Markers of survival and metastatic potential in childhood CNS primitive neuro-ectodermal brain tumours: an integrative genomic analysis

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See Comment page 753

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Summary

Background Childhood CNS primitive neuro-ectodermal brain tumours (PNETs) are very aggressive brain tumours for which the molecular features and best treatment approaches are unknown. We assessed a large cohort of these rare tumours to identify molecular markers to enhance clinical management of this disease.

Methods We obtained 142 primary hemispheric CNS PNET samples from 20 institutions in nine countries and examined transcriptional profiles for a subset of 51 samples and copy number profiles for a subset of 77 samples. We used clustering, gene, and pathway enrichment analyses to identify tumour subgroups and group-specific molecular markers, and applied immunohistochemical and gene-expression analyses to validate and assess the clinical significance of the subgroup markers.

Findings We identified three molecular subgroups of CNS PNETs that were distinguished by primitive neural (group 1), oligoneural (group 2), and mesenchymal lineage (group 3) gene-expression signatures with differential expression of cell-lineage markers LIN28 and OLIG2. Patients with group 1 tumours were most often female (male:female ratio 0·61 for group 1 *vs* 1·25 for group 2 and 1·63 for group 3; p=0·043 [group 1 *vs* groups 2 and 3]), youngest (median age at diagnosis 2·9 years [95% CI 2·4–5·2] for group 1 *vs* 7·9 years [6·0–9·7] for group 2 and 5·9 years [4·9–7·8] for group 3; p=0·005), and had poorest survival (median survival 0·8 years [95% CI 0·5–1·2] in group 1, 1·8 years [1·4–2·3] in group 2 and 4·3 years [0·8–7·8] in group 3; p=0·019). Patients with group 3 tumours had the highest incidence of metastases at diagnosis (no distant metastasis:metastasis ratio 0·90 for group 3 *vs* 2·80 for group 1 and 5·67 for group 2; p=0·037).

Interpretation LIN28 and OLIG2 are promising diagnostic and prognostic molecular markers for CNS PNET that warrant further assessment in prospective clinical trials.

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Introduction

Brain tumours are the most common paediatric solid neoplasms[1] and a leading cause of cancer-related morbidity and mortality in children.[2] Embryonal tumours are the largest group of malignant paediatric brain tumours and include medulloblastoma, atypical rhabdoid teratoid tumour, and CNS primitive neuro-ectodermal brain tumours (PNETs). Despite histological resemblance to medulloblastoma, patients with CNS PNETs fare poorly even with intensified therapy designed for patients with metastatic medulloblastoma.[3,4] By contrast with this disease, in which substantial progress has been made in molecular understanding[5,6] and clinical outcomes,[7] the molecular and cellular make-up of CNS PNET is largely unknown[8] and tumour treatments are often ineffective. To improve outcomes from CNS PNET, delineation of the cellular and molecular pathogenesis of CNS PNET will be important to inform diagnosis, prognosis, and design of tumour-specific treatments.

CNS PNETs are predominantly hemispheric tumours and make up about 3–5% of all paediatric brain tumours. Such cancers are histologically heterogeneous with variable neuronal, ependymal, or glial differentiation[9] and can be challenging to diagnose by routine histopathology.[10] Although diagnostic techniques and molecular-based tumour classifications have improved for atypical rhabdoid teratoid tumours[11] and medulloblastoma, the working classification for CNS PNET is not settled and thus therapeutic and molecular studies can be challenging. In recent studies, our research groups identified a distinctly aggressive subgroup of CNS PNETs that showed frequent amplification of an oncogenic miRNA cluster (*C19MC*).[12,13] However, the molecular composition of most CNS PNETs is unknown. Although genomic studies suggest substantial heterogeneity in DNA copy number profiles,[8,12,14] the significance of these findings in relation to clinical phenotypes is unclear. Similarly, gene-expression studies of small cohorts[12,15] have yielded few

insights into the clinical diversity of CNS PNETs. In this study, we undertook a multicentre, international collaboration with the aim of providing a concerted molecular analysis of a substantial number of primary CNS PNETs. To assess clinical significance of potential CNS PNET molecular subgroups, we examined whether subgroups differed in patient characteristics and outcome.

Methods

Participants and study design

We obtained 254 samples with an institutional diagnosis of CNS PNET from participating institutions including six registered Children's Cancer and Leukaemia Group centres in the UK and the Cooperative Human Tissue Network in Columbus, OH, USA (centres listed in the appendix).

CNS PNET tissue microarrays used in this study were constructed at the Hospital for Sick Children (Toronto, ON, Canada),[12] University of Nottingham (Nottingham, UK),[14] and the Institute of Cancer Research (Sutton, UK). All collected samples were initially reviewed for age of patient, location, and primary tumour occurrence (figure 1) and then subject to histopathologic review by CEH, who was masked to findings of previous assessments at the other centres. Samples were tested for loss of INI1 immunoreactivity or changes in *INI1* by sequencing or multiplex ligation-dependent probe amplification analyses to rule out misdiagnosed atypical rhabdoid teratoid tumours. We included only hemispheric tumours diagnosed as CNS PNET according to the 2007 WHO CNS tumour classification criteria[9] without mutations in *INI1*. For correlative analyses with clinical characteristics, we included only tumours with complete clinical information (full details of the patients and tumour information is listed in the appendix). We obtained tumour samples and clinical information with consent as per protocols approved by the hospital research ethics boards at participating institutions.

Procedures

All tumour samples confirmed to be CNS PNET and with snap frozen tumour material were processed for gene expression or DNA array analyses to initially establish tumour molecular subgroups. Tumour

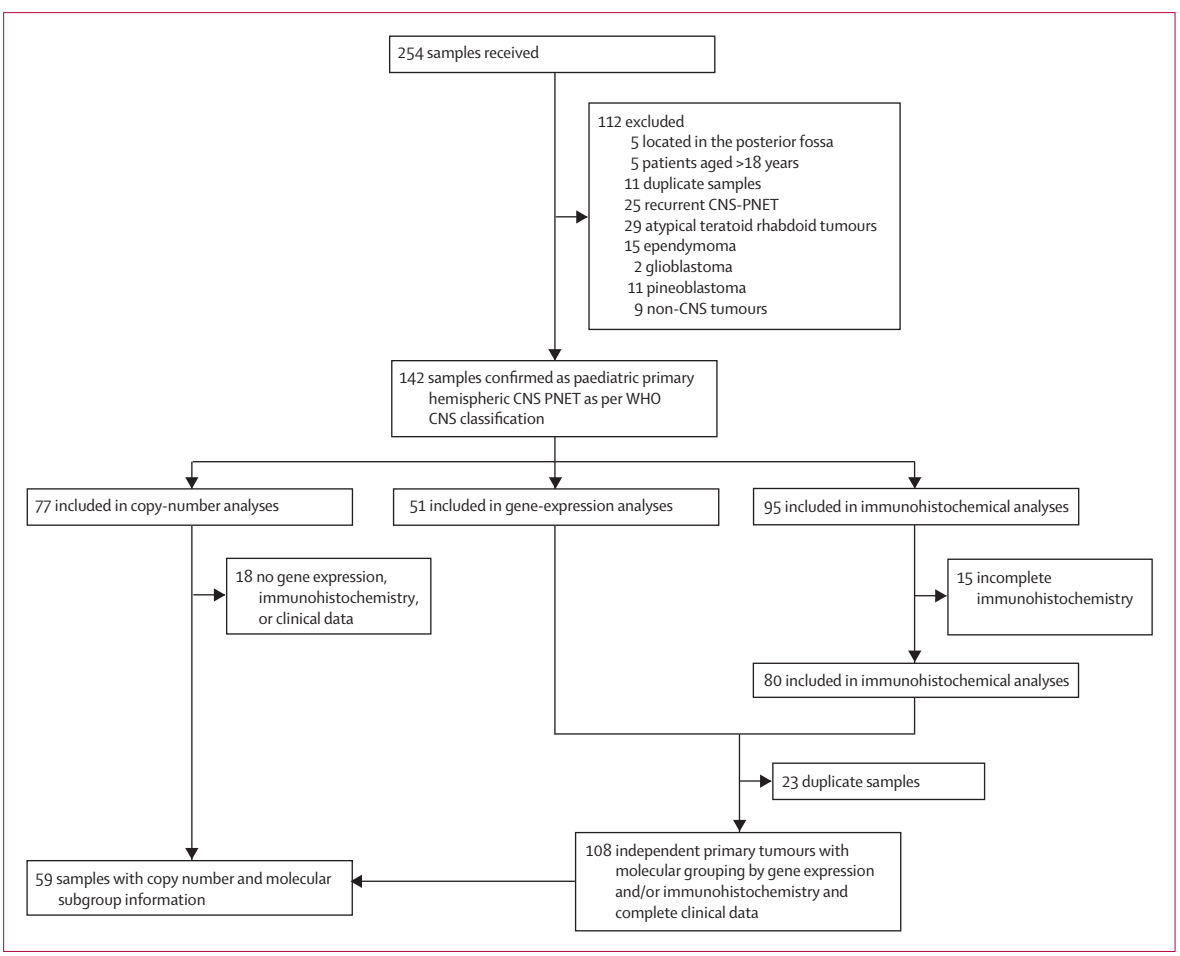

Figure 1: **Sample analysis**
PNET=primitive neuro-ectodermal brain tumour.

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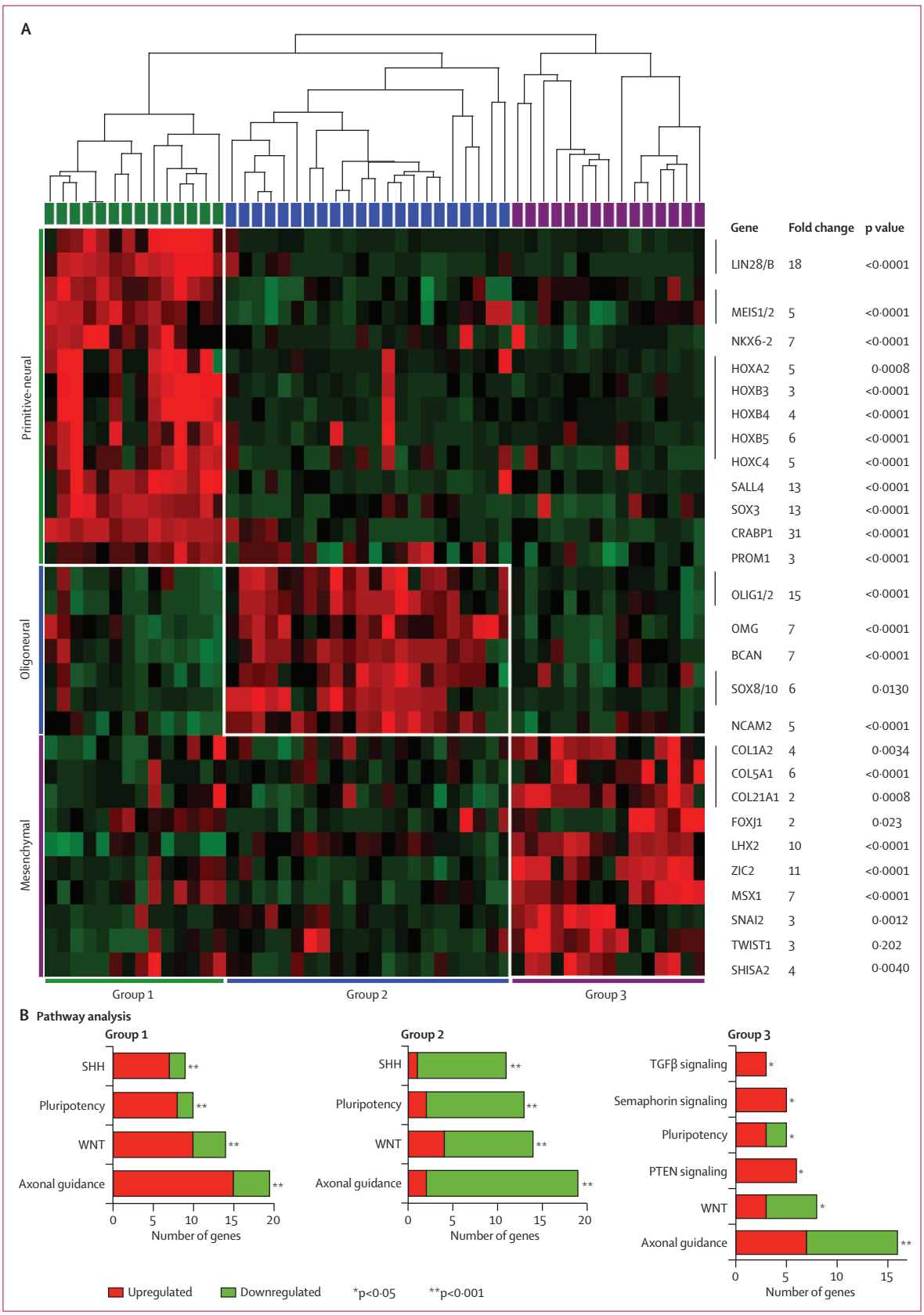

Figure 2: **Molecular subgroups of CNS primitive neuro-ectodermal brain tumours**

(A) Heat map of highly expressed cell lineage genes in each subgroup identified using a supervised *t* test adjusted for multiple testing (false discovery rate ≤0·05) relative to a hierarchical cluster map of all tumours; magnitude (fold change) and significance (p value) of cell lineage genes upregulated in each tumour subgroup is shown. (B) Significant changes in canonical pathways are presented from analyses of 343 genes in group 1, 276 genes in group 2, and 325 genes in group 3.

grouping for samples with only formalin-fixed paraffin-embedded (FFPE) materials available were determined with immunohistochemical analyses. All tumour samples with established molecular grouping information and clinical data for patients' demographics, metastatic status, and survival were then examined to determine whether molecular subgroups correlated with specific CNS PNET phenotypes.

To assess gene expression and DNA copy number profiles, we extracted RNA from 51 primary CNS PNET samples and DNA from 77 samples with standard methods. We used Illumina Omni 2.5M single-nucleotide polymorphism (SNP) for ultra-high resolution copy number analyses (interrogating 2·5 million SNPs) and Illumina HT-12.v4 gene-expression arrays (San Diego, CA, USA) to generate DNA copy number and gene expression profiles. We did DNA and RNA hybridisations at the Centre of Applied Genomics Facility at the Hospital for Sick Children, according to the manufacturer's protocol.

For the gene expression profiles, we did multiple unsupervised analyses to identify molecular subgroups of CNS PNETs. To define genes or pathways that characterise each CNS PNET subgroup, we then did supervised analyses of each subgroup relative to the others, and examined the most highly differentially expressed gene sets between subgroups for gene and pathway enrichment. To assess the clinical significance of identified molecular subgroups, we sought markers of each subgroup that could be examined by immunohistochemistry on a larger cohort of clinically well characterised tumours. We did quantitative RT-PCR analyses to validate group-specific gene clusters identified by supervised analyses and examined expression levels of individual genes across groups to identify the most robust, upregulated loci that can distinguish tumour subgroups.

To determine the relationship of copy number changes to molecular subgroups, we included 59 (77%) of 77 tumours with copy number profiles that had established molecular grouping.

The appendix shows details of molecular analyses done on individual tumour samples. All data are deposited in the Wellcome Trust, European Genome-Phenome Archive (accession number EGAS00000000116).

For gene-specific quantitative RT-PCR validation of array data, we amplified 10 ng cDNA synthesised from 1 µg of RNA (TaqMan Reverse Transcription Kit, Applied Biosystems, Burlington, ON, Canada) by use of specific TaqMan probes-primer sets (see appendix) and determined mRNA expression levels relative to actin with the ΔC_t method. We did all RT-PCR assays in triplicate. Immunohistochemical analyses of tumour tissue microarray or FFPE tumour slides were done by the Pathology Research Program laboratory at the University Health Network (Toronto, ON, Canada). We treated all tissue sections with heat-induced epitope retrieval and blocked for endogenous peroxidase and biotin. We assessed expression of markers for primitive neural, glial (nestin, glial fibrillary acidic protein [GFAP]) or neuronal (synaptophysin) differentiation—which are measurements conventionally used in histopathological diagnosis of CNS PNET—for all tumours. The antibodies used in this study were anti-LIN28 (Cell Signalling Technology, Boston, MA, USA), OLIG2 (Immuno-Biological Laboratories, Minneapolis, MN, USA), GFAP (DAKO, Burlington, CA, USA), and synaptophysin (Millipore, MA, USA). Antibody reactions were visualised with a Biogenix detection kit (BioGenex Laboratories, San Ramon, CA, USA). Immunoreactivity for LIN28, GFAP, and synaptophysin were scored manually on the basis of intensity (1 was low, 2 was moderate, and 3 was high) and distribution of stains (1 was ≤10%, 2 was 10–50%, and 3 was >50%). OLIG2 immunostains were quantified with the Aperio Scanscope (Aperio, Vista, CA, USA) system and the ImageScope software nuclear immunohistochemistry

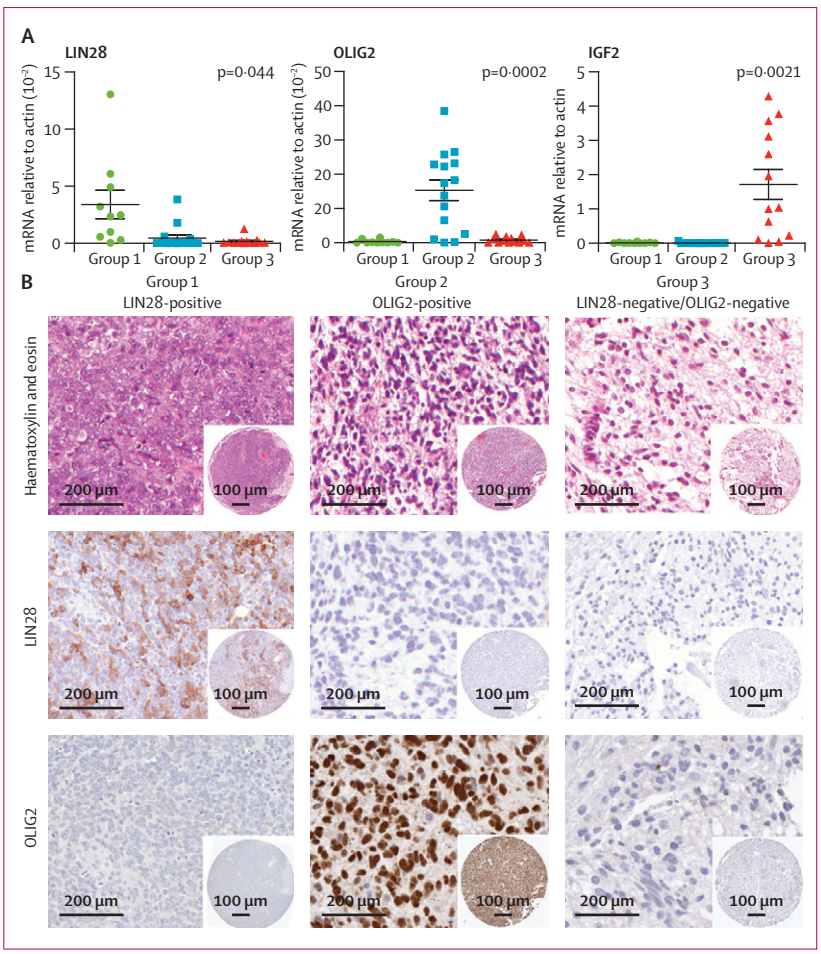

Figure 3: **Cell lineage markers of molecular subgroups of CNS PNETs**
(A) Quantitative RT-PCR analyses of 51 primary CNS PNETs profiled by gene expression arrays (appendix) showing enriched mean expression levels of LIN28 (group 1), OLIG2 (group 2), and IGF2 (group 3; three replicas) are shown with standard errors of mean (bars) and transcript levels shown as circles, squares, and triangles. (B) Characteristic immunohistochemical analyses from the validation of 72 samples of CNS PNET; LIN28 and OLIG2 immunostains (20× magnification) are shown in relation to a haematoxylin and eosin stain; insets (1× magnification) show corresponding tissue microarray cores. PNET=primitive neuro-ectodermal brain tumour.

For the **European Genome-Phenome Archive** see https://www.ebi.ac.uk/ega/

	Subgroup 1 (n=29)	Subgroup 2 (n=36)	Subgroup 3 (n=43)	p value	Comparison
Sex					
n	29	36	42		
Male	11	20	26		
Female	18	16	16		
Ratio	0·61	1·25	1·63	0·043*	Group 1 *vs* groups 2 and 3
Age at diagnosis					
n	26	32	42		
Median, years	2·9	7·9	5·9	0·005†	Groups 1 *vs* 2 *vs* 3
95% CI	2·4–5·2	6·0–9·7	4·9–7·8		
≤4 years	20	9	18		
>4 years	6	23	24		
Ratio	3·33	0·39	0·75	0·001‡	Groups 1 *vs* 2 *vs* 3
Metastasis status					
n	19	20	19		
M0	14	17	9		
M+	5	3	10		
Ratio	2·80	5·67	0·90	0·037*	Group 3 *vs* groups 1 and 2
Status					
n	26	26	34		
Dead	21	20	20		
Alive	5	6	14		
Ratio	4·20	3·33	1·43	0·13‡	Groups 1 *vs* 2 *vs* 3
Survival time					
n	20	23	23		
Median, years	0·8	1·8	4·3	0·019§	Groups 1 *vs* 2 *vs* 3
95% CI	0·5–1·2	1·4–2·3	0·8–7·8		

Some patients were not included in analyses because of a lack of specific clinical data; details of all patients are shown in the appendix. *Fisher's exact test. †ANOVA. ‡Pearson's χ^2. §Log-rank (Mantel-Cox) test.

Table 1: **Clinical and molecular characteristics of children with CNS primitive neuro-ectodermal brain tumours, by molecular subgroup**

algorithm. For tumours on tissue microarray, we established immunohistochemistry values on the basis of average staining score of at least two tissue cores, while tumours with FFPE slides were scored on the basis of the extent of staining in relation to the entire tumour section. Normal testicular tissue (human and mouse) was used as a positive control for LIN28 and oligodendroglioma tumour tissue was used as a positive control for OLIG2 immunostains; samples processed in parallel without primary antibodies were used as negative controls. DP and TC scored all immunohistochemistry stains while masked to cancer status, which were reviewed by AH and CEH. FISH was done on FFPE tissue microarrays or individual slides with established protocols. To confirm robustness of LIN28 and OLIG2 immunohistochemistry for subgrouping, we tested an initial cohort of 22 samples with subgroups established by gene expression studies for LIN28 and OLIG2 expression by immunohistochemistry (appendix) We used *MYCN* (2p24) and *p16* (9p21) specific PlatinumBright550 probe with corresponding *LAF* (2q11) and 9q21 PlatinumBright495 control probes (Kreatech, Stretton Scientific, Stretton, UK).

Statistical analysis

We classified CNS PNETs into molecular subgroups by unsupervised hierarchical clustering, non-negative matrix factorisation,[16] and principal component analyses of genes with the highest coefficient of variation with the Partek Genomics Suite version 6.5 (Partek, St Louis, MO, USA). We assessed genes enriched within tumour subgroups with a supervised *t* test adjusted for multiple hypotheses testing with the false-discovery-rate method. Ingenuity pathway analyses were done on supervised gene sets to identify canonical signalling pathways in each tumour subgroup. To establish regions of copy number gains and losses, inferred copy number data were generated with the Illumina Genome studio software and were imported into Partek for copy number variation partitioning-segmentation analyses with a SNP window of 150. We then determined significance of copy number alterations in tumour subgroups with Fisher's exact test. We used the log-rank analysis with the Kaplan-Meier method to compare survival times and χ^2 analyses to compare the proportion of survivors across tumour subgroups, whereas ANOVA was used to assess significance of tumour subgroups in relation to age. To analyse the significance of molecular subgroups in relation to sex and metastatic status at diagnosis, we compared features in an individual molecular subgroup to a pooled cohort of the other two molecular subgroups with Fisher's exact test. Adjustment for multiple testing was not done because patients with complete information available for every clinical parameter varied. A p value of less than 0·05 was regarded as significant for all analyses. All statistical analyses were done with SPSS version 19·0.

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. DP, SM, RGG, and AH had access to the raw data. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Unsupervised hierarchical and non-negative matrix factorisation clustering with 200–1000 genes consistently identified three distinct molecular subgroups of CNS PNET, with non-negative matrix factorisation analyses suggesting a strongest cophenetic coefficient at k=3 (figure 2, appendix). Principle component analyses suggested that group 1 tumours, which have frequent *C19MC* locus amplification, segregated distinctly, whereas group 2 and 3 tumours showed greater proximity and some overlap (appendix).

Supervised analyses revealed that the three subgroups showed significant differences in neural lineage and differentiation genes (figure 2). Expression profiles of group 1 were most significantly enriched for genes

associated with embryonic or neural stem cells. Notably, LIN28 and CRABP1,[17] which are implicated in stem-cell pluripotency, were among the most overexpressed genes with nearly 20–30 fold greater expression in group 1 as compared with group 2 and group 3. In group 2 tumours, OLIG1/2, SOX8/10, and BCAN (which are markers of oligoneural differentiation[18]) were the most upregulated genes, whereas group 3 tumours showed reduced expression of neural differentiation genes but upregulation of epithelial and mesenchymal differentiation genes including COL1A2, COL5A, FOXJ1,[19] and MSX1.[20]

Pathway enrichment analyses also suggested significant differences in the signalling gene profiles of every tumour subgroup (figure 2). Consistent with differential enrichment of lineage related genes in tumour subgroups, we noted significant differences in expression of axonal guidance genes among the CNS PNET subgroups. Genes involved in WNT signalling were upregulated in group 1 tumours and those involved in SHH signalling were downregulated in group 2 tumours, whereas TGF-β and PTEN signalling pathway genes were specifically upregulated in group 3 tumours.

Immunohistochemistry and quantitative RT-PCR analyses showed that *LIN28*, *OLIG2,* and *IGF2* were highly differentially expressed in CNS PNET group 1, group 2, and group 3 tumours, respectively (figure 3, appendix). IGF2 protein expression could not be reliably scored on tumour samples (appendix); however, immunohistochemical analyses for LIN28 and OLIG2 were robust and correlated with gene-expression levels as established by arrays and quantitative RT-PCR analyses. Immunohistochemical analyses on a test cohort of 22 tumours indicated that extent of cytoplasmic LIN28 and nuclear OLIG2 immunostaining also correlated with tumour subgroup assignment based on gene-expression profiles (appendix). LIN28 was expressed at high levels and OLIG2 was expressed at low levels in group 1 tumours, whereas group 2 tumours had high OLIG2 and little LIN28 immunopositivity. LIN28 and OLIG2 protein expression was low or absent in group 3 tumours (figure 3).

In the LIN28 and OLIG2 immunohistochemical analyses of an additional 72 primary CNS PNETs with only FFPE samples available for analyses, 15 tumours had inconclusive immunohistochemical analyses (appendix). Overall, we were able to assign 108 of 142 primary CNS PNETs to molecular subgroups on the basis of gene expression or immunohistochemical analyses of LIN28 or OLIG2 protein expression (or both measures; tables 1, 2, appendix). We classified 29 (27%) tumours as group 1, 36 (33%) as group 2, and 43 (40%) as group 3 (appendix). Group 1 tumours with high LIN28 expression generally also expressed high levels of nestin, but had little to no expression of GFAP. GFAP and synaptophysin expression varied substantially between each of the tumour groups and did not consistently correlate with LIN28 or OLIG2 expression. Notably, quantitative RT-PCR and expression analyses suggested that expression of other neuronal differentiation genes also do not differ significantly among the molecular subgroups of CNS PNETs (appendix). These findings collectively suggest the limitations of conventional markers to capture the molecular diversity of CNS PNETs.

CNS PNET subgroups have distinct DNA copy number patterns. Apart from the *C19MC* miRNA amplicon that we previously identified,[12] we noted few other recurrent high level copy number gains or amplification. Focal *MYCN* and *CDK4* amplification was detected in isolated tumours. Deletions centred on *CDKN2A/2B* were the most frequent copy number aberration noted (10 [12%] of 77 tumours; appendix). To establish whether there were characteristic copy number aberrations within the CNS PNET subgroups, we analysed the copy number patterns

	Subgroup 1	Subgroup 2	Subgroup 3	p value	Comparison
Age ≤4 years					
n	20	9	18		
Metastasis status					
n	15	6	4		
M0	11	5	4		
M+	4	1	0		
Ratio	2·75	5·00	4·00	0·48*	Groups 1 *vs* 2 *vs* 3
Status					
n	20	7	13		
Dead	16	6	10		
Alive	4	1	3		
Ratio	4·00	6·00	3·33	0·90*	Groups 1 *vs* 2 *vs* 3
Survival time					
n	15	6	6		
Median survival, years	1·0	0·8	2·7	0·70†	Groups 1 *vs* 2 *vs* 3
95% CI	0·7–1·3	0–4·8	1·9–3·5		
Age >4 years					
n	6	23	24		
Metastasis status					
n	4	14	15		
M0	3	12	5		
M+	1	2	10		
Ratio (analysis 1)	3·00	6·00	0·50	0·033*	Group 3 *vs* groups 1 and 2
Ratio (analysis 2)	..	6·00	0·50	0·014‡	Group 2 *vs* group 3
Status	6	19	21		
Dead	5	14	10		
Alive	1	5	11		
Ratio (analysis 1)	5·00	2·80	0·91	0·13*	
Ratio (analysis 2)	..	2·80	0·91	0·087‡	Group 2 *vs* group 3
Survival time					
n	5	17	17		
Median survival, years	0·5	1·8	4·8	0·004	Groups 1 *vs* 2 *vs* 3
95% CI	0·0–1·0	1·5–2·2	1·6–8·0		

Some patients were not included in analyses because of a lack of specific clinical data; details of all patients are shown in the appendix. *Pearson χ^2. †Log-rank (Mantel-Cox) test. ‡Fisher's exact test.

Table 2: **Clinical and molecular characteristics of children with CNS primitive neuro-ectodermal brain tumours, by molecular subgroup and age**

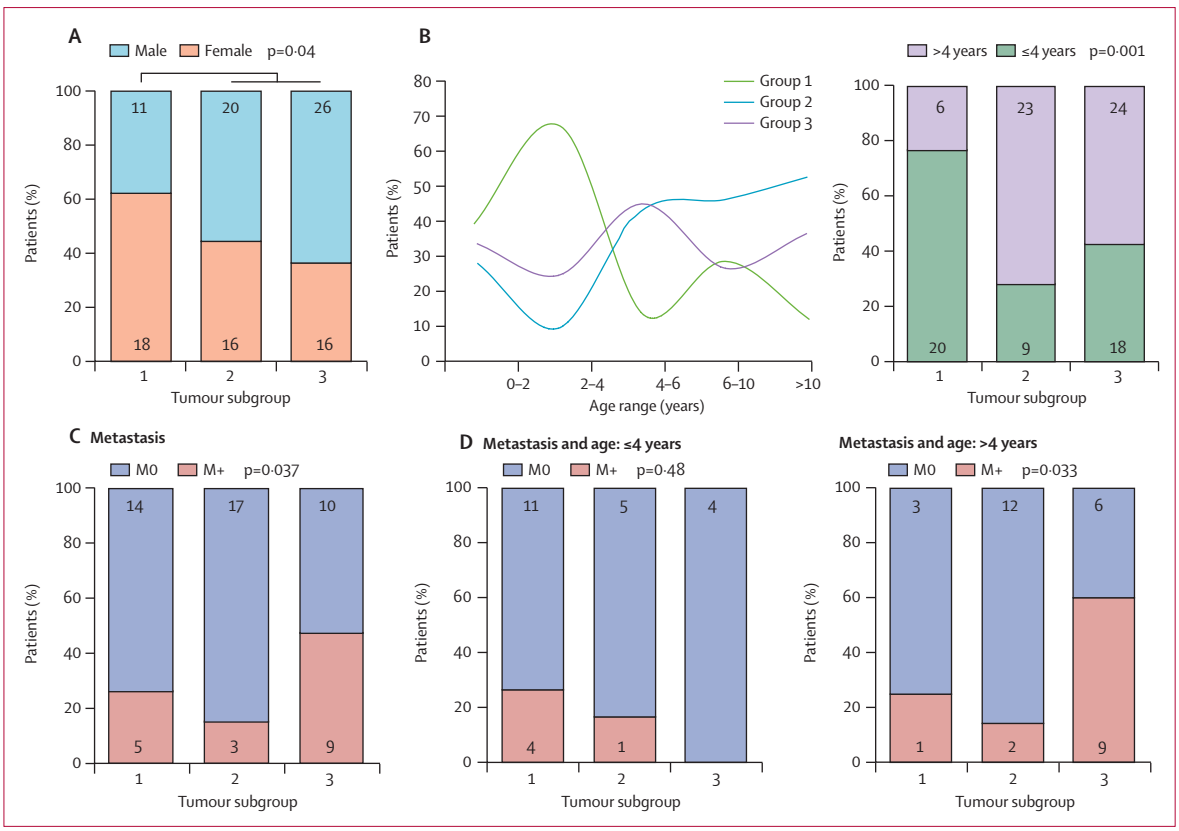

Figure 4: **Clinical phenotypes of molecular subgroups of CNS PNETs**
(A) Sex-specific and (B) age-specific correlations with tumour subgroup in 108 primary CNS PNET tumours (tables 1, 2, appendix). (C) Metastatic status at diagnosis (58 patients); p value from the two-sided Fisher's exact test (group 1 *vs* groups 2 and 3). (D) Metastatic status at diagnosis, stratified by age (58 patients); p values from Pearson's χ^2 (group 1 *vs* group 2 *vs* group 3). PNET=primitive neuro-ectodermal brain tumour.

of a subset of 59 tumours that could be subgrouped on the basis of LIN28 and OLIG2 gene or protein expression (appendix). Copy number analyses showed that, in addition to chr19q13·41 amplification and chromosome 2 gains, group 1 tumours had frequent gains of chromosome 3. Group 2 tumour copy number aberration profiles were characterised by more frequent gains of chromosome 8p (p=0·027), 13 (p=0·009), and 20 (p=0·039) compared with group 1 and 2 tumours. Notably, only group 2 and 3 tumours had frequent chromosome 9p loss centred on the *CDKN2A/2B* locus. Moreover, group 3 tumours showed frequent loss of chromosome 14 (p=0·009). Thus, CNS PNET subgroups correlate with distinct gene expression as well as genomic profiles.

To determine the clinical significance of CNS PNET molecular subgroups, we examined whether subgroups differed in patient characteristics and outcome. Of 108 patients for which tumour subgrouping could be established, demographic data on sex, age, survival, and tumour stage were available for 107, 100, 58 and 66 cases, respectively (tables 1 and 2; appendix). Molecular subgroups were associated with distinct clinical phenotypes. Sex and age distribution differed between the three molecular CNS PNET subgroups. Group 1 tumours were more often noted in female patients than were group 2 and group 3 tumours (figure 4, table 1). Patients with group 1 and group 2 tumours had bimodal age distributions with peak incidence at opposite age spectra, whereas patients with group 3 tumours had a single peak between 4–8 years. Patients with group 1 tumours were younger than were those with group 2 or group 3 tumours (table 1). 47 (47%) of 100 patients with data for age were 4 years old or younger; however young patients were significantly over-represented in group 1 as compared with group 2 and group 3 (p=0·001; figure 4, table 1).

Molecular subgroups of CNS PNET also had significant differences in incidence of tumour metastases. Patients with group 3 tumours had the highest incidence of disseminated disease at diagnosis (figure 4, table 1). Although metastatic disease is reportedly more frequently in younger children with embryonal brain tumours, analyses done with stratification for age (≤4 years *vs* >4 years) showed the incidence of tumour metastases differed significantly in CNS PNET subgroups diagnosed in older children (figure 4, table 2). More patients aged older than 4 years at diagnosis in group 3 presented with metastatic disease than did those in group 1 or group 2 (table 2). The proportion of

non-metastatic to metastatic tumours in this age group differed significantly in comparisons of group 3 to a combined cohort of group 1 and group 2 patients and to group 2 patients alone (figure 4, table 2).

Log-rank analysis of all tumour age groups showed that overall survival for patients in group 1 was significantly shorter than it was for patients in group 2 and group 3 (figure 5, table 1). With the exception of two longer term survivors, all patients in group 1 were deceased within 4·2 years of diagnosis. Because most group 1 tumours arise in younger children who are often treated heterogeneously with radiation-sparing therapeutic approaches due to worries of neurocognitive damage,[3,21] we examined whether the poor prognostic association of LIN28 expression in group 1 tumours held true for older children who are conventionally prescribed intensified treatment regimens with higher dose craniospinal irradiation. Moreover, because most infant brain tumour protocols enrol patients up to 3–4 years of age,[3,4,21] we stratified patients by age at the cutoff of 4 years, to remove age and potential treatment biases on survival. Although overall survival for all young patients was similarly poor, children older than 4 years of age with LIN28 group 1 tumours fared significantly worse (median survival of 0·5 years, 95% CI 0·0–1·0; p=0·004) than did patients older than 4 years of age in group 2 (1·8 years, 1·5–2·2) and group 3 (4·8 years, 1·6–8·0). These findings suggest that immunopositivity for LIN28 identifies a particularly high risk group of CNS PNET across ages.

Discussion

Advances in treatment for childhood CNS PNET have been difficult because of the low incidence of the disease,[9] incomplete understanding of the clinical and biological spectra of disease, and an absence of specific markers to aid histopathological diagnoses (panel).[8,10] In this study, we aimed to integrate gene expression, copy number, and immunohistochemical analyses to characterise 142 primary hemispheric CNS PNETs. Differential expression of cell lineage markers, LIN28 and OLIG2, distinguishes three molecular subgroups of CNS PNET and identifies CNS PNET subgroups at very high risk of metastases and treatment failures and distinct demographic features. Primitive neural group 1 tumours, with frequent *C19MC* amplification and high LIN28 expression, are distinctly aggressive tumours arising in young children. Oligoneural group 2 tumours, which have high OLIG2 expression, arise in older children and are frequently localised. Mesenchymal group 3 tumours, which have low LIN28 and OLIG2 expression, are associated with a high incidence of metastases and occur at all ages. Group 1 tumours were more frequently in females, whereas group 2 and group 3 tumours arose more frequently in males. These markers are promising molecular identifiers for childhood CNS PNET that could be applied to refine tumour diagnosis, classification, and treatment risk stratification.

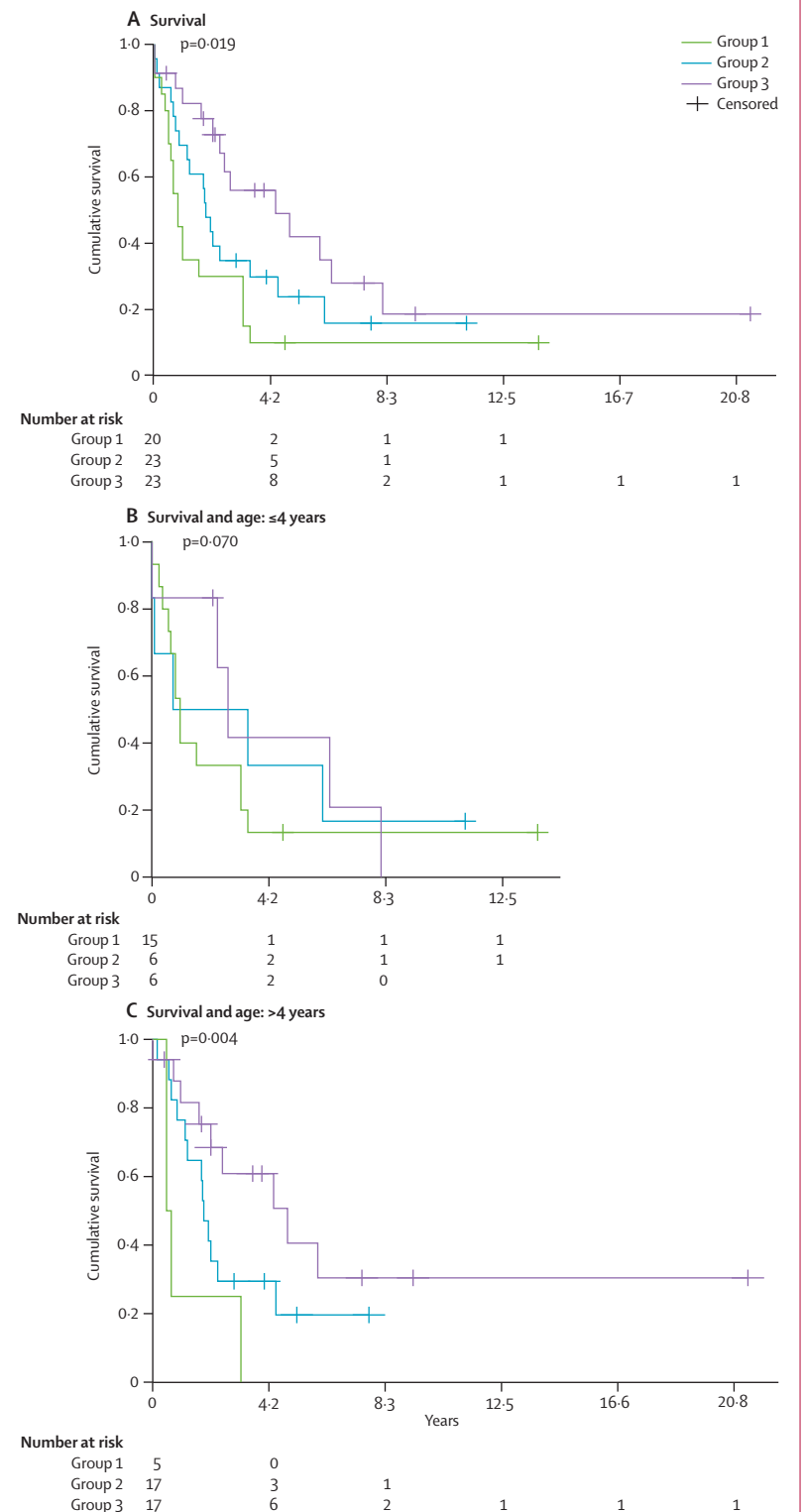

Figure 5: **Survival of molecular subgroups of primary CNS primitive neuro-ectodermal brain tumours**
(A) Overall survival (66 patients). Overall survival, stratified by age 4 years or younger (B; 27 patients) and age older than 4 years (C; 39 patients). p values from the log-rank test (group 1 *vs* group 2 *vs* group 3).

Panel: **Research in context**

Systematic review

We searched PubMed and Google Scholar for molecular studies of childhood CNS primitive neuro-ectodermal brain tumours (PNETs) published in English between Jan 1, 1985, and Dec 31, 2011, with the search terms "childhood PNET", "CNS-PNET", "supratentorial PNET", and "embryonal brain tumours" (because CNS PNETs were often included in molecular studies of medulloblastoma).

Interpretation

Present treatment strategies for CNS PNETs are largely designed based on the cancer's close histological similarities to medulloblastoma, although such therapy is not as efficacious in CNS PNETs. A tailored treatment for CNS PNET is needed to exploit their distinct biology; however, molecular studies of CNS PNETs have been restricted by rare disease incidence and scarcity of robust diagnostic markers. Apart from two recent studies[12,14] by our group and a previous review,[8] molecular studies of CNS PNETs have been restricted to small cohorts of CNS PNETs from various anatomical sites and diagnoses made on various histopathological criteria. Our integrated genomic analyses of primary CNS PNET tumour samples were restricted to the cerebral hemispheres and tumours that met the 2007 WHO CNS classification criteria for CNS PNET and were confirmed with genetic methods to be non-rhabdoid tumours. In keeping with previous clinical observations, we show that CNS PNET makes up a heterogeneous spectrum of tumours and defines three molecular subtypes of CNS PNETs with distinct survival and metastatic features. Our study provides the first molecular prognostic markers for CNS PNET, and is a substantial advance towards biology-driven treatment strategies for this disease.

Comprehensive clinical and biological data for a large cohort of primary CNS PNETs are not available. Previous molecular studies have often included a spectrum of CNS PNETs including rare variants, tumours arising in different anatomical locations such as pineoblastomas, and medulloblastoma.[8] Because the biological relation of CNS PNET arising in different anatomical locations is unclear, we restricted our study to tumours arising in the cerebral hemispheres, making up most childhood CNS PNET.

Although CNS PNETs are generally regarded as mainly a disease of younger children, analysis of our data suggests that more than 50% of CNS PNETs arise in older children (>4 years) and that CNS PNETs have an age-dependent distribution of molecular subgroups. The strong association of lineage-specific gene-expression signatures and age with specific tumour subgroups suggests molecular subgroups of hemispheric CNS PNET might derive from different precursor cell stages or type. Specifically, transcriptional signatures of group 1 tumours (enriched for *CD133*, *CRABP1*, *LIN28*, and *ASCL1*) and group 2 tumours (enriched for *OLIG1/2*) suggest that the former arose from early neural progenitors and the latter from oligoneural progenitors. The possible cellular origin of group 3 CNS PNETs, which are enriched for mesenchymal differentiation genes including *ZIC2*[22] and *LHX2*,[23] is less clear.

Studies of human brain tumours and brain tumour models suggest that cell lineage-related gene-expression signatures often correlate with and underlie clinical and biological heterogeneity in a spectrum of CNS tumours including malignant gliomas,[24] ependymoma,[25] and medulloblastoma.[26] In addition to age and sex, we noted significant differences in survival and metastatic tendency between the three CNS PNET subgroups. We noted poorest survival in the primitive neural group 1 tumours identified on the basis of LIN28 expression, irrespective of age or metastatic status. Together with previous findings that link *C19MC* amplification with a distinctly aggressive CNS PNET phenotype,[12,13] our study further emphasises CNS PNET with *C19MC* amplification and/or LIN28 expression as a unique clinicopathological entity and suggests LIN28 immunohistochemistry could be an important, new diagnostic tool for this distinct group of embryonal brain tumours.

Overall survival for group 2 and group 3 tumours, which more commonly presented in older children, did not differ significantly (p=0·087), but there was some suggestion that children older than 4 years with group 3 mesenchymal lineage tumours had better survival (11 [52%] of 21 children were alive at last assessment) than did those with group 2 tumours (five [26%] of 19 were alive at last assessment; table 2). This finding is surprising, because group 3 tumours had the highest incidence of metastases at diagnosis, which is linked to poorer outcomes in medulloblastoma and other embryonal brain tumours. These observations suggest greater sensitivity of group 3 tumours to medulloblastoma-type drugs, which are usually prescribed to older children with CNS PNET, and might suggest greater biological relatedness of group 3 CNS PNETs to medulloblastoma. However, treatment designed for high-risk medulloblastoma with higher-dose craniospinal irradiation, which is usually prescribed to older children with CNS PNET, might not offer additional therapeutic benefit for most group 2 CNS PNETs. Our findings underscore the clinical heterogeneity of CNS PNET arising in the cerebral hemisphere and suggest that group 2 and group 3 CNS PNET need different therapeutic approaches tailored to their specific biology.

Our study confirms the documented poor overall outcome of CNS PNETs across age groups and emphasises the need to seek new treatment strategies for this aggressive disease. Pathway enrichment analyses suggested that the non-canonical WNT pathway predominates in group 1 tumours and thus could be an attractive target for treatment. Group 1 tumours also

showed significant upregulation of the SHH signalling pathway, suggesting that novel SHH pathway inhibitors in clinical trials[27] might be attractive new drugs for this subgroup. Of note, CRABP1, a retinoid-binding protein known to change retinoic metabolism and confer all-trans retinoic acid resistance,[28] is very highly expressed in the group 1 CNS PNET. Thus retinoic acid, which is being tested in high-risk medulloblastoma and CNS PNET cooperative group clinical trials for older children, might be of little therapeutic benefit in group 1 CNS PNETs.

By contrast with group 3 tumours that showed upregulation of several canonical pathways, the oligoneural group 2 tumours showed downregulation of both SHH and WNT signalling pathways. Although we noted higher expression of PDGFRA and ERBB3 in this subgroup (appendix), pathway analyses did not show significant global enrichment of receptor tyrosine signalling pathways. However, these potential therapeutic pathways might emerge with studies of larger cohorts, and further delineation of CNS PNET subgroups. Consistent with the higher incidence of metastases noted in group 3 tumours, we identified substantial activation of semaphorin signalling genes in this tumour group. In addition to activated TGFβ signalling, group 3 tumours showed upregulation of PTEN signalling and IGF2 expression, thus making these pathways and genes attractive targets for potential subgroup specific therapies.

Our data show that hemispheric tumours diagnosed as CNS PNET in children can be differentiated into subgroups with distinct survival and metastatic characteristics on the basis of lineage markers, LIN28 and OLIG2. Because our study was restricted to hemispheric CNS PNETs, assessment of the significance of these molecular groupings to non-hemispheric CNS PNETs, such as pineoblastoma, will be important. Our study was limited by sample size and the absence of an independent validation cohort due to the rare incidence of CNS PNET. Nonetheless, we anticipate that LIN28 and OLIG2, which are the first molecular markers reported for CNS PNET to date, will help identify high-risk group 1 tumours for new therapies, allow tailoring of chemoradiotherapy for patients with group 2 and group 3 tumours (which differ strikingly in metastatic potential), and will help establish a working classification of CNS PNETs. Our report underscores the importance of concerted, collaborative efforts to study large retrospective cohorts of tumours and patients to accelerate biological and ultimately therapeutic studies of rare tumours.

Contributors

AH, DP, RGG designed the study. AH and RGG procured financial support. CH, RGG, AG, SMP, EB, AK, JC, LL-C, CE, HT, JF, S-KK, Y-SR, TVM, CCL, SLP, H-KN, CJ, SCC, JTH, XF, KMM, HN, and AH provided study materials or patients. DP, AH, SM, CH, and PZH analysed and interpreted the data. DP, AH and SM wrote the report; AH, SM, SMP and RGG reviewed and edited the final report. All authors approved the final manuscript.

Conflicts of interest

We declare that we have no conflicts of interest.

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References

1 Kohler BA, Ward E, McCarthy BJ, et al. Annual report to the nation on the status of cancer, 1975–2007, featuring tumors of the brain and other nervous system. *J Natl Cancer Inst* 2011; **103:** 714–36.
2 Turner CD, Rey-Casserly C, Liptak CC, et al. Late effects of therapy for pediatric brain tumor survivors. *J Child Neurol* 2009; **24:** 1455–63.
3 Timmermann B, Kortmann RD, Kuhl J, et al. Role of radiotherapy in supratentorial primitive neuroectodermal tumor in young children: results of the German HIT-SKK87 and HIT-SKK92 trials. *J Clin Oncol* 2006; **24:** 1554–60.
4 Pizer BL, Weston CL, Robinson KJ, et al. Analysis of patients with supratentorial primitive neuro-ectodermal tumours entered into the SIOP/UKCCSG PNET 3 study. *Eur J Cancer* 2006; **42:** 1120–28.
5 Northcott PA, Korshunov A, Witt H, et al. Medulloblastoma comprises four distinct molecular variants. *J Clin Oncol* 2011; **29:** 1408–14.
6 Cho YJ, Tsherniak A, Tamayo P, et al. Integrative genomic analysis of medulloblastoma identifies a molecular subgroup that drives poor clinical outcome. *J Clin Oncol* 2011; **29:** 1424–30.
7 Gajjar A, Chintagumpala M, Ashley D, et al. Risk-adapted craniospinal radiotherapy followed by high-dose chemotherapy and stem-cell rescue in children with newly diagnosed medulloblastoma (St Jude Medulloblastoma-96): long-term results from a prospective, multicentre trial. *Lancet Oncol* 2006; **7:** 813–20.
8 Li MH, Bouffet E, Hawkins CE, et al. Molecular genetics of supratentorial primitive neuroectodermal tumors and pineoblastoma. *Neurosurg Focus* 2005; **19:** E3.
9 Louis DN, Wiestler OD. WHO classification of tumours of the central nervous system, 4th edn. Lyon, France: International Agency for Research on Cancer, 2007.
10 Burger PC. Supratentorial primitive neuroectodermal tumor (sPNET). *Brain Pathol* 2006; **16:** 86.
11 Jackson EM, Sievert AJ, Gai X, et al. Genomic analysis using high-density single nucleotide polymorphism-based oligonucleotide arrays and multiplex ligation-dependent probe amplification provides a comprehensive analysis of INI1/SMARCB1 in malignant rhabdoid tumors. *Clin Cancer Res* 2009; **15:** 1923–30.
12 Li M, Lee KF, Lu Y, et al. Frequent amplification of a chr19q13·41 microRNA polycistron in aggressive primitive neuroectodermal brain tumors. *Cancer Cell* 2009; **16:** 533–46.
13 Korshunov A, Remke M, Gessi M, et al. Focal genomic amplification at 19q13·42 comprises a powerful diagnostic marker for embryonal tumors with ependymoblastic rosettes. *Acta Neuropathol* 2010; **120:** 253–60.
14 Miller S, Rogers HA, Lyon P, et al. Genome-wide molecular characterization of central nervous system primitive neuroectodermal tumor and pineoblastoma. *Neuro Oncol* 2011; **13:** 866–79.
15 Rogers HA, Miller S, Lowe J, et al. An investigation of WNT pathway activation and association with survival in central nervous system primitive neuroectodermal tumours (CNS PNET). *Br J Cancer* 2009; **100:** 1292–302.
16 Brunet JP, Tamayo P, Golub TR, et al. Metagenes and molecular pattern discovery using matrix factorization. *Proc Natl Acad Sci USA* 2004; **101:** 4164–69.
17 Skottman H, Mikkola M, Lundin K, et al. Gene expression signatures of seven individual human embryonic stem cell lines. *Stem Cells* 2005; **23:** 1343–56.
18 Ligon KL, Fancy SP, Franklin RJ, et al. Olig gene function in CNS development and disease. *Glia* 2006; **54:** 1–10.

19 Jacquet BV, Muthusamy N, Sommerville LJ, et al. Specification of a Foxj1-dependent lineage in the forebrain is required for embryonic-to-postnatal transition of neurogenesis in the olfactory bulb. *J Neurosci* 2011; **31:** 9368–82.

20 Houzelstein D, Auda-Boucher G, Cheraud Y, et al. The homeobox gene *Msx1* is expressed in a subset of somites, and in muscle progenitor cells migrating into the forelimb. *Development* 1999; **126:** 2689–701.

21 Fangusaro J, Finlay J, Sposto R, et al. Intensive chemotherapy followed by consolidative myeloablative chemotherapy with autologous hematopoietic cell rescue (AuHCR) in young children with newly diagnosed supratentorial primitive neuroectodermal tumors (sPNETs): report of the Head Start I and II experience. *Pediatr Blood Cancer* 2008; **50:** 312–18.

22 Elms P, Siggers P, Napper D, et al. *Zic2* is required for neural crest formation and hindbrain patterning during mouse development. *Dev Biol* 2003; **264:** 391–406.

23 Hoch RV, Rubenstein JL, Pleasure S. Genes and signaling events that establish regional patterning of the mammalian forebrain. *Semin Cell Dev Biol* 2009; **20:** 378–86.

24 Phillips HS, Kharbanda S, Chen R, et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell* 2006; **9:** 157–73.

25 Taylor MD, Poppleton H, Fuller C, et al. Radial glia cells are candidate stem cells of ependymoma. *Cancer Cell* 2005; **8:** 323–35.

26 Gibson P, Tong Y, Robinson G, et al. Subtypes of medulloblastoma have distinct developmental origins. *Nature* 2010; **468:** 1095–99.

27 Rudin CM, Hann CL, Laterra J, et al. Treatment of medulloblastoma with hedgehog pathway inhibitor GDC-0449. *N Engl J Med* 2009; **361:** 1173–78.

28 Boylan JF, Gudas LJ. The level of CRABP-I expression influences the amounts and types of all-trans-retinoic acid metabolites in F9 teratocarcinoma stem cells. *J Biol Chem* 1992; **267:** 21486–91.