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Diagnostic application of high resolution single nucleotide polymorphism array analysis for children with brain tumors

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Abstract

Single nucleotide polymorphism (SNP) array analysis is currently used as a first tier test for pediatric brain tumors at The Children's Hospital of Philadelphia. The results from 100 consecutive patients are summarized in the present report. Eighty-seven percent of the tumors had at least one pathogenic copy number alteration. Nineteen of 56 low grade gliomas (LGGs) demonstrated a duplication in 7q34, which resulted in a *KIAA1549-BRAF* fusion. Chromosome band 7q34 deletions, which resulted in a *FAM131B-BRAF* fusion, were identified in one pilocytic astrocytoma and one dysembryoplastic neuroepithelial tumor (DNT). One ganglioglioma (GG) demonstrated a 6q23.3q26 deletion that was predicted to result in a *MYB-QKI* fusion. Gains of chromosomes 5, 6, 7, 11, and 20 were seen in a subset of LGGs. Monosomy 6, deletion of 9q and 10q, and an i(17)(q10) were each detected in the medulloblastomas (MBs). Deletions and regions of loss of heterozygosity that encompassed *TP53*, *RBI*, *CDKN2A/B*, *CHEK2*, *NF1*, and *NF2* were identified in a variety of tumors, which led to a recommendation for germline testing. A *BRAF* p.Thr599dup or p.V600E mutation was identified by Sanger sequencing in one and five LGGs, respectively, and a somatic *TP53* mutation was identified in a fibrillary astrocytoma. No *TP53* hot-spot mutations were detected in the MBs. SNP array analysis of pediatric brain tumors can be combined with pathologic examination and molecular analyses to further refine diagnoses, offer more accurate prognostic assessments, and identify patients who should be referred for cancer risk assessment.

Keywords

Single nucleotide polymorphism; *BRAF*; *KIAA1549*; *FAM131B*; *TP53*; *MYB*; *QKI*; medulloblastoma; glioma; ependymoma; astrocytoma

Brain and other central nervous system (CNS) tumors account for the largest portion of solid tumor-related deaths among children (1), and their incidence appears to be on the rise in the United States (2). Brain tumors are classified into different subgroups based on histology and immunophenotype according to the World Health Organization (WHO) classification (3). The spectrum of brain tumors arising in children differs significantly from what is observed in adults, with pilocytic astrocytoma (PA) and medulloblastoma (MB) accounting for the largest portion of pediatric low grade and malignant brain tumors, respectively. These tumors rarely occur in adults, who have a higher incidence of meningioma and malignant gliomas, especially glioblastoma multiforme (GBM) (4,5). The structural alterations and sequence-based changes that characterize pediatric and adult brain tumors are often different and reflect differences in the underlying mechanisms of tumor formation (6–8).

Several hereditary cancer syndromes, the majority of which are characterized by incomplete penetrance or variable expressivity, predispose individuals to the formation of brain tumors. These syndromes include Li-Fraumeni, neurofibromatosis 1 (NF1), neurofibromatosis 2 (NF2), and Gorlin and Turcot syndromes (9). Although the vast majority of pediatric brain tumors are thought to be sporadic (10), certain cancers, such as rhabdoid tumor (RT), demonstrate a higher incidence of patients (35%) with germline mutations (11). A recent study suggests that 29% of pediatric cancers may be due to hereditary cancer predisposition syndromes (12,13).

Molecular characterization of pediatric brain tumors has contributed to our understanding and classification of these tumors (14,15). Notably, a variety of different platforms have been used to identify copy number alterations (CNAs) in pediatric brain tumors. The most commonly identified CNA in pediatric low grade gliomas (LGGs) is a duplication in 7q34 that results in a fusion protein (KIAA1549-BRAF) and constitutively active MAPK signaling (16–18). Aside from the 7q34 duplication, LGGs exhibit a relatively quiet copy number profile (16,19,20). Other frequent CNAs include an i(17)(q10) in MB (21) and frequent loss of 22q in pediatric meningiomas (22) and ependymomas (23,24). Monosomy 22 is not specific, however, because it is also typically seen as a primary abnormality in RT (25) and as an associated change in MB (21) and GBM (26).

The identification of novel or tumor-associated CNAs is increasingly used for classification of brain tumors into specific diagnostic or prognostic subgroups. For example, a MB with monosomy 6 as the only abnormality is consistent with the WNT pathway subgroup, which has an overall good prognosis (27,28). In contrast, the presence of an i(17)(q10), with or without *MYC* amplification, places the tumor into either a poor (Group 3) or intermediate (Group 4) risk category (28). In ependymoma, tumors with gains of the long arm of chromosome 1 have significantly worse outcomes than others (29,30). Similarly, amplification of a miRNA cluster (*miR371-373*), which is located in 19q13.41 in supratentorial primitive neuroectodermal tumors (sPNETs), is reportedly associated with a poor prognosis (31).

Identification of CNAs and molecular alterations become critical as targeted therapies are emerging for cancers that are driven by alterations in specific pathways. Patients with MB

and activation of the sonic hedgehog (SHH) pathway may benefit from small molecule inhibitors such as GDC-0449 (vismodegib; Genentech, San Francisco, CA) (32). For LGG, drugs that target the BRAF V600E mutation have shown efficacy both in vitro and in vivo (33,34). The majority of children with PA have a KIAA1549-BRAF fusion, and although this also results in increased MAPK signaling, these patients are not likely to respond to the first-generation BRAF inhibitors due to the altered structure of the fusion protein (35).

We implemented SNP array analysis for pediatric brain tumors as a diagnostic tool in the Cancer Cytogenetics laboratory at The Children's Hospital of Philadelphia (CHOP) in 2009. Herein, we report our findings from the analysis of brain tumor specimens from 100 patients who were analyzed with the Human610-Quad or the HumanOmni1-Quad genotyping bead SNP array. Clinically useful results from the SNP array analysis included clarification and subclassification of diagnoses, risk stratification of tumors into prognostic groups, and identification of genomic regions containing cancer predisposition loci.

Materials and methods

Clinical samples

Fresh or frozen brain tumor specimens were submitted for diagnostic testing to the Clinical Cancer Cytogenetics Laboratory at CHOP beginning in December 2009. A total of 101 specimens were collected over a 4-year period. These specimens represent brain tumors from 100 different patients, including one patient with both a primary and a recurrent tumor (11-274 and 11-274B). All patients were diagnosed and/or treated at CHOP with the exception of one patient who was a consultation case (10-399B). A total of 87 were newly diagnosed patients, and 14 were patients with recurrent brain tumors. Of the 100 cases included in this cohort, thirteen were previously reported (36). The clinical demographics are shown in Supplementary Table 1.

Illumina SNP array analysis

DNA was extracted from brain tumor specimens using the Gentra *Puregene* Tissue Kit from Qiagen (Valencia, CA). The Infinium HD assay was performed using the Illumina Human610-Quad and HumanOmni1-Quad genotyping bead arrays by the CHOP Center for Applied Genomics according to the manufacturer's instructions (Illumina, San Diego, CA). BeadStudio for the Human610-Quad array (Illumina) and GenomeStudio for the HumanOmni1-Quad array (Illumina) were used to analyze the SNP array data. Visual inspection of the LogR ratio and B allele frequency (BAF) plots were performed using the previously mentioned software tools to identify CNAs and regions of homozygosity (ROH). Criteria for reporting CNAs from our laboratory was previously described (36). Briefly, CNAs that encompassed at least 20 consecutive SNPs were included in the clinical reports. CNAs containing <20 SNPs were included in the report only if they occurred in a known cancer-associated gene. ROH, including copy-neutral loss of heterozygosity (CN-LOH), were included in the report if they were >5 Mb in size, with exceptions that allowed for the reporting of smaller ROH containing known cancer-associated genes. These reporting criteria were chosen based on previous experience of this laboratory with SNP array analysis of a variety of hematologic and solid tumor cases (36,37). Genomic positions for the

Human610-Quad and HumanOmni1-Quad arrays were based on the hg18 (March 2006) and hg19 (February 2009) builds of the human genome sequence, respectively (University of California, Santa Cruz (UCSC), <http://genome.ucsc.edu/>). Benign variants were determined by analysis of the Database of Genomic Variants (DGV) (<http://dgv.tcag.ca/dgv/app/home>), and in-house normal controls and were excluded from the results shown in Supplementary Table 2. For duplications, variants that were present in the DGV and in at least two in-house normal controls were considered to be benign. Similarly, heterozygous deletions were considered benign if they were germline and observed in the DGV and two in-house normal controls. No size limit was specified when evaluating these variants. Additionally, heterozygous deletions were considered likely to be germline if the BAFs were equal to zero and one. Percentages of mosaicism for both CNAs and CN-LOH were determined using the BAF (38).

Sequence analysis

BRAF sequence analysis of exons 11 and 15 was performed using one of two methods. In the majority of samples, *BRAF* exons 11 and 15 were amplified and sequenced as previously described (39). A minority of the tumors were analyzed for *BRAF* exons 11 and 15 with primers that contained M13 tags located at their 5' ends. Sequences for M13 tagged *BRAF* exon 11 and exon 15 primers were as follows: exon 11 forward primer 5'-GTAAAACGACGGCCAGTGTCCCTCT CAGGCATAAGGTAA-3'; exon 11 reverse primer 5'-GGA AACAGCTATGACCATGCGAACAGTGAATATTTCTTTGA T-3'; exon 15 forward primer 5'-GTAAAACGACGGCCAGTGGGAAAGCATCTCACCTCATCC-3'; exon 15 reverse primer 5'-GGAAACAGCTATGACCATGTTGAGACCTTCAA TGACTTTCTAGT-3'. The M13 tags were utilized in the sequencing reactions. *IDH1* exon 4 primers also contained M13 tags and sequences were as follows: exon 4 forward primer 5'-GTAAAACGACGGCCAGTGCCATCACTGCAG TTGTAGGTT-3' and exon 4 reverse primer 5'-GGAAA CAGCTATGACCATGGCAAATCACATTATTGCCAAC-3'. Primer sequences for exons 4-8 of *TP53* were obtained from the International Agency for Research on Cancer (IARC) (<http://p53.iarc.fr/ProtocolsAndTools.aspx>). All PCR reactions were performed using standard techniques (40), and products were treated with ExoSAP-IT (Affymetrix, Santa Clara, CA). Products were sequenced either with the same primers that were utilized in the PCR reactions (*BRAF*, *TP53*, *KIAA1549-BRAF*, *FAM131B-BRAF*) or with M13 primers when the PCR products were M13 tagged (*IDH1* and *BRAF*). Sequencing products were analyzed in the Nucleic Acid and Protein Core facility at CHOP with a 3730 DNA analyzer (Life Technologies, Carlsbad, CA) or in the Molecular Genetics Laboratory at CHOP with a 3130xl genetic analyzer (Life Technologies). All mutations found, including those of fusion transcripts, were confirmed by bidirectional sequencing. Sequences were analyzed either by visual inspection or with Mutation Surveyor v.4.0 software (SoftGenetics, State College, PA). *TP53* sequence variants were determined to be polymorphic or likely pathogenic based on the IARC database (41) and the Catalogue of Somatic Mutations in Cancer (COSMIC) (42).

RNA extraction and detection of fusion transcripts using reverse transcriptase PCR (RT-PCR)

RNA was extracted from frozen tissue specimens using either the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol, or by phenol/chloroform extraction as described previously (16). For all tumor samples, except for three (11-274, 11-326, and 12-058), approximately 1 ug of RNA was reverse transcribed into cDNA with the SuperScript III First-Strand Synthesis SuperMix (Life Technologies) according to the manufacturer's instructions. RNA extraction and detection of fusion products for samples 11-274, 11-326, and 12-058 is described by Roth et al. (Personal communication; J.J. Roth, April 1, 2014). All *KIAA1549-BRAF* fusions were detected with either primers located in exon 4 of *KIAA1549* and exon 16 of *BRAF* as described in Sievert et al. (16) or with a nested PCR reaction as described in Forshew et al. (43). Detection of the *FAM131B-BRAF* fusion product and β -*ACTIN* control was performed as described by Roth et al. (Personal communication; J.J. Roth, April 1, 2014). PCRs were performed with standard protocols (40) and PCR products were sequenced as stated previously.

Fluorescence in situ hybridization analysis (FISH)

For brain tumors that required FISH confirmation of SNP array findings, touch imprints were made from frozen tissue and analyzed as previously described (44). The number of nuclei analyzed for each specimen is recorded in Supplementary Table 2. Probes used for FISH analysis were all purchased from Abbott Molecular (Des Plaines, IL), except for LUC7L2 (RP11-236O18), which was purchased from the BACPAC Resources at the Children's Hospital Oakland Research Institute (Oakland, CA) (16), and *D9S2020* (WI-7383) and *PTCH* (provided by Robert Jenkins). All probes were labeled and processed as previously described (44).

Results

Clinical demographics and brain tumor distribution

The clinical demographics of the 100 patients (101 tumor specimens) included in this study are shown in Supplementary Table 1. The age of patients ranged from 2 months to 36 years, with an average age of 10 years. A total of 10 patients were 18 years or older at the time of surgery. Distribution of the patients' ages by tumor type is shown in Supplementary Figure 1. Sixty percent of the patients were male, and 40% were female (Supplementary Table 1). The majority of the tumors were primary lesions, whereas 14 were either single or multiple recurrent brain tumors (Supplementary Table 1). The diagnosis of the primary tumor was the same as that of the recurrent tumor in 13 of the 14 recurrent cases (Supplementary Table 1). Relevant medical histories also included four patients with NF1 (cases 10-286, 10-302, 12-229, and 12-379) and one patient who was diagnosed with Noonan syndrome (NS) (case 11-443).

A total of 101 tumor specimens from 100 patients included in this cohort were analyzed with the Illumina Human610-Quad genotyping bead array (27 specimens) or the Human-Omni1-Quad genotyping bead array (74 specimens) (Supplementary Table 2). Histopathologic classification of the tumors is shown in Figure 1. The most common types of brain tumors in

this study were LGG and MB, which is consistent with their incidence in the pediatric population (4,5). Other tumor types in this study included ependymoma, high grade glioma (HGG), meningioma, sPNET, and two additional tumors that could not be placed into one of these categories.

The detailed results from the SNP array analysis for the 101 tumors are shown in Supplementary Table 2. Overall, 87% of the tumors had at least one disease-associated or pathogenic abnormality. The number of CNAs identified per tumor ranged from 0 to >150. In complex cases with more than 100 CNAs, the alterations were not completely characterized. Normal population variants (NPVs) are not included in Supplementary Table 2. The SNP array results from 13 of these cases were previously reported (36).

Low grade glioma and neuronal-glial tumors

A total of 56 LGGs were analyzed using either the Human610-Quad or HumanOmni1-Quad array. A variety of diagnoses were included within the LGG category. The most frequent diagnosis was classic PA (25 tumors; Supplementary Table 1). Eight LGGs were reported as normal with no pathogenic CNAs identified. The most common CNA identified in the LGGs was a 2.0 Mb duplication in 7q34 that we and others have reported previously (16–18). This tandem duplication results in a KIAA1549-BRAF fusion protein, constitutive activation of the BRAF kinase, and increased MAPK signaling (16,17,43). This alteration was identified in one third of the LGGs (20 of 56), including approximately two thirds of the classic PAs (16 of 24) as well as in 4 additional LGGs including: 2 PA-diffuse variants (11-162 and 11-448); 1 pilomyxoid/PA (11-271); and 1 pilomyxoid astrocytoma (PMA) (12-034) as demonstrated in Figure 2 and Supplementary Tables 1 and 2.

A subset (16 of 56) of the LGGs demonstrated 1–14 whole chromosome gains (Figure 2; Supplementary Table 2), the majority of which were present in three copies. In three cases (11-018, 11-276, and 11-386), four and five copies were present. The most frequent gain involved chromosome 7 (13 tumors), as previously reported (45). In 5 of the 13 tumors, the gain of chromosome 7 occurred in conjunction with the 7q34 duplication (Figures 2 and 3A; Supplementary Table 2). A gain of chromosome 7 was also accompanied by a *BRAF* mutation (see *BRAF* mutation and fusion screening) in 3 of the 13 tumors (Figure 2). In addition to chromosome 7, chromosomes 5, 6, 11, and 20 were also gained in at least eight tumors. Interestingly, in one patient (case 12-222), the recurrent tumor demonstrated gains of chromosomes 5, 6, and 7, whereas the primary tumor analyzed 4 years previously (data not shown) harbored gains of chromosomes 5, 7, and 10. Whole chromosome losses were rarely observed in the LGGs (Figure 2).

Fluorescence in situ hybridization analysis was used in selected cases to confirm the SNP array findings as shown in Supplementary Table 2. Overall, the SNP array and FISH results were concordant. FISH analysis of one tumor (11-448) yielded an unexpected separation of signals with the dual-color *KMT2A/MLL* probe in 11q23. The split signal was suggestive of a translocation that interrupted *KMT2A/MLL*; however, the significance of this rearrangement was unclear in the setting of a LGG. This tumor also showed mosaic gains for a number of chromosomes as well as the 7q34 duplication (Supplementary Table 2).

SNP array findings other than CNAs involving 7q34 were noted in several LGGs (Supplementary Table 2). The only oligodendroglioma in our cohort, case 12-234, was from a 23-year-old male. This tumor displayed the characteristic heterozygous loss of 1p and 19q by SNP array analysis, which was present in ~50% of cells (Figure 3B,C; Supplementary Table 2) (46). This finding was confirmed by FISH. Case 12-234 was also found to harbor an IDH1 mutation. Tumor 10-210, a ganglioglioma (GG), displayed a mosaic 30 Mb deletion in 6q23.3q26 in ~75% of cells, with a proximal breakpoint in the last intron of the *MYB* gene. The distal end of this deletion occurred within the *QKI* gene and likely resulted in a fusion similar to the *MYB-QKI* fusion reported by Zhang et al. (Figure 3D; Supplementary Table 2) (20).

Heterozygous deletions of *TP53* were observed in two LGGs within our cohort: 11-247 and 10-286 (Figure 2; Supplementary Table 2). Additionally, tumor 12-175 displayed CN-LOH for the short arm of chromosome 17 in ~35% of cells (Figure 3E; Supplementary Table 2). *TP53* sequencing of tumor 12-175 yielded a heterozygous 2 bp deletion in exon 5 (c.547_548delTC), which is predicted to result in a truncated protein product (p.Ser183Argfs*2) (data not shown). This mutation was present in ~38% of the patient's tumor sample. To our knowledge, this *TP53* mutation has not been previously reported (41,42). Germline sequence analysis of *TP53*, which was conducted at an outside laboratory, did not reveal any mutations (data not shown). SNP array analysis was not performed on the patient's germline sample to rule out constitutional mosaic CN-LOH. A homozygous deletion of *CDKN2A* was observed in two LGGs: 10-286 and 12-229 (Figure 3F). In tumor 10-286, the deletion was located within a region of mosaic CN-LOH, which was present in ~50% of cells. In addition, tumor 10-286 was from a patient with NF1 (see SNP array findings in LGGs in the setting of cancer predisposition syndromes) and displayed heterozygous deletions that included *RB1* and *TP53* (Figure 2; Supplementary Table 2). A ROH encompassing *RB1* was observed in tumor 12-302 (Figure 2; Supplementary Table 2).

SNP array findings in LGGs in the setting of cancer predisposition syndromes

The risk of LGG, especially optic pathway glioma, is increased in patients with RASopathies, which is a group of genetic disorders associated with mutations in the *RASMAPK* signaling pathway genes (47). Our cohort included five patients with RASopathies. In three of four NF1 patients (10-286, 10-302, and 12-229), a clinical diagnosis was made by a clinical geneticist. A germline mutation in *NF1* was documented in the fourth patient (12-379) with clinical features of NF1 (data from an outside laboratory and not shown). The final RASopathy patient (11-443) received a clinical diagnosis of NS by a clinical geneticist (Supplementary Table 1).

We did not identify any additional *BRAF* alterations in the tumors from these patients, although *BRAF* fusions and mutations have been reported in gliomas from NF1 patients (48,49). Two of the NF1 patients, 12-229 and 12-379, demonstrated regions of CN-LOH or ROH in 17q that encompassed the *NF1* gene (Figure 2; Supplementary Table 2). Tumors from the remaining two NF1 patients, 10-286 and 10-302, did not display any detectable CNAs in *NF1*. Two NF1 patients, 10-286 and 12-229, displayed homozygous deletions of

CDKN2A, which has been associated with a poor prognosis in LGG (52). No pathogenic CNAs were identified in the glioma from the patient with NS.

***BRAF* mutation and fusion screening**

We performed RT-PCR to detect the *KIAA1549-BRAF* fusion transcript on the initial 12 LGGs. Of these 12 tumors, only tumor 11-162 demonstrated a positive result by RT-PCR, which was consistent with the SNP array results. In addition, RT-PCR to detect the presence of a *BRAF* fusion was performed on three tumors, 11-274, 11-326, and 12-058, which demonstrated variant deletions in 7q34. Tumor 12-058 was positive for the *KIAA1549-BRAF* fusion, whereas tumors 11-274 and 11-326 were positive for a *FAM131B-BRAF* fusion. Details of the SNP array results and fusion detection for both *KIAA1549-BRAF* (case 12-058) and *FAM131B-BRAF* (cases 11-274 and 11-326) fusions will be reported by Roth et al. (Personal communication; J.J. Roth, April 1, 2014).

In addition to performing SNP array analyses, we also screened the LGGs for mutations in exons 11 and 15 of *BRAF*. Sixteen tumors were analyzed by Sanger sequencing for *BRAF* exons 11 and 15, and 36 tumors were analyzed for mutations in exon 15 alone. No exon 11 mutations were detected, whereas mutations in exon 15 of *BRAF* were identified in five LGGs; none of these LGGs contained the 7q34 duplication. Four of the mutations, which were identified in a PA (case 12-318), fibrillary astrocytoma (FA) (case 12-264), pleomorphic xanthoastrocytoma (PXA) (case 12-093), and an indeterminate subtype LGG (case 11-018), were missense substitutions that resulted in a *BRAF* V600E alteration. The remaining *BRAF* mutation in our cohort was identified in a recurrent PA (case 12-222) and consisted of a three base pair insertion (c.1794_1796dupTAC), which is predicted to result in duplication of a threonine at position 599 (p.Thr599dup). This alteration, although infrequent, has been reported by several groups (48–51). Functional studies have shown this mutation to behave in a similar fashion to *BRAF* V600E, which results in activation of the MAPK signaling pathway and cellular transformation (49,50). The primary tumor, which corresponded to 12-222 (data not shown), demonstrated the same alteration, c.1794_1796dupTAC in *BRAF* exon 15.

High grade glioma

Primary high grade gliomas (HGGs) are rare in the pediatric population compared with adults (4,5). Nine HGGs were analyzed in this cohort, including four grade III and five grade IV tumors. SNP array analysis demonstrated CNAs in all HGGs, which were increasingly more complex than the CNAs identified in the LGGs. Two of the HGGs, 11-116 and 11-238, displayed CNAs that were too numerous to report.

Three of the four grade III gliomas (10-399B, 11-116, and 11-430) were characterized by whole chromosome gains (Supplementary Table 2). Loss of the long arm of chromosome 10, which contains the *PTEN* gene, was observed in 10-399B and confirmed by FISH. Homozygous deletion of *CDKN2A/B*, a non-random finding in pediatric HGG (52,53), was observed in two of the grade III gliomas: 11-116 and 12-305. The latter tumor, 12-305, also displayed loss of 16q.

The grade IV gliomas, in addition to whole chromosome gains and losses, displayed numerous complex changes, including chromothripsis (Figure 4). In contrast, tumor 11-447, a GBM, displayed a homozygous deletion of *CDKN2A* in conjunction with a *BRAF* V600E mutation, but was otherwise relatively simple, suggesting that it arose from a lower grade tumor. Homozygous deletions of *PTEN* and *RB1* were seen in tumor 13-023, as well as varying copy number gains of 1q, a finding noted previously in pediatric HGG (53,54). The complexity of CNAs displayed by the recurrent HGGs (11-116, 11-238, and 12-320) was likely due to the radiation treatment for previous tumors. No regions of amplification were observed in the HGGs.

BRAF and *IDH1* mutation screening was performed for all tumors, except 10-399B for which there was insufficient material. No *IDH1* mutations were found in exon 4, which was consistent with a previous report showing the lack of *IDH1* mutations in pediatric HGG (53). *BRAF* V600E mutations have been detected in 10% of pediatric HGGs (33), compared with 50% of non-PA grade I and grade II gliomas (45). *BRAF* exon 15 screening yielded one of nine HGGs (case 11-447) that was positive for a *BRAF* V600E mutation (Supplementary Table 2).

Ependymoma

Six ependymomas were included in this study: two of grade I, two of grade II, and two of grade III. All of the tumors were primary neoplasms. Two of the ependymomas, 10-400 and 12-122, had no significant CNAs, and an additional tumor, 12-246, displayed relatively few CNAs (Supplementary Table 2). The two myxopapillary ependymomas, 12-015 and 12-217, displayed a variety of whole chromosome gains, including gains of 7, 9, 16, and 18, which is a pattern that is reportedly associated with myxopapillary ependymomas (55). Tumor 12-276 was a grade II spinal cord ependymoma in a 12-year-old male. SNP array analysis revealed approximately 118 Mb of the genome to be homozygous, as well as amplification of *MYCN*. *MYCN* amplification was reported previously in adult spinal ependymoma (Supplementary Figure 2) (56). Notably, no gains of chromosome 1q or losses of chromosome 22q were observed in the ependymomas in this cohort.

Medulloblastoma

A total of 21 MBs were analyzed in the present study and at least one abnormality was identified in each tumor (Supplementary Table 2). High resolution copy number arrays and transcriptional profiling platforms have been used by several groups of investigators to subclassify MB into at least four distinct subgroups with different demographics and prognoses (27,28). It has been proposed that stratification of MBs into one of these subgroups can offer better risk assessment to patients as well as potentially guide treatment decisions. Whether this type of approach can be used on a prospective basis for individual patients has not been established yet; thus, we attempted to categorize each of the MBs into one of the four subgroups based on the SNP array data (Figure 5).

Monosomy 6 is characteristic of the WNT group of MBs. We observed two tumors (12-097 and 10-367) that demonstrated monosomy 6 on a background of relatively few other CNAs, which clearly placed these tumors into the WNT subgroup (Figure 5). Two additional

tumors (12-381 and 12-310) also demonstrated monosomy 6 along with a variety of additional CNAs, which included whole chromosome gains for almost the entire genome in tumor 12-310 and gain of chromosome 19 in 12-381. These additional alterations are more suggestive of aggressive tumors. Specifically, gain of chromosome 19 is often observed in a sPNET (57) or embryonal *tumor* with abundant neuropil and true rosettes (ETANTR) (58). Thus, although these tumors had monosomy 6, we did not categorize them as WNT pathway tumors.

The final WNT group tumor (13-003) in this cohort did not demonstrate monosomy 6, but instead showed a ROH for the entire long arm of chromosome 5 and the short arm of chromosome 19 (Figure 6). By report from an outside laboratory, this tumor displayed nuclear localization of beta catenin (CTNNB1), which is characteristic of the WNT group of MBs (59). The extended ROH in this tumor could unmask a mutation on the remaining allele in the *APC* gene, which maps to 5q21q22. *APC* mutations predispose individuals to both colon and brain tumors, especially in families with Turcot syndrome (60,61). Specific *APC* mutations result in the protein being unable to sequester CTNNB1 in the cytoplasm, which may explain the nuclear localization that was observed in this tumor. *SMARCA4* maps to 19p13.2 and somatic mutations have been reported in MB (62–64). Despite the unusual SNP array findings of this tumor, based on the immunohistochemistry results, we categorized this tumor as a WNT pathway MB.

Copy number changes that are associated with the SHH group of MBs include loss of the long arms of chromosomes 9 and/or 10, as well as *MYCN* amplification (28). Three tumors, 10-337, 10-375, and 12-225, demonstrated a heterozygous deletion of 9q (Supplementary Table 2; Figure 5), each of which included the *PTCH* gene. Gain of 9p was seen in tumor 10-375, which has also been reported in MBs (65,66). *MYCN* amplification was observed in one of the SHH tumors (12-225) on the background of other CNAs with unknown clinical significance. Overall, these tumors had relatively simple CNA profiles that displayed the key alterations found in the SHH group of MBs.

Group 3 and Group 4 tumors have numerous alterations in common, specifically the presence of an i(17)(q10), which was the most frequent alteration observed in our cohort of MBs (8 of 21). Thus, distinguishing between Group 3 and Group 4 tumors relies on the presence of other CNAs that are characteristic of each group. Two tumors (11-360 and 12-155) in our cohort were classified as Group 3 (Figure 5). In addition to the i(17)(q10), tumor 11-360 demonstrated a variety of discontinuous amplified regions on 8q, one of which contained the *MYC* gene, as well as other small CNAs including a ROH in 7q22.1q31.1. The second tumor in Group 3 (12-155) also displayed *MYC* amplification as well as a gain of 1q and loss of 10q, both of which are observed in Group 3 tumors (28).

Two tumors that harbored features of Group 4 were identified in this cohort. Both tumors, 11-141 and 13-060, occurred in female patients and displayed an i(17)(q10) as well as heterozygous loss of the X chromosome (Supplementary Table 2; Figure 5). Tumor 13-060 also demonstrated numerous CNAs that are common in Group 3 and 4 tumors, including gain of 7, monosomy 8, deletion 11p, and gain of 18q; however, the presence of monosomy X and *MYCN* amplification distinguish this tumor as belonging to Group 4 (28). Additional

MB subgroups have been proposed in limited institution studies (67). Four tumors (11-059, 11-438, 11-471, and 12-238) demonstrated the presence of an i(17)(q10) alone, or in combination with other features that are shared between Groups 3 and 4. The classification of these tumors could be refined by the addition of gene or protein expression studies, or these tumors may belong to an overlapping subgroup (Figure 5).

Overall, seven tumors were classified as MB based on pathologic findings that could not be further classified into one of the subgroups based on SNP array analysis. Some of these tumors did contain one or more characteristic CNAs of a particular subgroup; however, the other alterations observed did not overlap sufficiently with features of any one group. Finally, sequencing of exons 4–8 of *TP53* in 20 of 21 MB did not yield any mutations (Supplementary Table 2).

sPNETs

Four primary sPNETs were analyzed using a SNP array (Supplementary Table 2). Notably, these tumors lacked the presence of an i(17)(q10), which can aid in differentiating them from other embryonal tumors such as MB (68,69). Gain of 1q was seen in two of the sPNETs (cases 10-308 and 12-142). Tumor 10-308, which was reported by our group previously (36), demonstrated a gain of chromosome 2 and a region of amplification at 19q13.41, previously identified in CNS-PNET (31) and ETANTR (58), which harbors the *miR371-373* cluster. Overexpression of two miRNAs within this region, *miR-517c* and *miR-520g*, has been shown to promote oncogenicity both in vitro and in vivo (31). Tumor 11-435 displayed numerous discontinuous heterozygous deletions in 6q as well as chromothripsis of chromosome 22. Tumor 12-135 displayed a variety of CNAs that included heterozygous deletions of *NF2* and *CDKN2A*, as well as a gain involving *MYCN* and *ALK*. Overall, the alterations observed by SNP array analysis of the sPNETs in this cohort were complex, which is consistent with the aggressive behavior of this tumor.

Meningioma

Three meningiomas were included in this study, one of which was a recurrent tumor (case 12-394) (Supplementary Tables 1 and 2). Tumor 12-394 had no significant abnormalities. The remaining two meningiomas, 12-114 and 12-360, had numerous CNAs, including loss of chromosome 22q and *NF2*. A heterozygous deletion of *NF1* and *MNI* was also seen in tumor 12-360.

Miscellaneous tumors

One of the tumors could not be classified by histology or genetic alterations as a specific entity (Supplementary Table 2). This tumor, 10-316, demonstrated features of both a desmoplastic infantile ganglioglioma and a PNET. SNP array analysis of this tumor did not yield any significant findings that could aid in the diagnosis.

Tumor 12-244 was classified as a Ewing sarcoma/peripheral PNET (pPNET). Although SNP array analysis did not detect any abnormalities, FISH analysis on touch imprints of this tumor demonstrated separation of the *EWSR1* gene due to a translocation that is characteristic of Ewing sarcoma (70) (Supplementary Tables 1 and 2).

Clinical utility of SNP array analysis

Overall, 62 of 101 tumors demonstrated alterations that helped to confirm the diagnosis, allowed for subclassification of tumor type, enabled prognostic assessment, and/or led to a recommendation to the treating physician for further genetic testing to rule out a germline mutation in a cancer predisposition gene. Confirmation of the diagnosis based on SNP array results was made in 53 cases, with the most frequent example being identification of the 7q34 tandem duplication in LGG. Subclassification of tumors was possible in 15 cases, predominantly for MB (Figure 5). Prognostic assessment was made in 22 cases (e.g., the identification of *MYC* amplification in MB and thus the need for more aggressive therapy) (28). Finally, recommendations for germline testing based on SNP array findings were made in nine cases to rule out a germline *NF2*, *TP53*, *RBI*, or *PTEN* alteration. Follow-up information was available for one patient (TP53) who was negative for a germline mutation.

Discussion

The present study was designed to determine if real-time application of high resolution SNP array analysis could be used to aid in the diagnosis and prognosis of pediatric patients with brain tumors. This cohort included a wide variety of histologic types of pediatric brain tumors, reflecting the normal incidence of childhood brain tumors in the Pennsylvania, Delaware, and New Jersey tri-state area. Eighty-eight of 101 tumors demonstrated one or more CNAs that were considered to be pathogenic, whereas 13 tumors did not demonstrate any significant CNAs. This might have been due to the presence of nonneoplastic elements that masked low level aberrant clones, but may also reflect the lack of CNAs observed in subsets of brain tumors. Further characterization of the tumors by molecular methods maybe clinically indicated for these patients, including sequence analysis of *BRAF*, *IDH1*, or *TP53*.

Clinical utility of SNP array analysis

Although the majority of CNAs observed in this study were not tumor specific, the identification of non-random abnormalities was helpful in distinguishing tumors with overlapping histologic features. For example, the i(17)(q10) in MB can help distinguish these tumors from sPNET (68,69), atypical teratoid/ rhabdoid tumor (AT/RT) (25), and ETANTR (58,71,72). We did not have any AT/RTs in this cohort, because immunohistochemistry for SMARCB1 and sequence-based assays are used in our laboratory for differential diagnosis of this very aggressive embryonal tumor (11). One sPNET had trisomy 2 and amplification in 19q13, which has been associated with poor prognosis (31). Overall, the SNP array analysis provided useful information in over two thirds of the embryonal tumors in this series.

SNP array analysis was diagnostic for case 12-234, which was initially classified as gliomatosis cerebri, but was reclassified as an oligodendroglioma after SNP array analysis revealed the characteristic loss of 1p and 19q (46). In contrast, although we and others have identified a tandem duplication in 7q34 in the majority of PAs (16–18), it is now evident that this alteration is not specific for this subtype of LGG (73). We identified a *FAM131B-BRAF* fusion in a DNT, whereas all prior *FAM131B-BRAF* fusions have been identified in PA and diffuse astrocytoma (19,20,48). A deletion predicted to result in a *MYB-QKI* fusion was

identified in one GG in the present study, demonstrating that this fusion is not restricted to angiocentric glioma (8,20). These findings suggest that a molecular classification system may be useful in therapeutic targeting of these tumors, in addition to the histologic diagnosis.

Both copy number and molecular analyses can be useful for determining prognosis and, ultimately, in selecting appropriate therapy. *TP53* mutations, which have been identified in both WNT and SHH MBs, have proven to be a poor prognostic indicator in the SHH MBs (74). We did not identify any *TP53* mutations in the MBs in our cohort. DNA copy number gains and losses in MBs have proven to be useful in models predicting relapse versus no-relapse (75). MB patients with WNT pathway tumors, which are characterized by monosomy 6 and nuclear CTNNB1 staining, have the best prognosis (76), whereas tumors in the current MB consensus group 3 have the worst prognosis (67,77,78). Identification of key features that are indicative of high risk tumors, such as *MYC* or *MYCN* amplification (79), do aid in classifying the tumors when histology alone is inadequate. In our study, we were able to further classify two thirds of the MB into one of these subgroups.

The SHH group of MBs demonstrates increased signaling through the SHH pathway via a variety of mechanisms, including *PTCH*, *SUFU*, and *SMO* mutations. Small molecule inhibitors targeting members of this pathway (e.g., GDC-0449 vismodegib; Genentech, San Francisco, CA) are actively being investigated (80). A Phase I clinical trial of vismodegib in patients with recurrent or refractory MBs demonstrated that this therapy is feasible and safe in children, and anti-tumor effects were noted in one of three patients with SHH group MB (32). Although one patient with metastatic MB displayed only a transient and incomplete response to vismodegib (81), patients with basal cell carcinoma have demonstrated a 60% response rate to this drug (82), highlighting its therapeutic potential. In our study, we identified two patients with tumors in the SHH group of MBs that could benefit from this type of targeted treatment.

Similar targeted treatment is emerging in the field of LGG, with potential therapies directed at activated BRAF and MAPK pathway proteins. BRAF inhibitors targeted to the mutant V600E protein have shown efficacy in inhibition of mutant cells in vitro (34) and in murine xenografts (33). Phase I trials for BRAF V600E inhibition are currently under way (83). Conversely, Sievert et al. (35) showed that cells expressing *KIAA1549-BRAF* are resistant to vemurafenib (PLX4032; Plexxikon, Berkeley, CA), a small molecule inhibitor of BRAF V600E. Instead, these cells are responsive to a second-generation BRAF inhibitor, PLX PB-3 (Plexxikon) (35). Given the different responses of the *BRAF* mutant cells, it is critical to identify CNAs and sequence mutations to guide future therapeutic decisions. With the high frequencies of the *KIAA1549-BRAF* (20 tumors) and BRAF V600E alterations (six tumors) occurring in gliomas in our study, many patients stand to benefit from these targeted treatments. It is equally important to identify those patients who may benefit from reduction of therapy, especially radiation in young children. For example, limiting radiation doses in patients within the WNT group of MB could potentially help reduce secondary morbidity from the effects of treatment.

In addition to guiding diagnosis, prognosis, and treatment decisions, SNP array analysis can also be used to monitor CNAs that occur in both primary and recurrent tumors. This demonstrates proven clinical utility for hematologic malignancies, where response to therapy and secondary malignancies can be evaluated by molecular cytogenetic studies of serial bone marrow aspirates. One recurrent glioma in the present study, 12-222, had gains of chromosomes 5, 6, and 7. Interestingly, the primary tumor that was analyzed four years previously displayed gains of chromosomes 5, 7, and 10. This raises several intriguing possibilities. First, these tumors may arise as a result of sequential genetic alterations in different clones of cells. As shown in Figure 2, the pattern of gains within the LGGs was very similar, albeit not identical. Second, although we do not yet know the clinical significance of the extra chromosomal gains, especially in tumors with the 7q34 duplication, it was interesting to note that similar chromosomes are often gained in grade III gliomas. Attempts to identify the key genetic events in the recurrent HGGs, however, was complicated by the radiation effects and the large number of CNAs.

The use of SNP arrays in the clinical setting frequently results in the need for further investigation, especially among pediatric patients with solid tumors. One LGG in our study, case 12-175, demonstrated mosaic CN-LOH of 17p in approximately 35% of the tumor cells. Sequencing of TP53 (located in 17p13.1) revealed a frameshift mutation in exon 5 in w38% of cells. Genetic testing was especially warranted in this family since the patient's father was previously diagnosed with a brain tumor (GBM). Clinical sequence analysis of a blood sample showed that the mutation was not present in the patient's germline. To our knowledge, the blood DNA was not examined for CN-LOH. Thus, although the *TP53* mutation was likely acquired, it was not possible to determine if the CN-LOH was acquired or consistent with germline mosaic uniparental disomy (UPD). Similarly, several cases in our study demonstrated heterozygous or homozygous deletions of tumor suppressor genes that may predispose to cancer when inactivated in the germline, including *TP53*, *PTEN*, *RBI*, *CDKN2A*, *NF1*, and *NF2*. Further deletion and mutation studies were recommended when a germline deletion or ROH was suggested by the array analysis.

Similar to the mosaic case with 17p LOH described previously, genomic ROH, UPD, or regions of identity by descent (IBD) are easily visualized by SNP array analysis. With Illumina BeadChip arrays, the BAF will be either 0 or 1 when the ROH is present in the germline. This was the case in tumor 12-276, where large portions of the genome were in fact homo-zygous likely due to IBD. Identification of homozygous regions can prompt investigation into the genes contained within these regions and guide further genetic testing recommendations.

Our clinical cohort included five LGGs from patients with previously known cancer predisposition syndromes, including four patients with NF1 and one patient with NS. There were no alterations in *BRAF* by SNP array analysis or sequencing of the commonly mutated exon 15. Previous studies of CNAs in LGG suggest the mutual exclusivity of NF1 and BRAF MAPK pathway mutations, demonstrating that one hit in the pathway is sufficient for tumorigenesis (16,17); however, patients with NF1 have also reportedly demonstrated the presence of the *KIAA1549-BRAF* fusion (18), BRAF V600E mutation (49), or both the fusion and mutation (48). Combined SNP array studies and targeted sequencing studies are

therefore clinically indicated for patients diagnosed with these cancer predisposition syndromes.

Conclusions

The integration of SNP array data with targeted sequence analysis and histologic findings provides a more complete profile of the genomic alterations in pediatric brain tumors than can currently be achieved by standard cytogenetic analysis alone. The increasingly higher resolution of SNP arrays, as well as the concentration of probes located within known cancer-associated genes, will undoubtedly permit the identification of additional CNAs as this technology is used more routinely in diagnostic laboratories. Even with the development of next generation sequencing-based assays, SNP array studies will still provide information on copy number state and homozygosity that will help in the interpretation of sequence data, until such time that whole genome sequencing studies are available.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Distribution of classification of pediatric brain tumors included in this study. Ependymoma includes tumors from grades I, II, and III. LGGs include both grade I and grade II tumors. HGGs include both grade III and grade IV tumors. Two tumors did not fall within any of these categories and were classified as “Other.”

Figure 2.

CNAs and mutations in LGGs. Fusion positive tumors, *BRAF* mutations, CNAs involving selected genes, and whole chromosome aberrations are indicated as shown in the figure key. Tumors are ordered based on diagnosis, and final histopathologic diagnoses can be found in Supplementary Table 1. *CDKN2A*, *RB1*, *TP53*, and *NF1* CNAs were determined by SNP array. *Abbreviations:* Hmz, homozygous; Htz, heterozygous.

Figure 3.

SNP array findings in LGGs. (A) Gain of whole chromosome 7 and duplication in 7q34 in tumor 12-111. Breakpoints of the 7q34 duplication were located in the *KIAA1549* and *BRAF* genes. Deletion of 1p (B) and 19q (C) in the oligodendroglioma was observed in ~50% of cells (case 12-234). (D) Mosaic deletion in 6q, present in ~75% of cells from tumor 10-210, includes parts of the *MYB* and *QKI* genes. (E) Tumor 12-175 demonstrated CN-LOH of 17pterp11.2 in ~35% of cells. (F) Tumor 10-286 demonstrated mosaic CN-LOH of 9p in ~50% of cells with an additional homozygous deletion of 9p21.3, which includes *CDKN2A/B*. Exact breakpoints for CNAs can be found in Supplementary Table 2.

Figure 4. Chromothripsis in HGG. Whole genome view of tumor 12-118 displaying chromothripsis, which is most notable on chromosome 2 and is indicated by the arrow.

Figure 5. CNAs in MB identified by SNP array analysis. Chromosomal abnormalities are indicated according to the color key. Tumors are grouped based on classification into different subgroups of MB. *Abbreviation:* Htz, heterozygous.

Figure 6. SNP array analysis of a WNT group MB (case 13-003). Chromosomes 5 and 19 demonstrated ROH (BAF is equal to either 0 or 1).