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Cytokines in Pediatric Pilocytic Astrocytomas: A Clinico-Pathological Study

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Abstract: Pilocytic astrocytomas (PCA) are WHO Grade I tumors with a favorable prognosis. Surgical resection is usually curative. Nonetheless, progressive and/or metastatic disease occurs in 20% of patients. For these patients, treatment options are limited. The role of the immune system in PCA has not previously been reported. We hypothesize that the circulating cytokines contribute to tumorigenicity in PCA. This is an exploratory study with a focus on the identification of circulating cerebrospinal (CSF) cytokines associated with PCA. The primary objective is to demonstrate that CSF cytokines will be differentially expressed in the subset of PCAs that are difficult to treat in comparison to their surgically amendable counterparts. This is a single-institution, retrospective study of prospectively collected data. Patients with a confirmed histological diagnosis of PCA who have simultaneous intraoperative CSF sampling are included. Cerebrospinal fluid samples are subjected to multiplex cytokine profiling. Patient-derived PCA lines from selected patients in the same study cohort are cultured. Their cell culture supernatants are collected and interrogated using the sample multiplex platform as the CSF. A total of 8 patients are recruited. There were two patients with surgically difficult tumors associated with leptomeningeal involvement. Multiplex profiling of the cohort's CSF samples showed elevated expressions of IFN-γ, IL-2, IL-12p70, IL-1β, IL-4, and TNF-α in these two patients in comparison to the remaining cohort. Next, primary cell lines derived from the same PCA patients demonstrated a similar trend of differential cytokine expression in their cell culture supernatant in vitro. Although our findings are preliminary at this stage, this is the first study in pediatric PCAs that show cytokine expression differences between the two groups of PCA with different clinical behaviors.

Keywords: cerebrospinal fluid; cytokines; pilocytic astrocytoma

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1. Introduction

Brain tumors are the most common solid tumors in children, comprising 25% of all childhood cancers [1–3]. In this group, pilocytic astrocytomas (PCA) are the second most common, accounting for 21 to 23% of all pediatric brain tumors [3–7]. According to the

World Health Organization (WHO), PCA is classified as a Grade I tumor [3,8]. They can arise anywhere along the neuroaxis. Specifically, for PCA, the most common location is in the cerebellum, followed by the supratentorial compartment. Other places of origin include the brainstem and the spinal cord [1–3,5,9–12]. The male/female ratio is estimated to be 1.12 [11]. Broadly speaking, PCA has an extremely favorable overall prognosis with its 10-year overall survival rate as high as 96% [4,7,13]. The treatment of choice, whenever possible, is gross total surgical resection (GTR). For the majority of cases, no adjuvant radiation or chemotherapy is required [4,7]. These tumors seldom progress to higher grade gliomas, and dissemination is infrequent [14,15].

Nonetheless, recurrence and progressive disease occur in 20% of patients [7], and occasionally, tumors spread into the cerebrospinal fluid (CSF) may be encountered [7,16,17]. In this latter subset of patients, their tumors tend to be located at unresectable sites such as the optic tract, hypothalamus, and deep, midline regions adherent to important vasculature [7,18]. These lesions are notoriously difficult to treat, as they demonstrate a sustained tendency for progression [14] and often become a chronic disease with substantial morbidities [19]. Affected patients often suffer from life-long endocrinopathies, progressive cognitive and adaptive functioning impairments [20].

Recent studies have implicated inflammation as an important contributor in tumor behavior [21–23]. In particular, cytokines are known to coordinate various pro-inflammatory responses within the tumor microenvironment, acting on autocrine and/or paracrine pathways on both malignant and non-malignant cells [24]. Depending on the tumor microenvironment, cytokines can modulate anti-tumoral responses. Paradoxically, the same factors may induce cell transformation and malignancy instead during chronic inflammation [25]. Previous studies have demonstrated that expression changes in various cytokines have been observed in the CSF of brain tumor patients [26,27]. Nonetheless, it remains uncertain at this stage what the exact roles of these circulating CSF cytokines are in brain tumors; in particular, whether they are pro- or anti-oncogenic.

Currently, we are aware of the benefits of immunotherapy for various malignancies [28,29]. Here, treatment leverages the high specificity of the immune system to target and eliminate neoplastic cells while leaving healthy cells undamaged [30]. Extrapolating this concept into CNS malignancies, a thorough understanding of the immune environment of brain tumors will be a requisite to identify effective immune-based treatments [30]. For the purposes of this study, the authors postulate that immunotherapy may be helpful for the subset of therapeutically difficult PCAs. On a separate note, brain tumors by virtue of their location, are anatomically in contact with the circulating CSF [31,32]. In addition, CSF is a readily accessible body fluid rich in immune-related factors, allowing it to be reflective of the pathological state of the CNS [27]. Therefore, examining the CSF of brain tumor patients is likely to yield potential cytokines of interest that provide insight into their individual tumor environments. However, there is no previous study that specifically compared CSF cytokine profiles between different PCAs. Building on this knowledge, the authors hypothesize the following: firstly, CSF cytokines are differentially expressed in the subset of PCAs that are surgically difficult to treat, in comparison to their surgically amendable counterparts; and next, PCA tumor cells will secrete these same similar cytokines into their own microenvironment.

2. Materials and Methods

2.1. Study Design and Patient Demographics

This was a single-institution, retrospective study of prospectively collected data approved by the hospital ethics review board (Singhealth CIRB Ref: 2014/2079). All patients and their legal guardians provided signed informed consent for the research use of their medical data and biomaterials. This study was meant to be exploratory; with a key focus on the identification of circulating CSF cytokines associated with PCA in the study cohort. Inclusion criteria consisted of patients who underwent surgery by the Neurosurgical Service, KK Women's and Children's Hospital, and had proven histological diagnosis

of PCA. Patients who did not have a diagnosis of PCA; those who were operated on in other hospitals, with incomplete medical records, and/or who had insufficient CSF/tumor tissue were excluded. For the purposes of this study, we defined 0% of tumor remnant as the achievement of gross total resection (GTR), $\leq$ 10% tumor remnant as near total resection (NTR), and $\geq$ 30% remnant as subtotal resection (STR), based on postoperative radiological results.

2.2. Tumor Histopathological Diagnosis

All available archival histological glass slides, including immunohistochemical stains from the study cohort, were retrieved and reviewed by an in-house pathologist in relation to diagnostic criteria laid out in the current (2016) World Health Organization Classification of Tumors of the Central Nervous System [9].

2.3. Cerebrospinal Fluid Collection

Cerebrospinal spinal fluid was collected at the time of surgery as part of the operative procedure. For cytological examination, samples of CSF were mixed with up to 2 drops of BD Surepath™ preservative fluid (BD system, Franklin Lakes, NJ, USA), placed in a cytospin sample chamber, and centrifuged at 600 rpm for 6 min. Alcohol-fixed smears (95% ethanol for at least 60 min) were placed in an autostainer machine for Papanicolau staining, and air-dried smears were stained using the Hemacolour™ stain. Both cytospin samples and stained smears were then reviewed by our in-house pathologists for tumor cells.

2.4. Patient-Derived Tumour Cell Cultures and In Vitro Experiments

Pilocytic astrocytoma tumor samples were finely minced, washed in Dulbecco's Phosphate Buffered Saline (Capricorn Scientific, Germany) prior to culturing in culture media containing DMEM-F12A with L-Glutamine (Capricorn Scientific, Ebsdorfergrund, Germany) supplemented with 10% fetal bovine serum (Capricorn Scientific, Ebsdorfergrund, Germany) and 2% actinomycin (Naclai Tesque, Kyoto, Japan). Cell cultures were maintained in a 37 °C, humidified incubator containing 5% CO_2 and 95% air. Subsequently, viable cells were sub-cultured via trypsinization and expanded. Only cells cultured at less than 8 passages were used in the subsequent experiments for this study.

2.5. Molecular Investigations of Tumour and Patient-Derived Tumour Cell Lines

Ribonucleic acid (RNA) was extracted from FFPE tissue using Reliaprep FFPE Total RNA Miniprep System (Promega, Madison WI, USA) according to the manufacturer's instructions. Next, RNA concentration was measured by 260 nm absorbance using biophotometer plus spectrophotometer (Eppendorf, Hamburg, Germany). Approximately 100 to 350 ng of extracted RNA from each sample was used for the one-step RT-PCR using GoTaq 1-Step probe RT-PCR kit (Promega, Madison, WI, USA). Primers and probe flanking *KIAA1549-BRAF* breakpoint (68bp)–5′-CGTCCACAACTCAGCCTACA-3′ 5′-CCTCCATCACCACGAAATCCTT-3′; 5′-TCGGATGCCCAGACTTG-3′ (probe)-were used to detect the presence of breakpoint sequence of the exon 15 of KIAA1549 and exon 9 of BRAF using Rotor-gene Q real-time cycler (Qiagen, Düsseldorf, Germany) [33]. The temperature profile for the one-step RT-PCR was 45 °C for 15 min, 95 °C for 5 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min with yellow signal detection in every cycle at 60 °C. The specific amplification curve comparable to the positive control indicated the presence of the *KIAA1549-BRAF* (type 15-9) fusion transcript. Efforts to correlate the molecular profiles of patient-derived tumor cell lines helped to verify there was a minimal genetic drift from their original tumors [34].

2.6. Real-Time Quantitative Polymerase Chain Reaction for mRNA Expression

Ribonucleic acid (RNA) was extracted from patient-derived PCA cell lines using RNeasy Mini Kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's instructions prior to lysing cells in Trizol® Reagent (Life Technologies, Carlsbad, CA, USA).

Reverse transcription was performed using GoScript TM Reverse Transcriptase (Promega, Madison, WI, USA) with a starting material of 1µg per sample. The prepared cDNA of 25 ng was subjected to a real-time quantitative polymerase chain reaction (RT-qPCR) using SoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Irvine, CA, USA) on the CFX 96TM Real-Time Detection System (Bio-Rad Laboratories, Irvine, CA, USA) according to the manufacturer's instruction. The following primer sequence was used for the gene of interest, GFAP (forward primer: 5′-CTGGAGGTTGAGAGGGACAA-3′; reverse primer: 5′-CAGCCTCAGGTTGGTTTCAT-3′) with expression normalized against the following housekeeping gene. RPL13A (forward primer: 5′- CATAGGAAGCTGGGAGCAAG-3′; reverse primer: 5′-GCCCTCCAATCAGTCTTCTG-3′). ACTB (forward primer: 5′-TGACCCAGACATGTTTGAGA-3′; reverse primer: 5′- TACGGCCAGAGGCGTACGG-3′) was used as an extra validation housekeeping gene. Threshold cycles (Ct) were calculated automatically using the CFX Manager™ Software (Version 3.1) (Bio-Rad Laboratories, Irvine, CA, USA).

2.7. Cytokine Multiplex Assay: CSF and Cell Supernatant Samples

The LUNARIS^{TM} Human 11-Plex Cytokine Kit (AYOXXA Biosystems, Cologne, Germany) was used to measure cytokine concentration in the CSF and primary cell culture supernatants. This was a multiplex assay based on beads-on-a-chip technology with a classical sandwich immunoassay principle, followed by a fluorescence readout. The calibration curve was generated according to the manufacturer's instructions. Briefly, 7 standards were prepared by serial dilution (1:4) of the Human Cytokine Standard using Assay Diluent 2. The standard consisted of the following analytes: GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 (p70), and TNF-α. The LUNARIS^{TM} BioChips were first washed. Following that, 5 µL of standard, blank, and experimental samples, which were pre-diluted in Assay Diluent 2 (1:2), were loaded into the appropriate wells. After incubation of approximately 3 h at room temperature, the BioChips were washed again. Next, 10 µL of detection antibody solution was added per well and incubated for 60 min at room temperature. The BioChips were washed again, followed by the addition of 10 µL of streptavidin-phycoerythrin reagent per well. After 30 min of incubation, the final washing step was performed. The BioChips were then air-dried and imaged using the LUNARIS^{TM} Reader (LRS-001). The quantification of the readout was performed using the LUNARIS^{TM} Analysis Suite Software. Protein concentration was reported as ρg/mL for each analyte.

2.8. Statistical Analysis

All CSF- and cell supernatant experiments were performed in a minimum of 3 technical replicates. Data were presented as mean ± standard deviation (SD). Statistical analyses were performed using either Analysis of Variance (ANOVA) test or a Student's *t*-test, depending on the analysis required. Differences between sample means were considered statistically significant when the *p*-value was less than 0.05 (*) or less than 0.001 (**). Graphs were generated using GraphPad Prism version 8.3.1 (GraphPad Software, SD, USA).

3. Results

3.1. Study Recruitment

Based on the study's criteria, a total of 8 patients were recruited. There were 5 males and 3 females. Their ages ranged from 1 year to 19 years old (median age 9.88 years old). Six patients underwent either GTR or NTR for their tumors. There were 2 patients with leptomeningeal disease (LMD) at the time of diagnosis (Patients 2 and 5). In comparison to the others, these two patients (Patients 2 and 5) were challenging to manage, and their clinico-pathological details were described in the following as illustrative cases (Table 1).

Table 1. Summary of study cohort's clinico-pathological information. Of notes, Patient 2 is labeled as *2aˆ* and *2bˆ*, respectively, as she underwent two surgeries. Patient 5 (labeled as 5*) was first diagnosed 10 years earlier. (Abbreviation: EOR = extent of resection).

Patient Number	Location of Tumor	Presence of *KIAA-BRAF* Fusion Gene (Yes/No)	BRAF V600E Mutant Positive (Yes/No)	LMD (Yes/No)	Adjuvant Treatment before Surgery (Yes/No)	CSF Cytology Positive for Tumour Cells (Yes/No)
1	Suprasellar	No	No	No	No	No
2aˆ	Left frontotemporal	*Yes (15-9)*	*Yes*	*Yes*	*No*	***No***
2bˆ	Left frontotemporal	*Yes (15-9)*	*Yes*	*Yes*	*Yes (Chemotherapy)*	***No***
3	Third ventricle	No	Yes	No	No	No
4	Third ventricle	No	No	No	No	No
*5**	Right thalamus, brainstem, and temporoparietal	*No*	*Yes*	*Yes*	*Yes (Radiation)*	***No***
6	Posterior fossa	No	No	No	No	No
7	Posterior fossa	Yes (15-9)	No	No	No	No
8	Posterior fossa	Yes (16-9)	No	No	No	No

3.1.1. Patient 2: Large Frontotemporal PCA with Intratumoral Arterio-Venous Shunting

A 3-year-old female presented with cachexia, progressive loss of vision, and developmental delay. Magnetic resonance imaging (MRI) of her neuroaxis reported a large, heterogeneously enhancing mass centered in the left frontotemporal lobe extending into the right frontal lobe and left orbit, with central necrosis and intratumoral arterio-venous shunting. There was also a nodular leptomeningeal extension into the internal auditory canal. Significant mass effect with midline shift to the right and obstructive hydrocephalus was seen. She underwent two tumor debulking surgeries approximately 6 months apart. In between the surgeries, she did chemotherapy, consisting of carboplatin and vincristine [32]. The second surgery proceeded with another 40% of the tumor being removed. Significant blood loss and hemodynamic instability halted the surgery. The decision was made not to proceed in view of the patient's safety. She has since recovered back to her baseline neurological status (Figure 1).

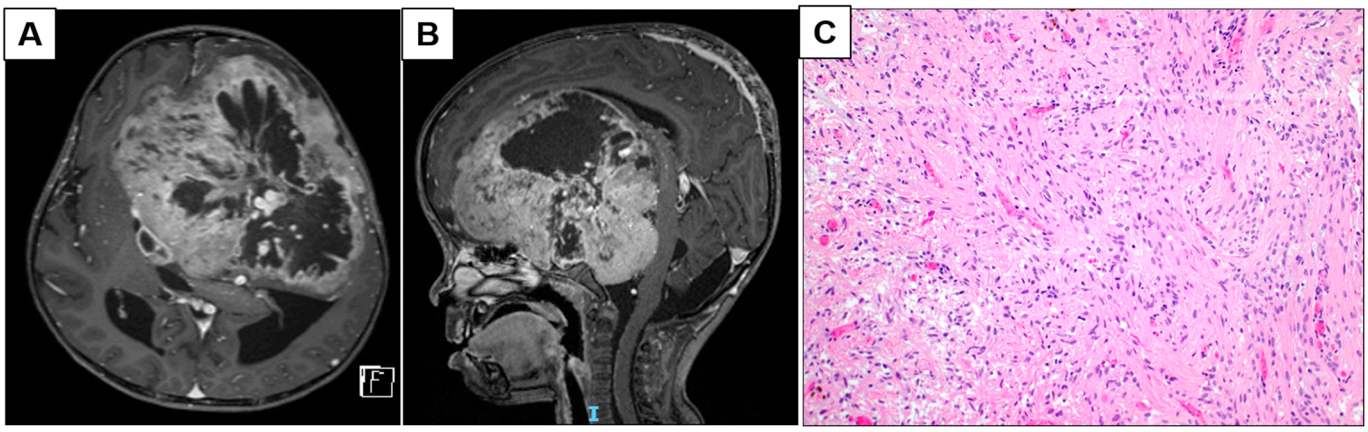

Figure 1. Representative T1-weighted, post-contrast MRI images of Patient 2's tumor in axial (**A**) and sagittal (**B**) views, respectively. They depict a large, frontotemporal heterogeneously enhancing solid-cystic tumor extending into the right frontal lobe and left orbit. Significant mass effect with right midline shift and obstructive hydrocephalus is also observed. (**C**) A hemotoxylin and eosin-stained photomicrograph of Patient 2's from her first surgery. This is a PCA that comprises bipolar cells with bland nuclei. Rosenthal fibers are present. (magnification ×400).

3.1.2. Patient 5: Progressive Intraventricular PCA with Leptomeningeal Disease

This is a previously well 9-year-old male who initially presented with an unsteady gait, left hemiparesis, and symptoms of raised intracranial pressure. An urgent MRI demonstrated a multi-lobulated cystic lesion involving the right thalamus, midbrain, and hemipons. There was also tumor extension across the right cerebellar peduncle to the right temporoparietal lobe. Areas of nodular enhancement were seen. Resultant hydrocephalus associated with periventricular lucency was observed. Subtotal excision of the lesion and insertion of a ventriculoperitoneal shunt was performed. Histopathology reported a pilocytic astrocytoma with visible infiltration into pia-arachnoid layers. Ki-67 index was 1 to 2% (Figure 2). The tumor remnant was subjected to intensity-modulated radiation therapy (IMRT) of 42 Gy over fractionated into 25 sessions. Approximately 10 years later, surveillance scans showed a solid-cystic lesion in the right postero-temporal region, associated with extensive leptomeningeal disease involving the skull base, along the optic apparatus, supra- and infratentorially (Figure 3). The decision was made for excision of the right cerebellopontine lesion for histological reassessment and adjuvant chemotherapy after.

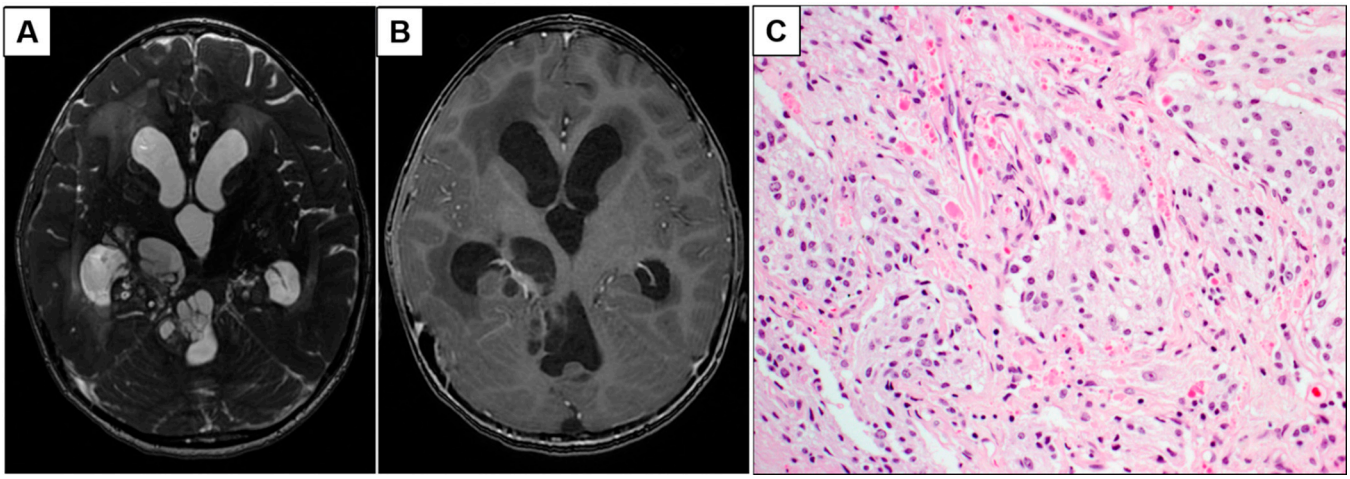

Figure 2. Representative MRI brain images of Patient 5 in (**A**) T2-weighted, axial, and (**B**) T1-weighted, post-contrast views when he was first diagnosed at 9 years old. There is a lobulated cystic mass with minimal solid components involving the right thalamus, midbrain, hemipons, cerebellar peduncle, and medial temporal lobe. Obstructive hydrocephalus is also noted. (**C**) A hemotoxylin and eosin-stained photomicrograph of Patient 5's tumor during his first surgery. The lesion comprises bipolar cells with bland nuclei. Eosinophilic granular bodies are present. (magnification ×400).

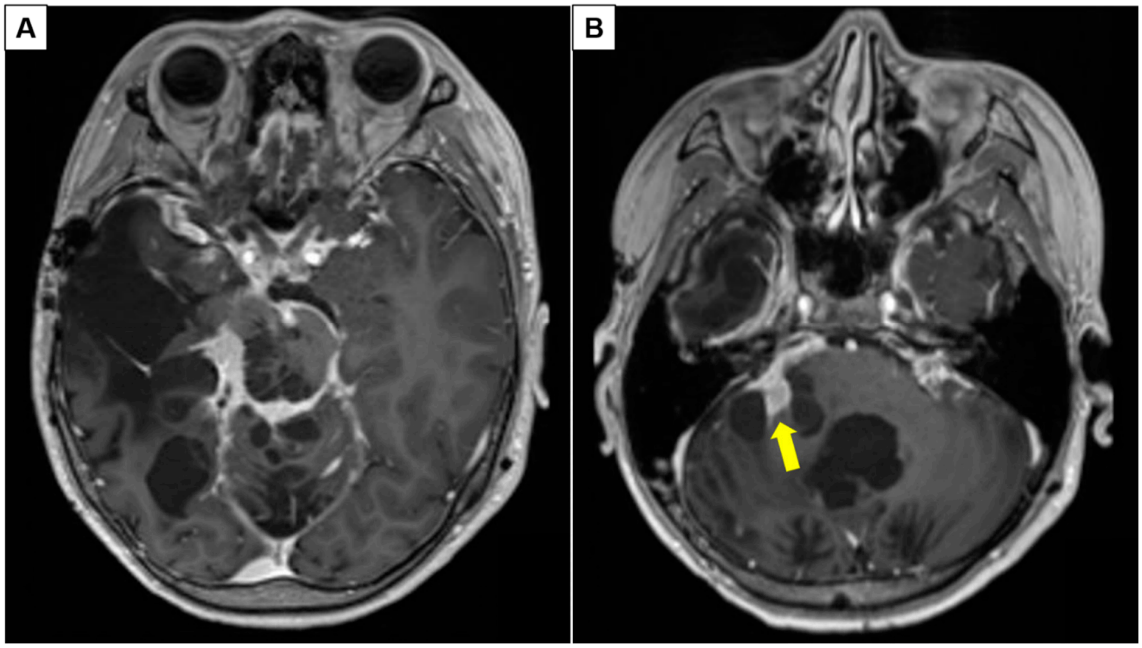

Figure 3. Representative MRI brain images of Patient 5 performed 10 years after his initial diagnosis. (**A**) T1-weighted, post-contrast image in axial view shows multiple confluent intra-axial multiloculated cystic lesions with enhancing solid components involving the right thalamus, basal ganglia, frontal, temporal, and midbrain. (**B**) T1-weighted, post-contrast image in axial view with a solid-cystic lesion along the right cerebellopontine lesion (yellow arrow). This lesion was excised in entirety for reduction of local mass effect and histological reassessment.

3.2. Selected CSF Cytokines Are Differentially Expressed in Therapeutically Challenging PCAs

Multiplex profiling of CSF specimens from the 8 patients observed statistically significantly higher expressions of IFN-γ, IL-2, IL-12p70, IL-1β, IL-4, and TNF-α for Patients 2 and 5, in comparison to the other patients. The remaining cytokines GM-CSF, IL-5, IL-6, and IL-8 demonstrated equivocal findings; neither did they show any trend nor statistical significance (Figure 4). Of note, results from a separate study with CSF collected from non-tumor patients were used to corroborate these findings (Appendix A and Figure A1).

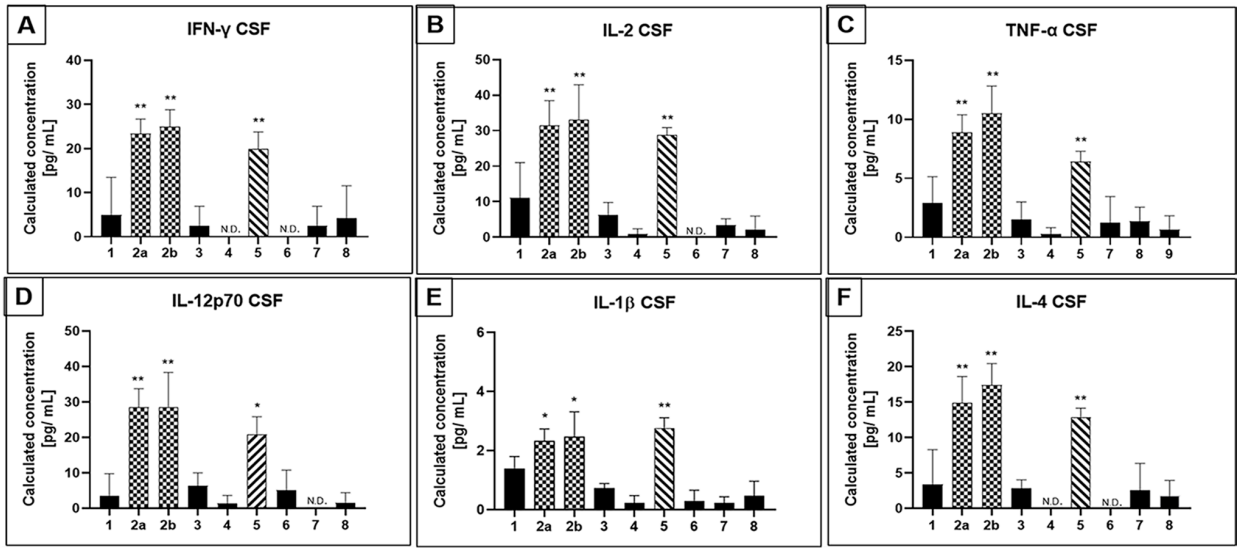

Figure 4. Graph results of CSF cytokine profiling that show differential expression with statistical significance for Patients 2 and 5 versus the rest of the patients. These include (**A**) IFN-γ, (**B**) IL-2, (**C**) TNF-α, (**D**) IL-12p70, (**E**) IL-1β, and (**F**) IL-4. (Abbreviation: N.D. = not detected). * $p < 0.05$; ** $p < 0.001$.

3.3. Patient-Derived Tumour Cells Express PCA Molecular Markers and Secrete Cytokines In Vitro

Patient-derived PCA cell lines were successfully cultured from Patients 2 (both surgeries), 3, and 5. They were examined for GFAP mRNA expression and KIAA1549-BRAF gene fusion. The results concurred with the patients' corresponding primary tumors (Table 2). Following that, cytokine profiling of their cell supernatants in culture demonstrated similar trends to the corresponding tumors, except for IL-4. However, only IL-12p70 statistically significant for Patient 2 (cell lines from both surgeries) and Patient 5. For this experiment, cytokine expression was compared to the blank (cell culture media only) for the statistical analysis (Figure 5).

Table 2. Summary of molecular markers validated in primary cell lines in tandem with their corresponding tumors. For GFAP expression, immunohistochemistry was used for the tumors as part of routine histological examination. GFAP mRNA expression using RT-qPCR was used for the cell lines as described in 'Materials and methods'.

Patient Number	Primary Tumor Positive for *KIAA-BRAF* (Yes/No)	Patient-Derived Cells Positive for *KIAA-BRAF* (Yes/No)	Primary Tumor Expresses GFAP (Yes/No)	Patient-Derived Cells Express GFAP (Yes/No)
2a	Yes (15-9)	Yes (15-9)	Yes	Yes
2b	Yes (15-9)	Yes (15-9)	Yes	Yes
3	No	No	Yes	Yes
5	No	No	Yes	Yes

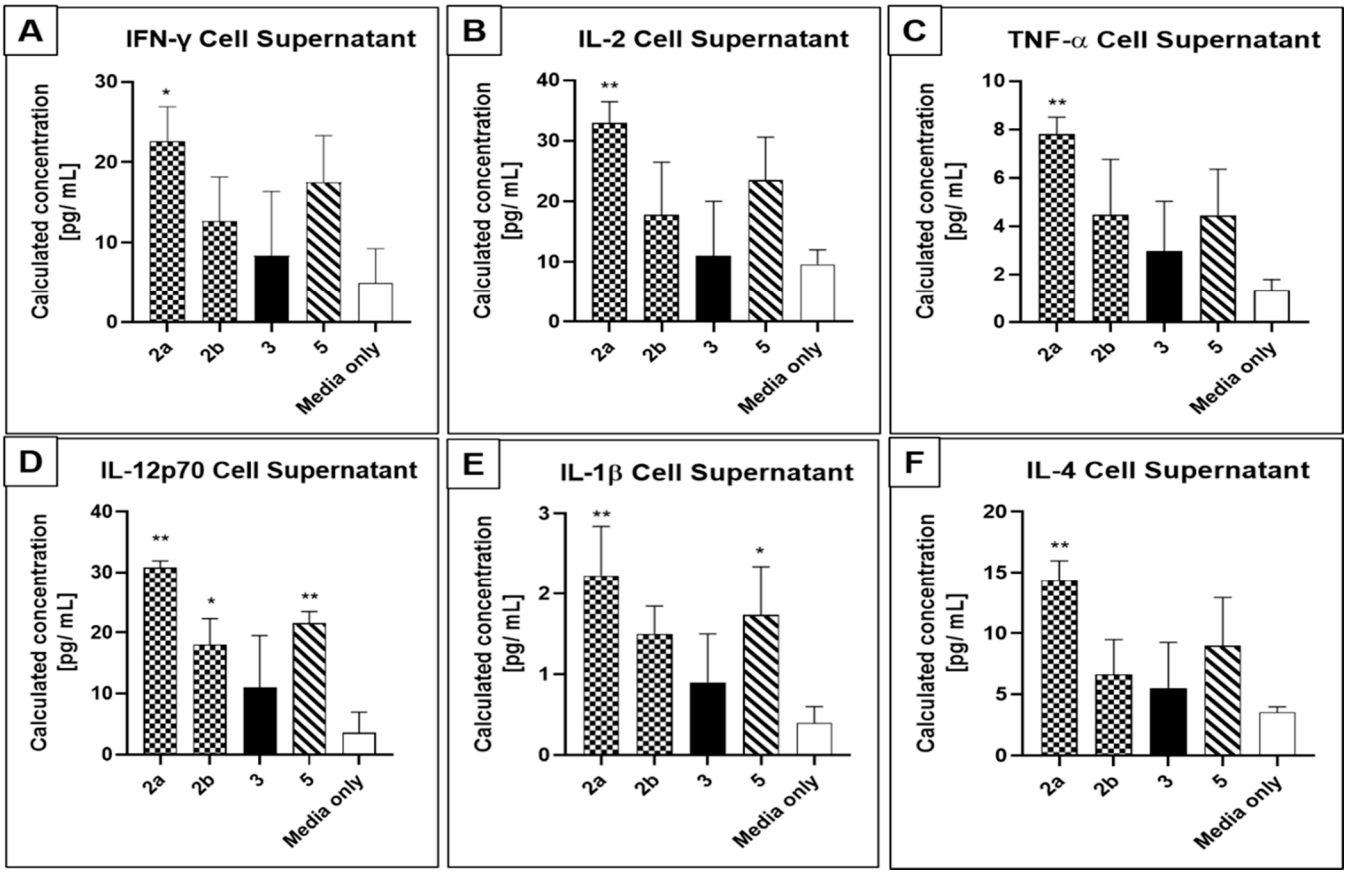

Figure 5. Graph results of in vitro cell culture supernatant cytokine profiling that show differential expressions for Patients 2 and 5, versus the rest of Patient 3 and a blank (cell culture media only). These include (**A**) IFN-γ, (**B**) IL-2, (**C**) TNF-α, (**D**) IL-12p70, (**E**) IL-1β, and (**F**) IL-4. After analysis, only IL-12p70 demonstrates statistical significance that is similar to the CSF findings. * $p < 0.05$; ** $p < 0.001$.

4. Discussion

For the majority of pediatric PCA, maximal safe surgical resection is recommended as the first-line treatment for tumors in areas of the brain amenable to complete removal [7,10]. Advancements in research have provided molecularly targeted diagnosis and treatment of PCA [2]. Examples include the identification of the KIAA1549-BRAF fusion gene in the majority of PCAs [35,36] and BRAF V600E mutation in the minority [37]. Nonetheless, targeted treatment with regards to the subset of surgically challenging PCAs, as highlighted in our cohort, remains a significant clinical gap. Specifically, for children with brain tumors who survive their disease after surgery and adjuvant treatment, many have long-term cognitive disabilities that limit their ability to live independently, progress fully in their education, or pursue a vocation [38]. For many of such patients, these post-chemoradiation treatment morbidities usually manifest long after the primary tumor is cured, even beyond the fourth decade of life [39]. Reports in the literature show that childhood cancer survivors' brains suffer progressive neurological deficits in visual processing [40,41], visual-motor functioning [42], attention and executive functioning [43]. Given that these so-called 'low-grade' tumors behave as a chronic disease with an indolent course, it is hence imperative that the choice of therapeutic intervention must be balanced against its potential for impact on the child's long-term growth and development [44].

Neuroinflammation has been implicated as an important player in tumor development [21–23]. In particular, cytokines and their inflammation-related partners have been demonstrated to coordinate pro-inflammatory responses within the tumor microenvironment, acting on autocrine, and/or paracrine pathways on malignant and non-malignant cells [24]. Broadly speaking, cytokines are small glycoproteins that bind to cell surface receptors and regulate the development, survival, and function of immune cells. Evolving data has observed their involvement in various intracranial pathologies, including traumatic head injury, post-hemorrhagic hydrocephalus, demyelination disease and so forth [45,46]. Although cytokines are ubiquitous, their individual expressions vary according to the context of the disease and state of each patient's immune system. Specifically, in brain tumors, cytokines have been extensively studied as potential therapeutic agents to manipulate the immune response to tumor cells [47]. In recent years, we are now aware that they can also function in the tumor microenvironment to induce cell proliferation, invasion, angiogenesis, and suppression of certain immune functions [48]. The latter postulates that since the CNS is not an immune-privileged site, therefore, regulatory immune cells and cytokines protect against excessive inflammation that involves the brain [49]. Aspects of neuroinflammation will vary within the context of disease, injury, infection, or stress [50]. It follows that, as brain tumors progress, perilesional tissue damage and hypoxia will induce regulatory cytokines and immune cells to counteract inflammation [51,52]. Putting it altogether, these factors can firstly contribute to the immunosuppressive behavior of the tumor itself and, next, blunt an anti-tumor immune response.

At this point in time, the option of immunotherapy is not available to the subset of PCA patients whose tumors are difficult to eradicate. Understanding the immune environment in which pediatric brain tumors exist is a first step to identifying effective immune-based therapies for these diseases [30]. As no matched normal brain tissue is collected as part of the surgical procedure, we did not compare cytokine expression between tumor cells and their non-tumor counterparts for each patient. More importantly, the immune system response is disease-context dependent. Under such circumstances, cytokine expressions are expected to overlap in different medical conditions. For the purposes of our study, the key aim is to assess for differences in cytokine expression between surgically amendable PCAs and those that are not. A blank media control is used to ensure there is no background expression from the growth factors in the cell culture media. Putting it together, we are able to demonstrate that firstly, patients with PCAs have circulating cytokines in their CSF; and selected cytokines have higher expression in the two patients with tumors that were difficult to treat. Following that, we demonstrated that similar cytokines are expressed by the patient-derived cell via an in vitro approach.

One such cytokine is interferon-gamma (IFN-γ), a pleiotropic, homodimer molecule formed by the noncovalent association of two 17 kDa polypeptide subunits [53]. This cytokine is considered to be a major effector of immunity and has been traditionally associated with anti-proliferative, pro-apoptotic, and anti-tumor mechanisms [54]. Nonetheless, there is growing evidence that IFN-γ facilitates contributing to tumor initiation by firstly promoting changes in tumor cell phenotype towards increased fitness for growth in the immunocompetent host; and next, contributing to homeostatic or cancer-triggered mechanisms to establish an immunosuppressive tumor microenvironment [55,56]. Currently, biological mechanisms by which IFN-γ functions are not fully understood, it is likely that the effect depends on multiple processes. IFN-γ primarily activates the JAK–STAT pathways that lead to the induction of the expression of multiple genes. In cancer cells, alterations in gene expression that are caused by IFN-γ are presumably associated with increased immunogenicity, which thereby induces immune stimulation [56]. Furthermore, some studies have established that IFN-γ is closely related to IL-12, which triggers NK and T cells, favoring cell-mediated immunity [57–59]. This is an interesting relationship that our current study intends to pursue in-depth to look for potential translational targets.

In tandem with IFN-γ, other cytokines produced in chronic inflammation, such as TNF-α and IL1-β, are also reported to promote cancer growth and progression. For instance, as a pro-inflammatory cytokine, TNF-α is secreted by inflammatory cells, which may be involved in inflammation-associated carcinogenesis [60]. These cytokines can steer cells toward more malignant phenotypes via multiple mechanisms, including induction of DNA damage response, angiogenesis, and activation of signaling pathways that promote cancer cell survival and, or proliferation [61,62]. In contrast to the rest, IL-2 plays a vital role in the growth as well as differentiation of T cells, B cells, natural killer cells, and many other cell types [61]. IL-2 is a T cell-derived common cytokine and was the first cytokine to be successfully used in the treatment of cancer. This was because it can promote T cells as well as NK cells. IL-2 can induce T cell proliferation, differentiation, and activation [62].

Overall, more research is needed to understand the immuno-mechanisms of PCAs, which are crucial pre-requisites for treatment optimization. Although this is a retrospective study with limited numbers, it is a novel attempt at exploring the possibility of immune factors involved in the oncogenesis of PCAs. Results from this proof-of-concept study allow us to pursue in-depth studies focusing on elucidating the relationship between PCAs and their corresponding cytokines of interest. The future work for this project includes, firstly, the comprehensive interrogation of each patient's tumor to assess for immune infiltration will be corroborated with the circulating CSF cytokines to identify possible relationships between them. Another consideration is to compare the immune profiles between the subset of challenging PCAs and other high-grade astrocytomas. Next, functional studies are performed to validate their biological significance in PCA tumorigenesis. In addition, a larger, prospective study that also includes the collection of blood from each patient as well, is already in place.

5. Conclusions

In summary, our study demonstrates there is differential cytokine expression in PCAs that are difficult to treat in comparison to those that are surgically amendable. Although our findings are preliminary, this is the first dedicated study exploring the involvement of immune-related factors in pediatric PCAs. This is important as a first step for further investigation of their translational role, especially in a subset of challenging pediatric PCAs. In the meantime, the authors advocate continued efforts with international collaborators to work together for better disease understanding in affected children.

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Appendix A

This is a separate, ongoing study based in the same institution. As part of this study's protocol, CSF from non-tumour patients after informed consent are included. These are patients who require CSF diversion surgeries for other intracranial pathologies. They include 3 patients diagnosed with post-traumatic hydrocephalus (1), congenital hydrocephalus (1), and post-haemorrhagic hydrocephalus (1). Once again, CSF samples are interrogated via a multiplex immunoassay kit that includes the cytokines of interest from the main study. Here, samples are prepared in duplicates for each patient and examined as per the manufacturer's instructions. Results are tabulated, analysed and compared against the samples of interest from the main study (namely, Patients 2a, 2b and 5) (Figure A1).

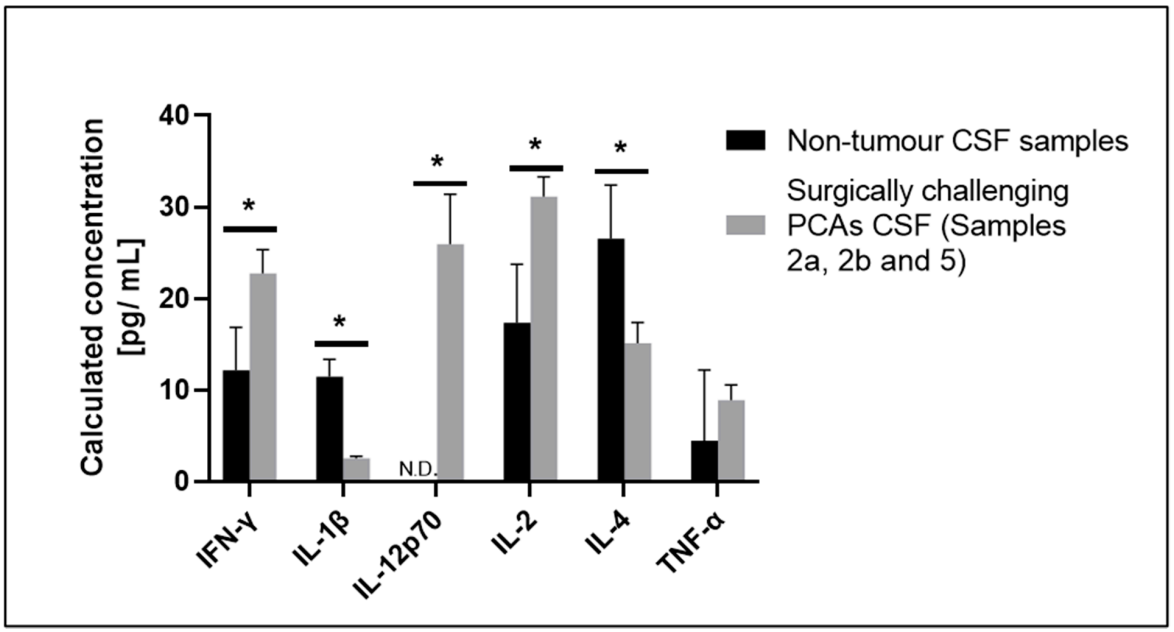

Figure A1. Graph showing comparison of the CSF cytokine expression combinatorial raw values between the non-tumour samples and surgically challenging PCA samples (Patients 2a, 2b and 5). * $p < 0.05$.

In this subgroup investigation, we observe differentially expressed CSF cytokines between non-tumour samples versus the patients with the surgically challenging PCAs. For IFN-γ, IL-12p70, IL-2 and TNF-α, their expressions in the non-tumour CSF patients are lower than those in the surgically challenging PCAs. This trend is similar to the results in Figure 4 from the main study. In contrast, IL1-β and IL-4 have higher expression in the non-tumour group versus the surgically challenging group. Given that firstly, immune-mediated expression is context-dependent in neuroinflammation; and next, cytokine expressions tend to vary between different intracranial pathologies, our findings are not entirely unexpected [45,46]. Nonetheless, the observations from this subgroup analysis suggest that there are distinct cytokines in the surgically challenging PCAs, that differ from our non-tumour CSF cohort and the PCA patients amenable to treatment.

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