratentorial primitive neuroectodermal tumor, a finding validated by semi-quantitative multiplex PCR [32], and in 5 of 10 medulloblastomas, although these results were not confirmed by another method [22].

A novel t(6;9) translocation between *MYB* and the nuclear transcription factor *NFIB* has recently been identified in adenoid cystic carcinomas (ACCs) of the breast and salivary glands [33,40]. The terminal exon and 3'UTR of *MYB* are lost in the translocation; this region contains the binding sites for several microRNAs (miR-15a, miR-16-1, miR-150) that negatively regulate *MYB* transcriptional activity. Upregulation of *MYB* (RNA and protein) was seen in ACCs, and levels of *MYB* target genes were also elevated in comparison to normal salivary gland tissue. The initial report identified fusions in all examined ACCs, while a recent study of salivary gland tumors found fusions in 28% primary and 35% metastatic ACCs [33,40]. *MYB* overexpression appears to be a key alteration in ACC development; increased expression was seen in 85% fusion-positive ACCs. It was also found in 60% of fusion-negative ACCs, although the mechanism for this is currently unknown.

The present series of LGGs included 23 diffuse gliomas; DAs, oligodendrogliomas, oligoastrocytomas, and angiocentric gliomas, and 34 PAs from three anatomic sites. Three diffuse LGGs showed novel *MYB* abnormalities at the genomic level, but *MYB* CNAs were not demonstrated in PAs or in a series of pediatric HGGs. Our data demonstrated two possible genomic mechanisms for *MYB* upregulation; *MYB* amplification in two DAs and loss of the 3' region of *MYB* in a single angiocentric glioma were both associated with

increased expression at RNA and protein levels. Both examples of *MYB* amplification contained the promoter region and transactivating domain, and translated products would be predicted to retain their transcription factor function. The nature of genomic loss in the angiocentric glioma is similar to the situation seen within *MYB-NFIB* fusions in ACCs, where *MYB* upregulation occurs through loss of micro-RNA binding regions within the 3'-UTR of *MYB*. Discrete copy number loss at 6q24-25 by metaphase comparative genomic hybridization (CGH) has previously been reported in 1 of 8 angiocentric gliomas [44], and this may represent the same region of loss demonstrated here.

Array data for both DAs with *MYB* amplification indicated additional regions of amplification on 6q23. iFISH confirmed the presence of double minutes at these loci and showed that both amplicons were not continuous with the *MYB* amplicon. The amplicon situated centromeric to *MYB* in DA1 contains the non-coding gene *AJ606331*. This is postulated to encode a short RNA, and expression has been documented in human testis and placenta [10]. The amplicon telomeric to *MYB* in DA2 included the first exon of *MAP7* and the terminal exon of the mitogen-activated protein kinase *MAP3K5*. Most of *MAP3K5* exon 30 encodes the 3'UTR, this contains many stop codons, so would not be expected to produce a protein, even if it were transcribed. The significance of these disparate additional amplifications remains to be determined. The close proximity of the amplified regions led us to search for possible gene fusions between *MYB* and *MAP7*/*MAP3K5*. No fusion could be found between these potential candidates, but our data do not entirely rule out the existence of such a fusion, particularly if a complex rearrangement is present. Chromosomal instability was considered as a possible cause for the genetic rearrangements in the two DAs with *MYB* amplification, but no significant differences were found when comparing the number of genetic gains and losses between these cases and a representative group of LGGs with no CNAs.

MYB upregulation was not confined to the diffuse gliomas with *MYB* amplification or deletion; immunohistochemistry disclosed increased nuclear expression of *MYB* in 41% of PAs and 19% of HGGs. These are lower frequencies than that for diffuse LGGs (60%), in which *MYB* immunoreactivity was also more pronounced; all tumors with strong nuclear immunoreactivity in nearly all tumor cells (++) cases) were found among diffuse gliomas. In some diffuse LGGs without *MYB* CNAs, *MYB* expression at the RNA level was increased, and in others and in all *MYB*-immunopositive PAs *MYB* expression was not elevated, suggesting multiple mechanisms account for *MYB* upregulation across pediatric gliomas. With one exception from the cerebral cortex, all *MYB*-immunopositive PAs were cerebellar, and the *MYB*-immunopositive oligodendroglial tumors did not show 1p/19q loss. Otherwise, LGGs with *MYB* upregulation did not demonstrate any distinctive clinical or genetic features.

This series of LGGs includes 10 cerebral DAs and 8 cerebellar PAs from our previous study of *KIAA1549-BRAF* gene fusions and MAPK pathway activation in LGGs. In total, we have now presented genetic data on 89 pediatric LGGs, including 24 diffuse gliomas – 15 cerebral DAs, 7 oligodendrogliomas or oligoastrocytomas, and 2 angiocentric gliomas, 58 PAs – 48 from the cerebellum and 10 from other sites, 6 pilomyxoid gliomas – 4 supratentorial and 2 spinal, and one PXA. In accord with other reports in the literature, this dataset highlights the following clinicopathological / genetic associations; tumors with *KIAA1549-BRAF* or *SRGAP3-RAF1* fusion genes are all PAs, most commonly from the cerebellum or pilomyxoid gliomas (one hypothalamic pilomyxoid astrocytoma and one spinal tumor) [13,18,20,21,27,52], tumors with *BRAF*p.V600E activating mutations are histologically varied (DA, PA, or PXA) and sometimes harbor concomitant deletion of *CDKN2A* [12,41,51], *IDH1/2* mutations are not a feature of pediatric diffuse gliomas

[11,60], and 1p/19q loss occurs in oligodendroglial tumors (oligodendroglioma or oligoastrocytoma) at a much lower frequency in children than in adults [25,42,47].

This study provides data on MYB abnormalities in a large series of pediatric LGGs that have been comprehensively analyzed for a range of genetic abnormalities already implicated in LGG development. Novel *MYB* amplifications were disclosed at a relatively low frequency (14%) in DAs, but they supplement very few genetic aberrations so far reported for these enigmatic WHO grade II tumors. Further, our immunohistochemical data indicate that upregulation of MYB can occur through mechanisms other than gene amplification or deletion of the 3'UTR, and that it can be a feature of other types of LGG and even some HGGs, suggesting a broader role for MYB in gliomagenesis.

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Fig. 1.

DNA CNAs at chromosome band 6q23 identified by Affymetrix SNP 6.0 analysis; gains are shown in red, losses in blue. (a) Two diffuse astrocytomas (DA1, DA2) showed gains within *MYB* and each had an additional region of amplification centromeric or telomeric from *MYB*, respectively. One further tumor (AG1) showed a focal deletion of the 3' terminal region of *MYB*. (b) Detailed mapping of the regions of copy number change within *MYB* showed that gained regions terminated within exon 9 in DA1 and within the UTR of exon 16 in DA2. Exon 16 alone was lost in AG1. (The start and end of segmented regions of gain or loss are shown by black bars, horizontal black lines are used to connect between segments and do not denote a confidence interval.)

Fig. 2.

Focal amplifications at 6q23 identified by iFISH. Multiple copies of *MYB* (red) were seen as double minutes within DA1 (a) and DA2 (b), in combination with control signals to 6p (green). Multiple copies of the amplified region at 6q23 that included *AJ606331* are seen as double minutes (red) within DA1 (c), in combination with control signals to 6p (green). Multiple copies of the amplified region at 6q23 that included *MAP7* and *MAP3K5* are seen as double minutes (red) within DA2, in combination with control signals to 6p (green) (d). Co-localization of *MYB* and the additional amplified regions within double minutes were confirmed using probes to target *MYB* (red) and *AJ606331* (green) in DA1 (e) and *MYB* (red) and *MAP7* and *MAP3K5* (green) in DA2 (f).

Fig. 3. MYB immunoreactivity (IR) in low-grade gliomas. Two broad categories of MYB IR were demonstrated; (i) strong nuclear IR of nearly all tumor cells as demonstrated by this diffusely infiltrating astrocytoma, in which normal glia and blood vessels are immunonegative (a, b), and weak to moderately strong nuclear IR of scattered tumor cells in this cerebellar pilocytic astrocytoma (c, d), and this anaplastic astrocytoma (e,f).

Table 1a

Clinical data and summary of results for diffuse cerebral LGGs

Code	Age	Sex	6q23 CNA by array	Additional CNA by array	RAF gene fusion	Mutation screening	MYB CNA by FISH	RT-PCR MYB Exons 8-9	MYB IHC
DA1	8	F	Gain	-	-	-	Amplified	504	++
DA2	6	F	Gain	-	-	-	Amplified	12	+
DA3	6	F	-	-	-	-	-	156	++
DA4	6	F	-	-	- ^a	-	-	147	++
DA5	11	M	-	-	-	-	-	3	++
DA6	4	M	-	-	-	-	-	-	+
DA7	5	M	-	-	-	-	-	-	+
DA8	9	M	-	-	-	BRAF p.V600E	-	-	+
DA9	7	F	-	-	-	-	-	-	0
DA10	3	M	-	-	-	-	-	-	0
DA11	9	F	ND	ND	-	-	-	-	0
DA12	3	M	-	-	-	-	-	-	ND
DA13	17	M	-	Gain of 7 ^b	-	-	Polysomy	-	ND
DA14	4	M	-	-	-	-	-	-	ND
OA1	6	F	-	-	-	-	-	-	++
OA2	14	M	-	-	-	-	-	-	0
OA3	14	M	-	-	-	-	Polysomy	-	0
OA4	2	F	-	-	-	-	-	-	0
OA5	5	M	-	Loss of 1p/19q	-	-	-	-	0
O1	3	M	-	-	-	-	-	4	+
O2	15	M	-	Loss of 1p/19q	-	-	-	-	0
AG1	3	M	Loss	-	-	-	-	2306	++
AG2	11	M	-	-	-	-	-	909	+

DA, diffuse astrocytoma; OA, oligoastrocytoma; O, oligodendroglioma; AG, angiocentric glioma. Age, age at diagnosis in years. CNA, copy number abnormality; ND, not done.

^aDA4 was previously reported to contain a *KIAA1549-BRAF* fusion [13], but this was not confirmed on repeated analysis for this study.

^b whole chromosome gain. Mutation screening included *BRAF* exon 15, *KRAS* exons 2 and 3, *IDH1* exon 4 and *IDH2* exons 4-5. Reverse-transcription PCR using primers within *MYB* exons 8-9, expressed as a normalized ratio. Negative results showed ratios equal to or less than a (normal) control. IHC, immunohistochemistry: ++, strong nuclear immunoreactivity in nearly all tumor cells; +, scattered tumor cells with weak to moderately strong nuclear immunoreactivity; 0, no immunoreactivity in tumor cells.

Table 1b

Clinical data and summary of results for pilocytic astrocytomas

Code	Site	Age in years	Sex	7q34 CNA by array	RAF gene fusion	Mutation screening	MYB CNA by FISH	MYB IHC
PA1	Cb	3	M	–	KIAA1549-BRAF Ex 15-Ex 9	–	–	+
PA2	Cb	5	F	Gain	KIAA1549-BRAF Ex 15-Ex 9	–	–	+
PA3	Cb	13	M	Gain	KIAA1549-BRAF Ex 15-Ex 9	–	–	+
PA4	Cb	10	M	Gain	KIAA1549-BRAF Ex 16-Ex 9	–	–	+
PA5	Cb	12	F	Gain	KIAA1549-BRAF Ex 16-Ex 9	–	–	+
PA6	Cb	7	M	Gain	KIAA1549-BRAF Ex 16-Ex 9	–	–	+
PA7	Cb	15	F	Gain	KIAA1549-BRAF Ex 16-Ex 9	–	–	+
PA8	Cb	19	M	Gain	KIAA1549-BRAF Ex 19-Ex 9	–	–	+
PA9	Cb	2	F	–	–	KRAS p.G12A	–	+
PA10	Cb	3	F	–	–	–	–	+
PA11	Cb	6	M	–	–	–	–	+
PA12	Cb	18	F	Gain	KIAA1549-BRAF Ex 15-Ex 9	–	Polysony	0
PA13	Cb	7	M	Gain	KIAA1549-BRAF Ex 15-Ex 9	–	–	0
PA14	Cb	7	M	Gain	KIAA1549-BRAF Ex 16-Ex 9	–	–	0
PA15	Cb	2	F	Gain	KIAA1549-BRAF Ex 16-Ex 9	–	–	0
PA16	Cb	9	M	Gain	KIAA1549-BRAF Ex 16-Ex 9	–	–	0
PA17	Cb	10	M	Gain	KIAA1549-BRAF Ex 16-Ex 9	–	–	0
PA18	Cb	2	F	Gain	KIAA1549-BRAF Ex 16-Ex 9	–	–	0
PA19	Cb	4	F	Gain	KIAA1549-BRAF Ex 16-Ex 9	–	–	0
PA20	Cb	1	F	Gain	KIAA1549-BRAF Ex 16-Ex 9	–	–	0
PA21	Cb	4	F	Gain	KIAA1549-BRAF Ex 16-Ex 11	–	ND	ND
PA22	Cb	3	F	–	KIAA1549-BRAF Ex 16-Ex 11	–	ND	ND
PA23	Cb	14	F	–	KIAA1549-BRAF Ex 16-Ex 11	–	–	ND
PA24	Cb	15	F	Gain	KIAA1549-BRAF Ex 18-Ex 10	–	–	0
PA25	Cb	13	F	– <i>a</i>	SRGAP3-RAF1 Ex 11-Ex 8	–	–	0
PA26	Ce	2	M	Gain	KIAA1549-BRAF Ex 15-Ex 9	–	–	+

Code	Site	Age in years	Sex	7q34 CNA by array	RAF gene fusion	Mutation screening	MYB CNA by FISH	MYB IHC
PA27	Ce	14	M	–	<i>KIAA1549-BRAF</i> Ex 15-Ex 9	–	–	0
PA28	Ce	5	F	–	<i>KIAA1549-BRAF</i> Ex 15-Ex 9	–	–	0
PA29	Ce	4	M	Gain	<i>KIAA1549-BRAF</i> Ex 16-Ex 9	–	–	0
PA30	Ce	18	F	Gain of 7 ^b	–	–	–	0
PA31	Ce	10	F	–	–	–	–	0
PA32	Th	5	F	ND	<i>KIAA1549-BRAF</i> Ex 15-Ex 9	–	ND	ND
PA33	Th	6	M	Gain	<i>KIAA1549-BRAF</i> Ex 16-Ex 9	–	–	0
PA34	Th	13	F	ND	–	<i>BRAF</i> p. V600E	–	ND

PA, pilocytic astrocytoma; Cb, cerebellum; Ce, cerebral cortex; Th, thalamus. CNA, copy number abnormality.

^aPA25 contained 3p25 copy number gain by array analysis;

^bwhole chromosome gain. ND, not done. Mutation screening included *BRAF* exon 15, *KRAS* exons 2 and 3, *IDH1* exon 4 and *IDH2* exons 4-5. IHC, immunohistochemistry: ++, strong nuclear immunoreactivity in nearly all tumor cells; +, scattered tumor cells with weak to moderately strong nuclear immunoreactivity; 0, no immunoreactivity in tumor cells.

Table 2

Primers for DNA qPCR

Gene	Primer	Sequence	Conc (nM)	Amplicon (bp)
<i>MYB</i> exon 8	Forward	CCTGAGAAGGAAAAGCGAAT	300	194
	Reverse	GAATGGATATATGGAGCCTTGC	300	
<i>MYB</i> exon 16	Forward	CTTTCTCCATCAGCCTTGTAGCAG	500	76
	Reverse	GGAAGATGTCATCTGCTCCTCCAT	500	
<i>ZNF80</i>	Forward	CTGTGACCTGCAGCTCATCCT	300	120
	Reverse	TAAGTTCTCTGACGTTGACTGATGTG	300	
<i>GPR15</i>	Forward	GGTCCCTGGTGGCCTTAATT	300	101
	Reverse	TTGCTGGTAATGGGCACACA	300	

Table 3

Primers for RT PCR

Gene	Primer
<i>MYB</i> exon 8 forward	TGCTCCTAATGTCAACCGAGA
<i>MYB</i> exon 9 reverse	GCGCTTTCTTCAGGTAGGG
<i>GAPDH</i> forward	CTCTGCTCCTCCTGTTCGAC
<i>GAPDH</i> reverse	GCCCAATACGACCAAATCC
<i>B2M</i> forward	GGGATCGAGACATGTAAGCAG
<i>B2M</i> reverse	CAAGCAAGCAGAATTTGGAA