Molecular Profiling of Pediatric and Adult Glioblastoma

A Spotlight on Mismatch Repair Proteins

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ABSTRACT

Objectives*: Although glioblastoma (GBM) is rare in the pediatric population, it is the most common cause of death among children with central nervous system neoplasms. Recent molecular profiling of these neoplasms has demonstrated distinct differences in comparison to their adult counterparts. Moreover, many pediatric GBMs occur within the context of cancer predisposition syndromes, such as constitutional mismatch repair deficiency syndrome (CMMRD). Children with CMMRD who develop GBM exhibit a high tumor mutational burden and may benefit from treatment with immune checkpoint inhibitors.*

Methods: *We performed next-generation sequencing and immunohistochemistry for mismatch repair proteins in our cohort of pediatric and adult GBMs to further characterize the molecular profiles of these groups.*

Results: *We examined a total of 11 pediatric and 11 adult GBMs. Pediatric patients had a higher number of alterations compared to their adult counterparts. They also had a higher frequency of alterations in the mismatch repair genes, which can be detected by immunohistochemistry (IHC). We also identified one pediatric patient with CMMRD syndrome.*

Conclusions: *Our study highlighted the distinct molecular differences between pediatric and adult GBM. We also demonstrated that pediatric patients have a higher frequency of alterations in the mismatch repair genes, which may render them susceptible to treatment with immune checkpoint inhibitors. These alterations can be detected using routine IHC and should be performed on all pediatric GBM.*

Key Points

- We performed next-generation sequencing in our cohort of pediatric and adult glioblastomas to highlight the different molecular profiles of these groups.
- We suggest that mismatch repair immunohistochemical stains should be used as a quick, simple method to identify pediatric glioblastoma patients who may benefit from immune checkpoint inhibitors.
- We highlight the importance of using the methods described to screen pediatric patients with glioblastoma for constitutional mismatch repair deficiency syndrome.

Glioblastoma (GBM) is the most aggressive glial tumor and is classified by the World Health Organization as grade IV.[1,2] The median age at diagnosis is 64 years, but GBM can occur at any age.[3] In the pediatric population, GBM is the most common cause of death among children with central nervous system neoplasms.[4] The median survival for these patients is less than 2 years with the current standard of care, which includes surgical resection followed by chemotherapy and radiation.[1]

Although they are histologically indistinguishable, the molecular biology of adult and pediatric GBM differs dramatically.[5,6] In recent years, large molecular profiling studies have allowed for classification of GBMs into molecular subgroups. Mutations in the epigenetic modulator gene isocitrate dehydrogenase 1 (*IDH1*), for example, has a key role as a driver mutation in adult GBM. Other alterations, such as *TP53*, *ATRX*, *TERT* promoter variants, and *MGMT* promoter methylation, have been discovered to have key roles in prognosis and response to therapy in the adult population. Pediatric cases may harbor *TP53* variants but often lack these

other alterations. Further molecular characterization of these rare tumors is essential for the identification of targeted therapies and further subclassification, especially in the pediatric population.

Pediatric GBMs can occur within the context of cancer predisposition syndromes, such as constitutional mismatch repair deficiency (CMMRD) syndrome, especially in geographic regions with high rates of consanguinity.[7] CMMRD syndrome results from germline biallelic variants within one of the 4 DNA mismatch repair (MMR) genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*), most commonly *PMS2*.[8] Children with CMMRD present with a variety of malignant neoplasms and typically present with the first malignancy at a median age of 7.5 years.[9] The most common malignancies are in the brain, gastrointestinal tract, and hematologic, and children with these malignancies often do not reach adulthood. These cases can be confusing clinically because patients may have café-au-lait macules, the hallmark feature of neurofibromatosis type 1, further complicating and often delaying the initial diagnosis of these patients.[10] Children with CMMRD who develop GBMs have been found to exhibit a high tumor mutational burden (TMB) and may benefit from treatment with immune checkpoint inhibitors.[4,6] This finding emphasizes the importance of early diagnosis.

In this study, we performed next-generation sequencing (NGS) of both adult and pediatric GBMs to characterize the molecular profiles of these groups in our patient population. We also evaluated the utility of MMR immunohistochemical (IHC) staining in those cases that harbored MMR genetic variants to determine whether there was concordance between the presence of deleterious mutations and the loss of protein expression.

Materials and Methods

GBM Patient Selection

Institutional review board approval, including a Health Insurance Portability and Accountability Act (HIPPA) waiver, was obtained before beginning this study. CoPath software was used to search for and identify cases for our study. A total of 22 GBM specimens were selected, including 11 adult and 11 pediatric cases diagnosed between 2004 and 2018. Patients younger than 21 years were considered pediatric cases. Although the majority of our patients fell within this traditional cutoff for pediatric patients, suggested by the American Academy of Pediatrics, we decided to include an additional 24-year-old patient (pediatric GBM case 8 [pGBM 8]) because this patient was initially treated by a pediatric neurologist and neurosurgeon. This patient also had a midline GBM that harbored a *H3F3A* K28M mutation, which is more common in the pediatric population. The adult cohort consisted of both *IDH*-mutant and *IDH* wild-type GBMs.

IHC for MMR Proteins

The original H&E-stained slides from each case were reviewed, and representative areas were selected from the corresponding formalin-fixed, paraffin-embedded block for each case. A tissue microarray was constructed with all cases in duplicate 2.0-mm cores. Immunohistochemistry was performed by the immunohistochemistry laboratory of University Hospitals Cleveland Medical Center for MLH1, MSH2, MSH6, and PMS2 ▮Table 1▮. Briefly, unstained 4-µm sections were prepared from paraffin blocks and baked for 30 minutes at 60°C in a Boekel Lab oven. The slides were then processed using a BenchMark Ultra Automated Immunostainer (Roche). The slides were deparaffinized, antigen retrieved, incubated in primary antibody, and subsequently counterstained on board the automated instrument. All detection was done using OptiView DAB IHC Detection Kits (Roche).

Next-Generation Sequencing

DNA was extracted from formalin-fixed, paraffin-embedded tumor samples using the Gentra Puregene Tissue Kit (Qiagen). NGS libraries were prepared using the Life Technologies Oncomine Comprehensive v3 Assay (Thermo Fisher). Targeted amplicon-based ion-semiconductor NGS was performed on the Ion Personal Genome Machine (Thermo Fisher) to examine single-nucleotide variants, copy-number alterations, and small insertions and deletions within 161 genes relevant to solid tumors. Variant calling was performed using the

▮Table 1▮
Antibodies Used in this Study

Antibody	Dilution	Source	Clone	Incubation, min	Instrumentation
MLH1[a]	Predilute	Roche	M1	24	BenchMark Ultra
MSH2[b]	Predilute	Roche	G219-1129	12	BenchMark Ultra
MSH6[a]	Predilute	Roche	SP93	12	BenchMark Ultra
PMS2[c]	Predilute	Roche	A16-4	32	BenchMark Ultra

[a]Antigen retrieval was performed with Cell Conditioning 1 (Roche), a Tris-based buffer with a slightly basic pH (8.5), for 64 min at 100°C.
[b]Antigen retrieval was performed with Cell Conditioning 1 for 40 min at 100°C.
[c]Antigen retrieval was performed with Cell Conditioning 1 for 92 min at 100°C.

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Ion Reporter Software version 5.6 (Thermo Fisher) with alignment to human genome assembly GRCh37 (hg19). Two of the pediatric cases, pGBMs 9 and 11, also had Foundation Medicine sequencing reports available. These reports were reviewed in conjunction with our NGS data.

Alterations detected are reported in Supplementary Table 1 (all supplementary material can be found at *American Journal of Clinical Pathology* online). Reference mRNA sequences and variant allele frequencies are listed in the table. For pGBM 11, the alterations listed were detected by Foundation Medicine. Reference mRNA sequences and variant allele frequencies were not provided by Foundation Medicine.

Results

In the pediatric cohort, the mean age at the time of diagnosis was 14 years and ranged from 4 to 24 years. In this group, 54.5% (6/11) of patients were female and 45.5% (5/11) were male. In the adult cohort, the mean age at the time of diagnosis was 52 years and ranged from 36 to 70 years. In this group, 64% (7/11) of the patients were male and 36% (4/11) were female.

We detected genomic alterations in all pediatric GBMs, with an average of 5.6 alterations per sample and a median of 4 (**Table 2** and Supplementary Table 1). The most frequently mutated gene was *TP53* in 54.5% (6/11) of cases. *H3F3A* was mutated in 27% (3/11) of samples, all of which were midline GBMs harboring the K28M variant. We also detected MMR genetic alterations in 27% (3/11) of the pediatric cases. This correlated with samples that had the highest number of alterations, with an average of 12.7 genomic alterations per sample.

Table 2
Genetic Variants Within Pediatric Glioblastoma Cases

	Pediatric Glioblastoma Cases										
Gene	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
TP53		X		X				XX	XX	X	XX
MMR	X				X						XX
SETD2	XX										X
NOTCH 1-3					X	X	X				X
H3F3A		X						X	X		
BRCA 1/2	X		XX								XX
NF1				XX	X						
TSC1	X		X								
RB1					X						
POLE			X								
ATRX				X							
Other mutations	3				1	1	2		2		9

X, an alteration in the gene; XX, >1 alteration in the gene.

Table 3
Genetic Variants Within Adult Glioblastoma Cases

	Adult Glioblastoma Cases										
Gene	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
TP53	X		X	X						X	
IDH1	X			X	X						
TERT		X									
PTEN		XX	X			X			X		
H3F3A											
ATRX	X										
EGFR						X	X	X			
BRAF								X			
RB1										XX	
NF1										X	
KRAS					X						X
Other mutations		1			1			1			

X, an alteration in the gene; XX, >1 alteration in the gene.

In the adult cohort, genomic alterations were also detected in all samples, with an average of 2.5 alterations per sample and a median of 2 (**Table 3** and Supplementary Table 1). None of the adult cases showed alterations in the MMR genes. The most frequently mutated gene was *TP53* (36%, 4/11). *PTEN* mutations were identified in 27% (3/11) of cases. Other genomic alterations included *IDH1* (27%, 3/11), *EGFR* (27%, 3/11), and *KRAS* (9%, 1/11) amplifications. A *TERT* promotor mutation was identified in 1 case.

Compared with adult GBM, pediatric GBM was enriched not only for variants within MMR genes but also for other genes involved in DNA repair (*BRCA1, BRCA2, POLE, SLX4, FANCD2, FANCI, RAD50, ATM*, and *CHEK2*). MMR IHC staining was preserved in all adult cases. In the pediatric cohort, 27% (3/11) showed loss of staining with at least one antibody; pGBM 1 demonstrated preserved MSH2 staining and loss of MSH6 **Image 1**. In addition, pGBM 5 demonstrated loss of both MLH1 and PMS2 staining **Image 2**, and pGBM 11 showed complete loss of PMS2 staining **Image 3**.

Discussion

In this study, we analyzed the molecular profile of our cohort of pediatric and adult GBMs. The overall molecular findings highlight the distinct molecular landscape of pediatric GBMs in comparison to their adult counterparts. The pediatric patients also had a higher TMB than the adult patients.

The pediatric cohort demonstrated a significant number of alterations in MMR genes. We noted that pGBMs 1, 5, and 11 all demonstrated loss of MMR IHC

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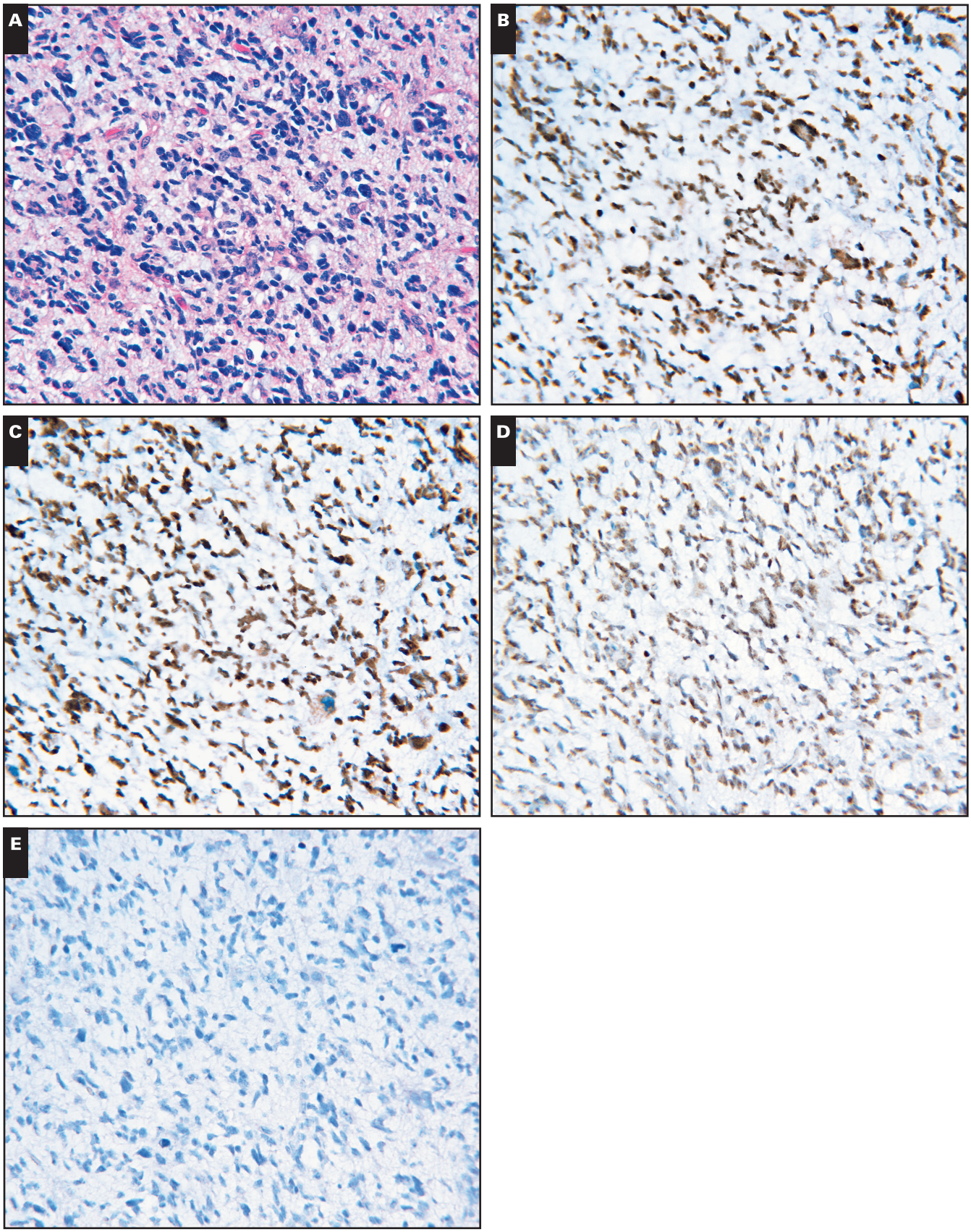

Image 1 **A**, H&E-stained section of pediatric glioblastoma case 1 harboring *MSH2* p.Gln218* mutation and corresponding immunohistochemistry showing retained MLH1 (**B**), PMS2 (**C**), and MSH2 (**D**) staining and loss of MSH6 (**E**) staining.

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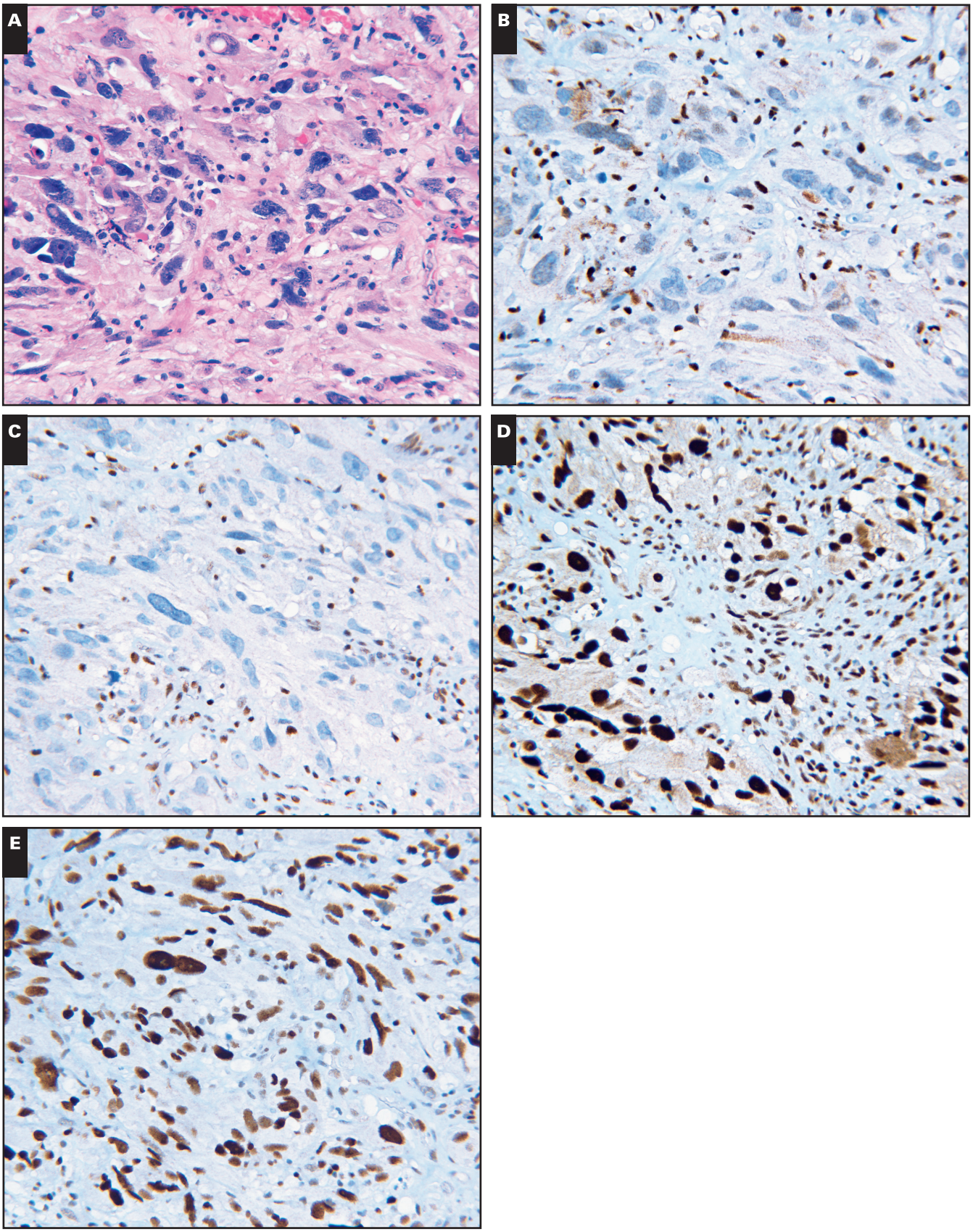

Image 2 **A**, H&E-stained section of pediatric glioblastoma case 5 harboring *MLH1* p.Ser698* mutation and corresponding immunohistochemistry showing loss of MLH1 (**B**) and PMS2 (**C**) staining in tumor cells and retained MSH2 (**D**) and MSH6 (**E**) staining.

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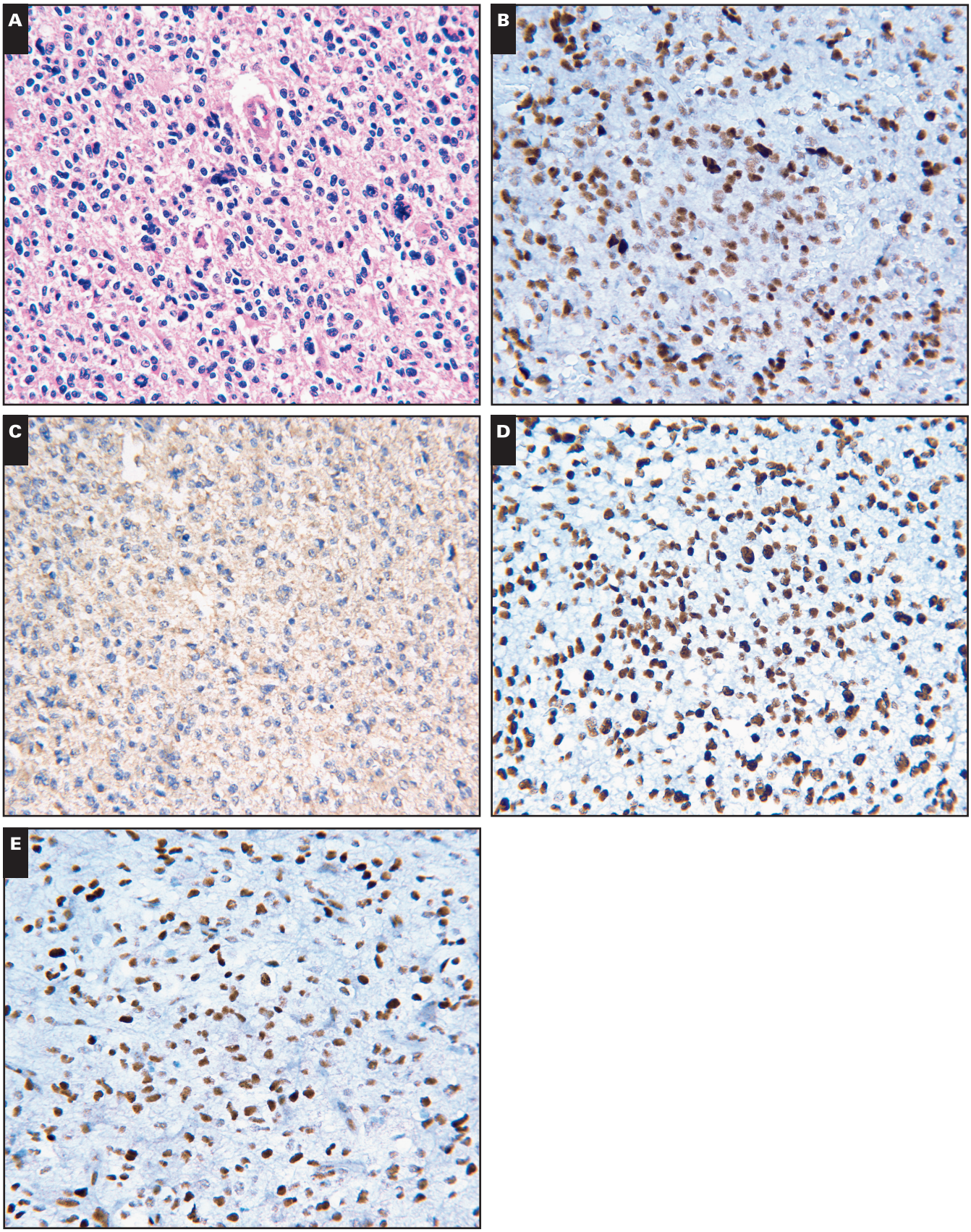

Image 3 **A**, H&E-stained section of pediatric glioblastoma case 11 harboring a germline *PMS2* mutation and corresponding immunohistochemistry showing retained MLH1 (**B**), MSH2 (**D**), and MSH6 (**E**) staining and complete loss of PMS2 (**C**) staining.

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staining and had corresponding MMR gene mutations identified by NGS. In pGBM 1, MSH2 staining appeared intact and MSH6 staining was lost. This is likely due to the patient's *MSH2* mutation resulting in a nonfunctional MSH2 protein that is still antigenic and recognized by the *MSH2* IHC antibody. However, there was loss of staining in the corresponding heterodimer, MSH6. In pGBM 5, a *MLH1* mutation was identified by NGS. IHC was consistent, showing loss of MLH1 and PMS2 staining.

In pGBM 11, a 4-year-old male patient had café-au-lait macules and a suspected diagnosis of neurofibromatosis type 1. The patient's family history was significant for lung cancer, colon cancer, bladder cancer, and Hodgkin lymphoma. IHC showed loss of PMS2 staining in both tumor and normal tissue. NGS identified a *PMS2* mutation and a high TMB. This raised the possibility of a germline biallelic *PMS2* mutation resulting in CMMRD. The patient received germline testing at an outside hospital that confirmed a diagnosis of CMMRD. He was treated with surgical resection and radiation and enrolled in a clinical trial at an outside hospital but died 16 months after his initial diagnosis. A complete autopsy was performed at our hospital and showed extensive involvement by GBM. The tumor involved numerous areas of the brain, including the right and anterior frontal lobes, right and left anterior forebrain, right and left basal ganglia, corpus callosum, right hippocampus, and right and left cerebellar hemispheres Image 4.

Children with GBM have an overall poor prognosis, and primary management includes surgery,

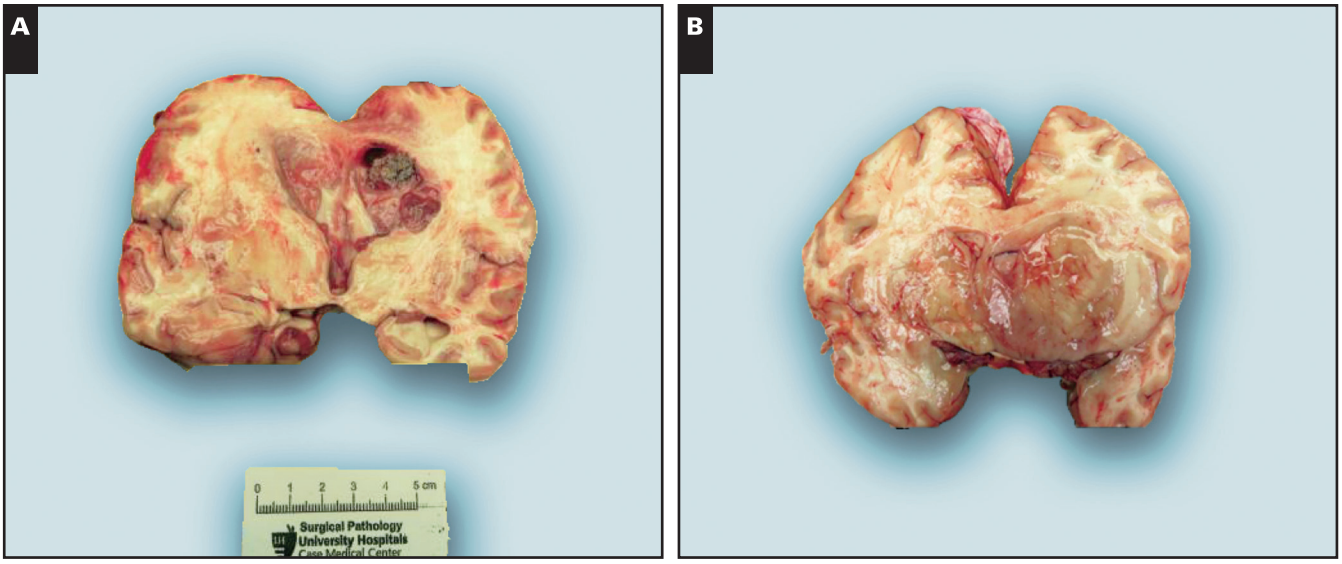

Image 4 Gross autopsy photos from pediatric glioblastoma (GBM) case 11. **A,** Residual GBM involving the left hemisphere with an area of necrosis. **B,** Residual GBM involving both the left and right hemispheres.

Table 4

Treatment Summary and Clinical Outcome of Pediatric Patients

pGBM Case	Patient Age at Diagnosis, y	Surgery Type	Radiation	Temozolomide	Clinical Outcome
1	12	ST	X	X	Deceased 15 mo after diagnosis (infection and respiratory failure)
2	13	B	X	X	Deceased 17 mo after diagnosis (ventricular shunt infection)
3	14	T	Unknown	Unknown	Treated at outside hospital; no follow-up information
4	19	T	Unknown	Unknown	Treated at outside hospital; no follow-up information
5	21	T	X	X	Disease free at 5 y after diagnosis
6	9	ST	Unknown	Unknown	Treated at outside hospital; no follow-up information
7	20	T	X	X	Disease free at 7 y after diagnosis
8	24	B	X	X	No follow-up information available
9	12	T	X	X	Deceased 10 mo after diagnosis (respiratory failure)
10	8	B	Unknown	Unknown	Unknown, discharged with palliative care 3 mo after diagnosis
11	4	ST	X		Deceased 16 mo after diagnosis

B, biopsy; pGBM, pediatric glioblastoma; ST, subtotal resection; T, total resection; X, received corresponding treatment.

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following by radiation and chemotherapy.[11] Our patient cohort was mainly treated with this standard protocol Table 4. Tumors resulting from CMMRD have been shown to have a survival rate similar to their sporadic counterparts.[12] Because these tumors are hypermutated, immune checkpoint inhibitors have emerged as possible treatment for these patients.[4] Nivolumab, which inhibits receptors such as programmed cell death protein 1 (PD-1), a T-cell coinhibitory receptor, and its ligand (programmed cell death ligand [PD-L1]), has been reported in the literature as a potential treatment option. Two separate case studies have demonstrated a profound radiologic response in a total of 3 CMMRD patients with GBM treated with nivolumab.[13,14] Patients with somatic mutations in MMR genes may also benefit from treatment with immune checkpoint inhibitors, given their high TMB. In our pediatric cohort, 2 patients had suspected somatic mutations in MMR genes (pGBMs 1 and 5) and demonstrated a high TMB in comparison to those patients without MMR mutations. Immune checkpoint inhibitors have also been suggested as cancer-preventive treatments in those patients with known CMMRD; however, further investigation is needed.[9]

Identification of CMMRD patients has other important clinical implications that extend beyond treatment. Patients with CMMRD, along with the patient's parents and siblings, should be referred to medical genetic counselors for evaluation.[10] Because CMMRD results from biallelic germline variants, each parent has 1 mutated allele. This means that the patient's siblings each will have a 25% chance of inheriting 2 mutated alleles, resulting in CMMRD. Siblings will also have an additional 50% chance of inheriting 1 mutated allele, resulting in Lynch syndrome.[14] This information may lead to earlier and/or more frequent preventive cancer screenings. Some literature even suggests that whole-body magnetic resonance imaging, as used in patients with Li-Fraumeni syndrome, may be the best cancer screening tool for patients with CMMRD.[9] Anti-inflammatory drugs, such as aspirin or ibuprofen, have been suggested as cancer-preventive treatments for these patients. This information may also have a profound impact on future family planning.

MMR expression has also been reported as an important factor in GBM response to temozolomide (TMZ). TMZ is an alkylating chemotherapy agent that is commonly used in the treatment of GBM. On average, TMZ increases the life expectancy of patients by 1 to 2 months. The toxicity of TMZ is mediated primarily by MMR-dependent processing at O^6-methylguanine base lesions produced by TMZ. The *MGMT* gene encodes a DNA repair enzyme that removes O^6-guanine adducts from DNA, decreasing the effectiveness of TMZ.[15] *MGMT* promoter methylation resulting in epigenetic silencing and loss of DNA repair enzyme capabilities is a recognized mechanism by which tumor cells become more sensitive to TMZ. Because MMR proteins are also an essential part of the mechanism of action of TMZ, recent studies have demonstrated that decreases in MMR protein levels contribute to TMZ resistance.[16] Therefore, MMR protein expression may be a useful marker for predicting response to TMZ.

Of note, in pGBM 10, an 8-year-old male patient had a history of an anaplastic ependymoma diagnosed at 15 months old and was suspected of having a radiation-induced GBM. NGS identified a *TP53* mutation, which is consistent with recent literature suggesting that radiation-induced gliomas frequently harbor *TP53* mutations. No other mutations were identified, and this case had the lowest TMB within the pediatric cohort. Lopez et al[17] identified a low TMB in their cohort of radiation-induced gliomas. These tumors also notably lack mutations in commonly mutated GBM genes such as *IDH1*, *IDH2*, *H3F3A*, *TERT,* and *PTEN*, suggesting that radiation-induced GBMs have a distinct molecular profile.

In our study, we highlighted the distinct molecular differences between pediatric and adult GBMs. We also suggest that MMR IHC could be incorporated into the diagnostic work-up of select pediatric patients with GBM as a quick and easy-to-interpret method to screen for those patients who may have mutations in MMR genes. Detection of these mutations is helpful for identifying patients with CMMRD and thus identifying families that may benefit from screening for Lynch syndrome. In addition, patients with both germline and somatic mutations may benefit from treatment with immune checkpoint inhibitors. We suggest that all pediatric patients with GBM should be screened using immunohistochemistry. NGS can be performed as a confirmatory testing method. Although pediatric patients with GBM have an overall poor prognosis, this method can help provide essential diagnostic, prognostic, and therapeutic information for these patients.

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