Pediatric primary central nervous system germ cell tumors of different prognosis groups show characteristic miRNome traits and chromosome copy number variations

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Abstract

Background: Intracranial pediatric germ cell tumors (GCTs) are rare and heterogeneous neoplasms and vary in histological differentiation, prognosis and clinical behavior. Germinoma and mature teratoma are GCTs that have a good prognosis, while other types of GCTs, termed nongerminomatous malignant germ cell tumors (NGMGCTs), are tumors with an intermediate or poor prognosis. The second group of tumors requires more extensive drug and irradiation treatment regimens. The mechanisms underlying the differences in incidence and prognosis of the various GCT subgroups are unclear.

Results: We identified a distinct mRNA profile correlating with GCT histological differentiation and prognosis, and also present in this study the first miRNA profile of pediatric primary intracranial GCTs. Most of the differentially expressed miRNAs were downregulated in germinomas, but miR-142-5p and miR-146a were upregulated. Genes responsible for self-renewal (such as POU5F1 (OCT4), NANOG and KLF4) and the immune response were abundant in germinomas, while genes associated with neuron differentiation, Wnt/β-catenin pathway, invasiveness and epithelial-mesenchymal transition (including SNAI2 (SLUG) and TWIST2) were abundant in NGMGCTs. Clear transcriptome segregation based on patient survival was observed, with malignant NGMGCTs being closest to embryonic stem cells. Chromosome copy number variations (CNVs) at cytobands 4q13.3-4q28.3 and 9p11.2-9q13 correlated with GCT malignancy and clinical risk. Six genes (BANK1, CXCL9, CXCL11, DDIT4L, ELOVL6 and HERC5) within 4q13.3-4q28.3 were more abundant in germinomas.

Conclusions: Our results integrate molecular profiles with clinical observations and provide insights into the underlying mechanisms causing GCT malignancy. The genes, pathways and microRNAs identified have the potential to be novel therapeutic targets.

Background

The reported incidence of primary germ cell tumors (GCTs) of central nervous system (CNS) in children is significantly higher in Taiwan, Japan and Korea compared to Western countries. The comparative incidences are 15.3% in Japan, 14.0% in Taiwan, 11.2% in Korea, 2.3% in USA, and 2.5% in German in various reported series [1-5]. There is still no explanation for this extreme geographic and ethnic difference between the three Asian series and the two Western series (p < 0.0001) [5]. Genomic differences need to be considered and evaluated.

Primary CNS GCT consists of several subtypes with different degrees of histological differentiation and malignancy. According to histological differentiation, related tumor markers, and secreted protein markers, these tumors can be classified into germinomas and nongerminomatous GCTs (NGGCTs), the latter including embryonal carcinoma (EC), yolk sac tumors (YST), choriocarcinoma (CC), teratoma (mature teratoma,
immature teratoma, or immature teratoma with malignant differentiation) and mixed GCTs [6]. For NNGGCTs, except for benign mature teratoma, all of the other tumors present with diverse malignancies and therapeutic sensitivities when compared to germinomas and are grouped together as nongerminomatous malignant GCTs (NGMGCTs). NGMGCTs require more extensive drug and irradiation treatment regimens, have a higher recurrence rate and a lower survival rate [7,8]. Clinically, >50% of pediatric CNS GCTs are germinomas, while the majority of remaining tumors are NGMGCTs [5,9]. Histologically, germinoma is the most undifferentiated GCT and is composed of undifferentiated large cells that resemble primordial germinal elements. Among the NNGGCTs, the histological picture differs depending on the diagnosis. EC contains undifferentiated stem cells resembling the embryonic inner cell mass (ICM). YST and CC correspond to the extra-embryonic differentiation along mesoblast and trophoblast lines, respectively. This contrasts with teratomas, which consist of differentiated derivatives that include all three germ layers with or without incompletely differentiated tissue elements, like neuroepithelium, which resembles fetal tissue. CNS GCTs often present with more than one histological component and are then classified as mixed GCTs [7,10,11].

GCTs are presumed to arise from mutated primordial germ cells (PGCs) of genital ridge origin or dysfunction totipotent embryonic cells [12]. Investigation of the different genetic compositions in ECs and ES cells may provide clues about the reduced dependency on external cues for self-maintenance that exist among GCTs, thereby benefiting tumorigenesis research on ECs as well as applications for human ES cells (see also a review article by Werbowetski-Ogilvie et al. [13]). Global gene expression studies in human embryonic stem cells and human pluripotent germ cell tumors have shown that the gene expression patterns of human ES cell lines are similar to those of the human embryonal carcinoma cell samples but are more distantly related to those of seminoma samples [12,14]. Genes that are expressed at significantly greater levels in human ES and embryonal carcinoma cell lines than in control samples were pinpointed and are possible candidates for involvement in the maintenance of a pluripotent undifferentiated phenotype [12]. Wnt and Notch pathway genes are overexpressed in the pluripotent human embryonal carcinoma cell line NTERA2 and in embryonic stem cells [15]. These include members of the frizzled gene family (FZD1, FZD3, FZD4, FZD5, FZD6), which encodes receptors for the Wnt proteins, the Frizzled Related Protein family (SFRP1, SFRP2, FRZB, SFRP4), which encode soluble Wnt antagonists and also ligands and receptors of the Notch pathway (DIKL, Jagged1, Notch1, Notch2, Notch3) [15].

The histological differences between the various different GCTs are mirrored by their gene expression profiles [16,17]. Genomic studies have been conducted on GCTs, most notably on Caucasian adult gonadal ones [12,16]. However, only limited gene profiling studies have focused on primary pediatric CNS GCTs, and, to our knowledge, no transcriptome profiling work on Asian cases has been reported. A very recently paper studied global mRNA expression patterns in pediatric malignant GCTs arising from the testis, the ovary, the sacrococcygeal region and the brain, and then compared these with adult testicular tumors. These results showed that there is no segregation of GCTs with the same histology at different sites or at different ages, within the pediatric range. However, clear segregation of pediatric and adult tumors, most conspicuously among the YSTs, was observed [17]. The pediatric seminomas are significantly enriched for genes associated with a self-renewing pluripotent phenotype, whereas the pediatric YSTs are significantly enriched for genes associated with differentiation and proliferation [17]. These results suggest that the observed clinical differences between pediatric CNS GCTs from different ethnic backgrounds or prognosis groups may also be detected using genomic analysis.

MicroRNAs (miRNAs) are small RNAs of 18-24 nucleotides in length that are involved in the regulation of gene expression and hence a variety of biological processes through post-transcriptional RNA interference-based mechanisms. Mature miRNAs interact and inhibit target mRNAs and result in translational repression or mRNA cleavage [18-20]. In medulloblastoma (MB), an aggressive brain malignancy with a predominant incidence in childhood, a high throughput miRNA profiling analysis found that only a few miRNAs displayed upregulated expression, while most of them, such as miR-9 and miR-125a, were downregulated in the tumor samples, suggesting a tumor growth-inhibitory function [21]. Moreover, the same group identified miRNAs downregulated in human MBs with high Hedgehog (Hh) signaling, which is one of the pathogenesis mechanisms of MB [22]. Differential miRNAs, such as miR-184, have been identified and found to correlate with prognosis, differentiation, and apoptosis in pediatric neuroblastoma [23]. A high-throughput miRNAome analysis of adult gonadal GCTs has been published, and in each GCT subtype the miRNA patterns are quite different [24]. For GCTs in children, only limited miRNA data has been reported.

Genomic copy number variation (CNV) in GCTs of adulthood has been extensively investigated. Gain of 12p in up to 80% of cases of adult testicular GCTs [25,26]. In contrast, comparatively little genomic CNV investigation has been conducted on childhood GCTs. Using metaphase comparative genomic hybridization (CGH), a
wide range of CNVs has been described in pediatric GCTs, including gains on 1q, 2p, 3, 7, 8, 13, 14, 20q, 21, and X, as well as losses on 1p36, 4q, 6q, 11, 13 and 18; but none are seen consistently [27-29]. This may due to either the heterogeneity of the GCTs, or the different algorithms that were applied to identify the CNV regions. In 2007, Palmer et al. used 34 GCTs (22 yolk sac tumors (YSTs), 11 germinomatous tumors and one metastatic embryonal carcinoma), which had occurred in children from birth to age 16, for CNV analysis. Most of their cases were from the testis, the ovary and the sacrococcygeal region and only two germinomas and one YST brain BCT were included [30]. Gain of 12p was found to be present in 53% of primary MGCTs of children aged 5-16 and was also observed in four of fourteen YSTs affecting children less than 5 years old. The YSTs showed an increased frequency of 1p loss (p = 0.003), 3p gain (p = 0.02), 4q loss (p = 0.07) and 6q loss (p = 0.004) compared to the germinomatous tumors [30].

In this study, we applied genomic approaches to explore the molecular messages governing the ethnic and prognosis differences of CNS GCTs. Both mRNA and miRNome expression patterns were studied in pediatric primary CNS GCTs. To provide novel insights into GCT pathogenesis, the transcriptomes of all GCT cases were further compared to those of ES cell lines from both Caucasian and Taiwanese genetic backgrounds [12,23]. Copy number variations (CNVs) in different GCT subtypes were also measured to evaluate their possible influence on gene expression traits. Finally, the transcriptomes of our patients were organized into functional modules in order to identify the dominant biological processes and key genes in the germinomas and NGMGCTs; this sought to help explain the clinical observations associated with these tumors.

Results

Clinical aspects of primary pediatric CNS GCTs examined
In our series of 176 cases of primary pediatric CNS GCTs, 58.5% were germinoma and 41.5% were nongerminomatous GCTs (NGGCTs). Among the germinomas, 62.1% had a histological diagnosis, while the remaining 37.9% of cases had a presumptive diagnosis. For NGMGCTs, 90.3% had a histological diagnosis, with the remaining cases having a presumptive diagnosis. Each presumptive diagnosis of the GCTs was made according to the tumor’s clinical features, neuroimaging results, serum tumor marker level (alpha fetal protein [AFP], beta human chorionic gonadotropin level [beta-hCG]) and response to radiotherapy and/or chemotherapy. Subtypes of NGGCTs included mature teratomas (51.3%), various NGMGCTs including immature teratomas, mixed GCTs, pure YSTs, and tumors diagnosed by tumor markers (35.2%), and unclassified GCTs (1.7%) (Additional file 1-A). The 5-year, 10-year and 15-year overall survival rates for the germinomas and NGMGCTs were 82.2%, 74.5% and 74.5% for the germinomas and 66.1%, 45.4% and 30.3% for the NGMGCTs.

Kaplan-Meier estimator analysis and log-rank test revealed that the germinoma patients had a better overall survival than the NGMGCT patients (p = 0.0005; Figure 1A). Accordingly, therapeutic classification of the GCTs represents prognostic factor-based classification and management. However, the therapeutic classification of CNS GCTs is quite different between the CNS GCTs and extra-CNS GCTs, because of rareness of systemic metastasis of the CNS GCTs [9]. According to the clinical and therapeutic classification of CNS GCTs [9], in our series of CNS GCTs in children, 113 cases (63.6%), including 103 germinomas, 9 mature teratomas, and 1 mixed germinoma and mature teratoma, were categorized as members of the good prognostic group (GPG), 40 cases, including 12 immature teratomas and 19 mixed GCTs, were categorized as members of the intermediate prognostic group (IPG), and 14 cases, including 10 pure yolk sac tumors and 4 mixed GCTs dominated by yolk sac tumors, were categorized as members of the poor prognostic group (PPG) [9]. For the 21 cases that underwent genomic studies (Additional file 1-B), cases 1-12 could be categorized as members of the GPG and these included 9 pure germinomas, 2 mature teratomas, and 1 mixed germinoma- mature teratoma. Cases 13-18 could be categorized as members of the IPG and included 5 mixed GCTs and 1 immature teratoma. Cases 19-21 belonged to the PPG and included 3 mixed GCTs with YST component predominance (Additional file 1-B).

The MicroRNA signatures associated with the different pediatric CNS GCT prognostic groups
Global miRNA expression patterns (the “miRNome”) were analyzed in 12 cases (case 1-6, 12-14 & 16-18 in Additional file 1-B). Differentially expressed miRNAs that correlated with the germinoma group (GPG) and the NGMGCT group (IPG/PPG) were identified by 2-tailed Student’s t-test with a significance level of p < 0.05 plus ≥2-fold changes. Their discrimination ability was assessed by principle component analysis (PCA). Thus, patients within the different prognosis groups were separated by their distinct miRNA profiles (Figure 1B). A heat map of these miRNAs indicates the unique expression levels associated with each prognostic group (Figure 1C). Two miRNAs (hsa-miR-142-5p and hsa-miR-146a) are enriched in the germinoma group (GPG) and 19 miRNAs are enriched in the NGMGCT group (IPG/PPG) (Figure 1C). The differential expression levels of the miRNAs across the two different histological
Figure 1 MiRNome analysis of childhood CNS GCTs. (A) Overall survival rates of GCTs of different histological subtypes. In total, 161 patients were followed up for up to 20 years. These were then subjected to Kaplan-Meier survival analysis. Numbers in parentheses are case numbers of each tumor subtype. Vertical lines indicate the censored survival observations. (B) Principal component analysis (PCA) using the filtered miRNAs ($p < 0.05$ and fold change $\geq 2$). Each spot represents a single array. (C) A heat map shows the miRNAs enriched in the different prognostic groups. MiRNAs in red showed increased expression, while those in blue showed decreased. (D-E) Validation of miRNA array results by real-time PCR. The mean expression levels of the target miRNAs are compared to that of the U6 small nuclear RNA control. Results are expressed as the mean ± standard deviation (SD). (E) The miRNAs’ array hybridization signals are also shown (D).
categories and prognostic groups of the pediatric CNS GCTs were organized by array hybridization intensity (Figure 1D) and verified by quantitative PCR (qPCR) (Figure 1E). The expression levels of hsa-miR-142-5p, hsa-miR-335 and miR-654-3p were found to be different when the patients in these two different groups were compared (Figure 1D-E).

**Stem cell traits associated with the expression patterns of protein-coding gene within the NGMGCT group**

The expression patterns of the protein-coding genes of the same 12 cases described above together with 1 additional germinoma case (case 7 in Additional file 1-B), were also analyzed. In total, 399 probe sets were specifically enriched in the germinoma group (GPG) compared to 292 ones in NGMGCT group (IPG/PPG) with a strict positive false discovery rate (pFDR) threshold of q < 0.001 (Additional file 2). The discrimination ability of these probe sets was assessed by a multidimensional scaling (MDS) assay (Figure 2A). The top 50 transcripts most strongly expressed in the germinoma group (GPG) or the NGMGCT group (IPG/PPG) among the pediatric CNS GCTs are shown in Table 1 and 2, respectively. In the germinoma group (GPG), the presence of MMP-12, which is involved in promoting tumor metastasis, needs to be noted [31] (Table 1, labeled by a asterisk). Podoplanin, a significant lymphatic endothelial cell marker, is also found in the top 50 genes of this group. Podoplanin is expressed by cancer associated fibroblasts (CAFs) and has been shown to be correlated with a poor prognosis in lung adenocarcinomas [32]. In addition, POU5F1 (alias OCT4), a significant transcription factor involved in maintaining the stemness of ES cells [33], is also among the top 50 genes in this group (Table 1, labeled by asterisks). Among the members of the GP group, the NANOG and KLF4 stemness factors are overexpressed (q < 0.01, data not shown). These stemness genes can induce pluripotency in somatic cells and then reprogram them back to a pluripotent status so that they have the essential characteristics of embryonic stem (ES) cells [33,34]. Another pluripotency associated gene, DPP4 (developmental pluripotency associated 4), is also highly expressed in germinomas. Finally, spermatogenesis- and oogenesis-related genes, such as SPATA2 (spermatogenesis associated 2), SPEPS1 (sperm equatorial segment protein 1) and GTSF1 (gametocyte specific factor 1), were also found to be expressed more abundantly in germinomas than in NGMGCTs (Table 1).

In the NGMGCT group (IPG/PGG), genes involved in cell adhesion and migration, such as cadherin 11 (CDH11) and various collagens, are abundantly expressed (Table 2, labeled by asterisks). SNAI2 (alias SLUG) and TWIST2, two key regulators involved in neural crest development and epithelial-mesenchymal transition (EMT), are also highly expressed in this group; these proteins are known to contribute heavily to cell motility and tumor metastasis [35]. Finally, genes such as FZD7 and SFRP1, which are involved in the Wnt signaling pathway, are also highly expressed (Table 2).

It has been recognized that aggressive and poor prognostic glioblastomas, as well as other tumors, acquire characters reminiscent of embryonic stem cells (ESCs) and the degree of ESC gene expression correlates with patient prognosis [36]. It is possible that pediatric CNS GCTs, especially the poor prognosis NGMCGTs, are reminiscent of ES cells. We compared the gene expression patterns of pediatric GCTs to those of Caucasian and Taiwanese ESC lines. PCA analysis showed that NGMGCTs have a closer relationship to ES cells (Figure 2B). The ESC array data from five different data sets (GSE7234, GSE7896, GSE9440 (for the Taiwanese ESC lines) and GSE9832 and GSE13828 (for the Caucasian ESC lines) were all grouped together (Figure. 2B) and possible batch effects during array analysis were ignored. To provide quantitative insights, we calculated the relationships between the GCT subgroups and the ESCs by measuring the average linkage Euclidean distances between them. NGMGCTs were found to closerto the ESC than the germinomas (Figure. 2C).

The closer relationship between NGMGCTs and ESCs was verified further by hierarchical clustering. As shown in Figure. 2D, clearly the NGMGCTs and ESCs form one group while the germinomas form another. In total, 100 genes commonly show high-expression between NGMGCTs and ESCs (Figure. 2D). Among these genes the following are notable. IRS1 (Insulin receptor substrate 1) is an effector of sonic hedgehog mitogenic signaling in cerebellar neural precursors [37] and regulates murine embryonic stem cell self-renewal [38] (Figure. 2D, underlined and in bold). MID1 is a RING finger transcription factor involved in Opitz syndrome and is expressed strongly in undifferentiated cells in the central nervous system as well as the gastrointestinal and respiratory tract epithelium of human embryos [39]. Embryonic oncogenes such as NET1 (neuroepithelial cell transforming gene 1), HIF3A (hypoxia inducible factor 3, alpha subunit), ETS2, RUNX1T1, and the Wnt signaling pathway genes (FZD7 and SFRP1) also appear in this cluster (Figure. 2D). However, notably, two key EMT genes, SNAI2 (SLUG) and TWIST2, are uniquely expressed by NGMGCTs (Figure. 2D).

Among the genes commonly found to show abundant expression in both the ESCs and germinomas, the pluripotent stemness genes DPP4 and POU5F1 (OCT4) are significant (Figure. 2D, underlined and in bold). The array hybridization signal for POU5F1 is shown in Figure. 2E. The high expression of POU5F1, as well as that...
Figure 2 Gene expression analysis of the different GCT subgroups. (A) A multidimensional scaling (MDS) plot containing the differentially expressed genes (690 probe sets, \(q < 0.001\)). Each spot represents a single array. (B) A comparison of the transcriptome traits between ESCs and NGMGCTs by principal component analysis (PCA). (C) Relationships between ESCs, germinomas and NGMGCTs. Average linkage Euclidean distances between the tissues and ESC were calculated using genes distinguishing the filtrated 690-probe set. The confidence limits shown represent the standard error. (D) A heat map shows genes enriched in the ESCs and in the different prognostic groups (\(q < 0.001\)). (E-F) Real-time PCR validation of the microarray data. Mean expression levels of the examined genes were compared to that of the GAPDH control. Each bar represents a different individual (F). The genes' array hybridization signals are also shown (E).
| Probe Set ID   | UniGene ID | Gene Title                                                                 | Gene Symbol | Location   |
|---------------|------------|-----------------------------------------------------------------------------|-------------|------------|
| 207522_s_at   | Hs.513870  | ATPase; Ca++ transporting; ubiquitous                                       | ATP2A3      | chr1p13.3  |
| 1552487_a_at  | Hs.459153  | basocunin 1                                                                | BNC1        | chr15q25.2 |
| 219928_s_at   | Hs.511983  | calcium binding tyrosine-(Y)-phosphorylation regulated                      | CAYR        | chr1q11.2  |
| 219578_s_at   | Hs.547988  | cytoplasmic polyadenylation element binding protein 1                        | CPEB1       | chr15q25.2 |
| 1564491_at    | Hs.590784  | chromosome X open reading frame 18                                          | CXorf18     | chrQ27.2   |
| 206588_at     | Hs.131179  | deleted in azosperma-like                                                   | DAZL        | chr5p24.3  |
| 228057_at     | Hs.480378  | DNA-damage-inducible transcript 4-like                                      | *DDIT4L     | chr4q23    |
| 221630_s_at   | Hs.223581  | DEAD (Asp-Glu-Ala-Asp) box polypeptide 4                                   | DDX4        | chr5p15.2-p13.1 |
| 220004_at     | Hs.125507  | DEAD (Asp-Glu-Ala-Asp) box polypeptide 43                                  | DDX43       | chr6q12-q13 |
| 220493_s_at   | Hs.98586   | doublesex and mab-3 related transcription factor 1                           | DMR1        | chr9p24.3  |
| 232985_s_at   | Hs.317659  | developmental pluripotency associated 4                                    | *DPPA4      | chr3q13.13 |
| 210868_s_at   | Hs.412939  | ELOVL family member 6; elongation of long chain fatty acids                  | *ELOVL6     | chr4q25    |
| 1555299_s_at  | —          | endogenous retroviral family W; env(C7); member 1 (syncytin)                | ERVWE1      | chr7q21-q22 |
| 1533614_a_at  | Hs.652066  | hypothetical protein FL25694                                                | FLJ25694    | chr3q21.31 |
| 207899_at     | Hs.1454    | gastric inhibitory polypeptide                                              | GIP         | chr1q21.3-q22 |
| 222711_at     | Hs.524476  | gametocyte specific factor 1                                                | *GTSF1      | chr1q13.2  |
| 219863_at     | Hs.26663   | hect domain and RLD 5                                                      | *HERC5      | chr9q22.1  |
| 209398_at     | Hs.7644    | histone cluster 1; H1c                                                      | HIST1H1C    | chr6p13    |
| 214455_at     | Hs.553506  | histone cluster 1; H2bc                                                    | HIST1H2BC   | chr6p13    |
| 223861_at     | Hs.298312  | HORMA domain containing 1                                                   | HORMAD1     | chr1q21.2  |
| 217522_at     | Hs.660831  | potassium channel, subfamily V; member 2                                   | KCV2        | chr9p24.2  |
| 219955_at     | Hs.685462  | LINE-1 type transposase domain containing 1                                 | LITD1       | chr1p31.3  |
| 220660_at     | Hs.224183  | leucine zipper protein 4                                                    | LUZP4       | chr9q23    |
| 205668_at     | Hs.153563  | lymphocyte antigen 75                                                      | LY75        | chr2q24    |
| 229475_at     | Hs.651245  | maestrom homolog (Drosophila)                                               | MAEL        | chr1q24.1  |
| 207534_at     | Hs.73021   | melanoma antigen family B; 1                                               | MAGEB1      | chr9p21.3  |
| 206218_at     | Hs.113824  | melanoma antigen family B; 2                                               | MAGEB2      | chr9p21.3  |
| 214397_at     | Hs.25674   | methyl-CpG binding domain protein 2                                         | MB2D        | chr1q21.3  |
| 204580_at     | Hs.1695    | matrix metalloproteinase 12 (macrophage elastase)                          | *MMP12      | chr11q22.3 |
| 204702_s_at   | Hs.404741  | nuclear factor (erythroid-derived 2)-like 3                                | NFE2L3      | chr7p15-p14 |
| 220352_at     | Hs.657952  | sperm equatorial segment protein 1                                         | *SPESP1     | chr15q23   |
| 209626_s_at   | Hs.520259  | oxy steroid binding protein-like 3                                          | OSBP3L      | chr7p15    |
| 204870_at     | Hs.468675  | podoplanin                                                                 | *PDPN       | chr1p36.21 |
| 210265_x_at   | Hs.450254  | POU class 5 homeobox 1 pseudogene 3                                        | *POUSF1(OCT4) | chr12p13.31 |
| 225579_at     | Hs.274415  | PQ loop repeat containing 3                                                | PQLC3       | chr2p25.1  |
| 204086_at     | Hs.30743   | preferentially expressed antigen in melanoma                               | PRAME       | chr2q11.22 |
| 218700_s_at   | Hs.115325  | RAB7; member RAS oncogene family-like 1                                    | RAB7L1      | chr1q32    |
| 1558668_s_at  | Hs.351068  | spermatogenesis associated 22                                              | *SPATA22     | chr1p13.3  |
| 223883_s_at   | Hs.309767  | serine/threonine kinase 3                                                  | STK31       | chr7p15.3  |
| 1535999_s_at  | Hs.506504  | synaptonemal complex protein 3                                             | SYCP3       | chr1q2    |
| 39318_at      | Hs.2484    | T-cell leukemia/lymphoma 1A                                                | TCLI1A      | chr14q32.1 |
| 206413_s_at   | Hs.510368  | T-cell leukemia/lymphoma 1B                                                | TCLI1B      | chr14q32.1 |
| 215356_at     | Hs.646351  | tudor domain containing 12                                                | TDRD12      | chr19q13.11 |
| 223530_at     | Hs.144439  | tudor and KH domain containing                                            | TDRK1H      | chr1q21    |
of another stemness gene NANOG in germinomas, was verified by qPCR (Figure 2F). In contrast, SNAI2 (SLUG) is overexpressed in NGMGCTs (Figure 2E-F).

Relationships between abundant microRNAs and their target mRNAs
The most differentiating miRNAs between the histological subgroups were used to predict their mRNA targets. This was performed by examining whether there were any candidate miRNA target genes, the expression of which became significantly higher in a given group of tumors, which also showed a correlated reduction in the related miRNAs. This analysis yielded miRNA-target pairs that showed opposite expression patterns in the same prognostic group (Table 3). In the germinoma group, the expression levels of RUNX1T1 and THRB were inversely correlated with expression of miR-146a, and the levels of NRP1, SVIL, and PDGFRA were inversely correlated with the expression of miR-142-5p. Furthermore, RUNX1T1 is a putative target of both miR-142-5p and miR-146a (Table 3, underlined). In the NGMGCT group, inverse correlation expressions were also found between miRNAs and their candidate downstream targets (Table 3), specifically, miR-218, which is an intragenic miRNA of the overexpressed SLIT2 gene (Table 3, labeled by an asterisk).

The signature miRNAs in the same GCT prognosis group were found to target the same mRNAs. miR-503 and miR-543 both target PAFAH1B1 and RNF138, while miR-26a and miR-503 both target CREBL2 and DNAJA2 (Table 3, underlined). In addition, FRAT2 is a putative target of both miR-26a and miR-539, ATP11C is a target of both miR-26a and miR-543, NMT1 is a target of both miR-181c and miR-401, WNT2B is a target of both miR-218 and miR-503, N4BP1 is a target of both miR-335 and miR-503, and OSBPL3 is a target of both miR-410 and miR-543 (Table 3, underlined). Some mRNAs are even targeted by more than two miRNAs: NUP50 is targeted by three miRNAs (miR-26a, miR-218 and miR-503), while WAPAL is a target of four miRNAs (miR-26a, miR-335, miR-433 and miR-539 (Table 3, in bold and underlined, respectively). Thus it would seem that there are complex and highly interactive miRNA-mRNA genetic networks active in germinomas and NGMGCTs.

Functional module and pathway analysis as a framework for the interpretation of GCT biology
The gene list outlined above gave us preliminary insights into the functional consequences of detected differential gene expression. To understand more about how the gene expression profiles might be correlated with pathogenesis and the various clinical phenotypes as well as to provide quantitative evidence, the signature mRNAs were subjected to a Gene Ontology (GO) database search [40] in order to find statistically overrepresented functional groups within the gene lists. The WebGestalt web tool [41] was applied to provide statistical analysis and visual presentation of the results. The GO categories of biological processes that were statistically overrepresented ($p \leq 0.05$) among genes of the germinoma group are shown in Figure 3A. Genes CHEK2 and HUS1, which are involved in the DNA damage checkpoint, were significantly overexpressed in germinomas ($p = 3.45 \times 10^{-2}$; Figure 3A, panel 1). Another significant biological process associated with this group is related to the immune system processes ($p = 2.64 \times 10^{-2}$; Figure 3A, panel 2, where the involved immune response genes are shown). Other predominant processes in the GP group include genes pertaining to reproduction ($p = 2.74 \times 10^{-2}$) and male gonad development ($p = 1.24 \times 10^{-2}$; Figure 3A, panel 3).

In contrast, the principal functions of the p-regulated genes in the NGMGCT group (IPG/PPG) of pediatric GCTs include those related to small GTPase (Rho protein especially) mediated signal transduction (Figure 3B, panel 1), cell motility (Figure 3B, panel 2) and various genes associated with active differentiation processes, in particular neuron differentiation (Figure 3B, panel 3). Seven genes involved in the Wnt receptor signaling pathway are also significantly active in this group ($p = 1.07 \times 10^{-4}$; Figure 3B, panel 1). When the genes ($q < 0.001$) are subjected to a KEGG pathway database to obtain a similar module analysis using the DAVID 2008 web-based tool, the top-ranked canonical pathways in the NGMGCT group again include cell motility (such as Focal adhesion, ECM-receptor interaction and Gap junction), axon guidance and Wnt signaling (Figure 3C). Expression of Wnt pathway genes (such as FZD1, FZD3, FZD4, FZD5, FZD6 and SFRP1, SFRP2, FRZB, SFRP4) have been previously reported in a pluripotent human

### Table 1: Top 50 known genes in TW germinomas (Continued)

| Gene ID | Hs.156471 | Transcription factor CP2-like 1 | TFCP2L1 | chr2q14 |
|--------|-----------|---------------------------------|---------|---------|
| Gene ID | Hs.487510 | transmembrane protein 170 | TMEM170 | chr16q23.1 |
| Gene ID | Hs.458406 | undifferentiated embryonic cell transcription factor 1 | UTF1 | chr10q26 |
| Gene ID | Hs.371738 | WD repeat domain 21C | WDR21C | chr8q21.3 |
| Gene ID | Hs.709857 | WD repeat domain 66 | WDR66 | chr12q24.31 |
| Gene ID | Hs.335787 | zinc finger protein 42 homolog (mouse) | ZFP42 | chr4q35.2 |
| Probe Set ID | UniGene ID | Gene Title | Gene Symbol | Location |
|--------------|------------|------------|-------------|----------|
| 219935_at    | Hs.58324   | ADAM metallopeptidase with thrombospondin type 1 motif, 5 | ADAMTS5    | chr2q13.3 |
| 219087_at    | Hs.435655  | asporin    | ASPN        | chr9q22  |
| 205433_at    | Hs.420483  | butyrylcholinesterase | BCHE | chr3q26.1-q26.2 |
| 236179_at    | Hs.116471  | cadherin 11; type 2; OB-cadherin (osteoblast) | *CDH11 | chr16q22.1 |
| 212865_s_at  | Hs.409662  | collagen; type XIV; alpha 1 | *COL14A1 | chr8q23 |
| 202311_s_at  | Hs.709197  | collagen; type 1; alpha 1 | *COL1A1 | chr7q21.33 |
| 229218_at    | Hs.489142  | collagen; type 1; alpha 2 | *COL1A2 | chr7q22.1 |
| 208096_s_at  | Hs.47629   | collagen; type XXI; alpha 1 | *COL21A1 | chr6p12.3-p11.2 |
| 232458_at    | Hs.443625  | Collagen; type III; alpha 1 (Ehlers-Danlos syndrome type IV) | *COL3A1 | chr2q31 |
| 212489_at    | Hs.210283  | collagen; type V; alpha 1 | *COL5A1 | chr9q34.2-q34.3 |
| 221729_at    | Hs.445827  | collagen; type V; alpha 2 | *COL5A2 | chr2q14-q32 |
| 202575_at    | Hs.405662  | cellular retinoic acid binding protein 2 | CRABP2 | chr1q21.3 |
| 204619_s_at  | Hs.695930  | chondroitin sulfate proteoglycan 2 (versican) | CSPG2 | chr5q14.3 |
| 232090_at    | Hs.594880  | Dynamin 3 | DNM3 | chr1q24.3 |
| 204433_s_at  | Hs.183713  | endothelin receptor type A | EDNRA | chr4q31.23 |
| 204400_at    | Hs.24587   | embryonal Fyn-associated substrate | *EFS | chr1q43.2-q12 |
| 203184_at    | Hs.519294  | fibrillin 2 (congenital contractural arachnodactyly) | FBNA2 | chr5q23-q31 |
| 231130_at    | Hs.645700  | fibronectin 7 | FKBP7 | chr2q31.2 |
| 204359_at    | Hs.533710  | fibronectin leucine rich transmembrane protein 2 | FLRT2 | chr4q24-q32 |
| 222853_at    | Hs.41296   | fibronectin leucine rich transmembrane protein 3 | FLRT3 | chr2p11 |
| 243278_at    | Hs.656280  | forhead box P2 | FOXP2 | chr7q31 |
| 203706_s_at  | Hs.173859  | frizzled homolog 7 (Drosophila) | *FZD7 | chr2q33 |
| 205498_at    | Hs.125180  | growth hormone receptor | GHR | chr5p13-p12 |
| 227070_at    | Hs.631650  | glycosyltransferase 8 domain containing 2 | GLT8D2 | chr1q23 |
| 204237_at    | Hs.470887  | GULP; engulfment adaptor PTB domain containing 1 | GULP1 | chr2q32.3-q33 |
| 224997_s_at  | Hs.533566  | H19; imprinted maternally expressed transcript | H19 | chr11p15.5 |
| 215446_s_at  | Hs.102267  | Iysyl oxidase | LOX | chr5q24.2 |
| 204069_at    | Hs.526754  | Meis homeobox 1 | MEIS1 | chr2p14-p13 |
| 207480_s_at  | Hs.510989  | Meis homeobox 2 | MEIS2 | chr15q14 |
| 206201_s_at  | Hs.170355  | mesenchyme homeobox 2 | MEOX2 | chr7p22.1-p21.3 |
| 203637_s_at  | Hs.27695   | midline 1 (OptZIP/BBP syndrome) | MID1 | chrXp22 |
| 222722_at    | Hs.708130  | osteoglycin | OGN | chr9q22 |
| 213568_at    | Hs.253247  | odd-skipped related 2 (Drosophila) | OSR2 | chr8q22.2 |
| 225975_at    | Hs.591691  | protocadherin 18 | PCDH18 | chr4q31 |
| 203131_at    | Hs.74615   | platelet-derived growth factor receptor, alpha polypeptide | PDGFRB | chr4q11-q13 |
| 212915_at    | Hs.434900  | PDZ domain containing RING finger 3 | PDZR3 | chr5p13 |
| 227419_s_at  | Hs.204947  | placenta-specific 9 | PLAC9 | chr10q22.3 |
| 218080_s_at  | Hs.136348  | peristin; osteoblast specific factor | POSTN | chr13q13.3 |
| 234852_at    | Hs.657841  | Paired related homeobox 1 | PRX1 | chr11q24 |
| 208131_s_at  | Hs.302085  | prostaglandin D2 synthase | PTGIS | chr2q13.13 |
| 214043_at    | Hs.446083  | protein tyrosine phosphatase, receptor type D | PTPRD | chr9p23-p24.3 |
| 225946_at    | Hs.696433  | Ras association (RalGDS/AF-6) domain family member 8 | RASSF8 | chr12p12.3 |
| 232060_at    | Hs.654491  | receptor tyrosine kinase-like orphan receptor 1 | ROR1 | chr1p33-p32 |
| 205529_s_at  | Hs.368431  | runt-related transcription factor 1; translocated to 1 | RUNX1T1 | chr8q22 |
Table 2: Top 50 known genes in TW NGMGCTs (Continued)

| miRNA | Mapping | Intragenic | Predicted Targets | p value |
|-------|---------|------------|------------------|---------|
| 203022_at | Hs.736730 | secreted frizzled-related protein 1 | *SFRP1 | chr8p12-p11.1 |
| 203023_at | Hs.188824 | snail homolog 2 (Drosophila) | *SNAI2 (SLUG) | chr8q11 |
| 203024_at | Hs.371147 | transforming growth factor beta 1 induced transcript 1 | TGFB1I1 | chr16p11.2 |
| 203025_at | Hs.708196 | twist homolog 2 (Drosophila) | *TWIST2 | chr2q37.3 |

Table 3: Signature miRNAs and their predicted targets in the opposite prognostic group

| miRNA | Mapping | Intragenic | Predicted Targets | p value |
|-------|---------|------------|------------------|---------|
| 142-5p | 17q22 | ADAMT55, BCHE, DCHS1, FIGN, FLJ10357, FLRT2, FZD7, HDAC4, MEIS2, NRP1, PDGFRB, PTPRD, RUNX1T1, SCD, SVIL | 2.09E-10 |
| 146a | 5q33.3 | C5orf23, PTGFRN, RPS3P, RUNX1T1, SRR, THR3 | 5.89E-07 |
| let-7e | 19q13.43 | (NA) | (NA) |
| 26a | 3p222 | CTDSPL | (NA) |
| 181c | 19p13.12 | C17orf63, EPB41, NMT1 | 1.18E-03 |
| 154 | 1q32.2 | (NA) | (NA) |
| 218 | 4p15.31 | *SLIT2, NUP50, SFBMT1, WDR66, WNT2B, ZDHHC23, ZNF313 | 1.82E-02 |
| 335 | 7q32.2 | N4BP1, PHTF2, SLCA45A3, WAPAL | 3.34E-04 |
| 376a | 1q32.31 | BNC1, MAN1C1 | 1.45E-02 |
| 410 | 1q32.31 | AGPAT7, DLG3, NMT1, OSBPL3, RGS16, ROD1, ZNF2 | 2.27E-04 |
| 433 | 14q32.2 | RTL1, PCGB, WAPAL | 4.50E-02 |
| 503 | Xq26.3 | CREBL2, DNAJA2, KIAA1333, MBP, N4BP1, NUP50, PFAH1B1, RNF138, WNT2B | 1.41E-07 |
| 539 | 14q32.2 | CCDC88A, FRAT2, LBA1, MYC1L, PSME3, SNAP29, WAPAL, XPO6 | 4.75E-05 |
| 543 | 14q32.31 | ARFGEF2, ATP11C, CSNK1D1, CTF8, OSBPL3, PFAH1B1, PPTC7, RNF138, SDF2L1 | 2.38E-05 |

NA: No miRNA target could be found in the opposite group.
Underlined: Genes targeted by 2 microRNAs.
In bold and underlined: Genes targeted by more then 2 microRNAs.

embryonal carcinoma cell line and in an embryonic stem cell [15], which supports the reliability of our functional module analysis. FZDI, FZD4, FZD7 and SFRP1 are also in our gene list (Table 2 and Additional file 2). The detailed locations of the signature genes are indicated in Additional file 3 and Additional file 4.

Chromosome locations of the differentially expressed genes and cytogenetic analysis of the GCTs

Gene set enrichment analysis (GSEA) was performed by DAVID for all chromosomal arms using the entire gene list. NGMGCTs were found to shows significantly transcript expression in the 7q21 cytoband region, which contains 3 NGMGCT genes: GNG11 (guanine nucleotide binding protein (G protein), gamma 11), GNAI1 (G protein alpha inhibiting activity polypeptide 1) and FZDI (frizzled homolog 1). In germinomas, genes were overexpressed at Xq27.1, 14q32.1 (TCL1A & 1B), 1p36.11 (CCDC21, ZNF593, FAM46B and Ciorf135), 12q13.13, 6p21.33 (ABC1F, HIST1H2BK and C6orf136) and 20q13.1-q13.2 (Figure 3D). The POU5F1 (OCT4) germinoma gene, as well as SLC4A8, LOC57228 and CIorf44, are overexpressed at chr12q13.13. The spermatogenesis associated gene SPATA2, as well as PTPN1, are overexpressed at 20q13.1-q13.2 (Figure 3D). It is likely that gene expression changes are attributable to underlying chromosomal aberrations. To identify such a correlation, we examined the cytogenetic abnormalities present in each GCT prognosis subtype. Copy number variation (CNV) analysis was performed on 15 pediatric CNS GCT cases (7 pure germinomas, 3 pure mature teratomas and 5 NGMGCTs; Additional file 1-B) in order to detect chromosomal aberrations. A data set containing 125 Human 1 M HapMap samples (generated by the ParTek Inc.) was used as a copy number baseline. The aberrant chromosome regions in each tested individual are summarized in Additional file 5. As shown in Figure 4, 3 out of 5 NGMGCT cases have a reduced DNA copy number between 4q13.3-4q28.3 (S1) and 9p11.2-9q13 (S2). The protein-coding genes and miRNAs located in these changed regions are shown in Table 4. BANK1, CXCL9, CXCL11, DDIT4L, ELOVL6 and HERC5 are
Figure 3 Altered functional modules in the different pediatric GCT prognostic groups. (A-B) Gene set enrichment analysis according to the Gene Ontology (GO) classification. Probe sets differentiating good prognostic CNS GCTs from intermediate/poor prognostic CNS GCTs were subjected to the GO database search via the DAVID 2008 interface. The number of genes, gene symbols, their percentages and the p-values for each category that show significance (p < 0.05) and are enriched in either the good (A) or the intermediate/poor (B) prognostic group are listed. (C) KEGG pathways significantly enriched in the TW NGMGCT genes. The number of genes, their percentages in terms of total genes, and the p-values for pathways that are significantly over-represented (p < 0.05 by the DAVID 2008 tool) are listed. (D) Distribution of signature genes on the chromosome cytobands.
within 4q13.3–4q28.3 and are relatively more abundant in germinomas (Table 4 and Additional file 2). DDIT4L, ELOVL6 and HERC5 are also among the top 50 most dominant genes in germinomas (Table 1).

Discussion and Conclusions

GCT is a specific type of CNS tumor with several subtypes. The two major forms of these tumors, germinoma (GPG) and NGMGCT (IPG/PPG), present with different clinical behaviors, differences in sensitivity to therapeutic regimens and different outcomes. The overall survival of patients with germinomas is significantly better than that of patients with NGMGCTs in our series (Figure 1A) and this is similar to other previously reported series [42,43]. To explore the molecular difference between these two different histological/therapeutic prognostic groups, we have identified with confidence a number of differently expressed miRNAs and mRNA; these permit an interpretation of the clinical survival variations and downstream hypothesis testing. The various divergent biological functions that correlate with the clinical observations are also revealed.

Among these miRNAs, miR-142-5p and miR-146a are upregulated in the pediatric germinomas (GP group) when compared to the NGMGCTs (IPG/PPG). Up to the present, no miRNA profile of pediatric GCTs has been published. A miRNome report on adult gonadal GCTs showed that, for each GCT subtype, the miRNA patterns are quite different [24]. In their dataset, miR-142-5p and miR-146a are also more abundant in adult seminomas than in gonadal ECs [24]. In addition, let-7e, miR-133b, miR-218 and miR-654-3p are also abundant in both pediatric NGMGCTs and adult ECs (Figure 1C) [24]. However, the notable discrepancies are miR-181c and miR-218, the expression levels of which are more abundant in adult testicular seminomas but are lower in pediatric intracranial germinomas (Figure 1) [24]. The unique expression pattern of these miRNAs in pediatric CNS GCTs may reflect the differences in pathogenesis mechanisms between adult and pediatric GCTs [17], or, alternatively, the variation in genetic makeup between Western and Taiwanese patients.

We also correlated the transcript levels of miRNAs to their candidate targets in order to identify microRNA-mRNA target pairs (Table 3). It has been shown that some miRNAs, such as miR-1, can downregulate the transcript levels of a large number of target genes in mammalian cells [18]. Two large scale proteomic studies published very recently have shown that, although some microRNA target proteins are repressed without

Figure 4 Chromosomal aberrations in the TW germinomas, mature teratomas (MTs) and NGMGCTs. The red bars on the right side of the chromosome idiograms indicate gain in these chromosomal regions, while blue bars indicate chromosomal loss. Two common copy number variation (CNV) regions (S1 & S2) in 3 out of 5 NGMGCT cases are highlighted.

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detectable changes in mRNA levels, more than a third of translational repressed targets also display detectable mRNA destabilization and, for the more highly repressed targets, mRNA destabilization usually makes up the major component of repression [19,20]. Gene expression microarrays can therefore be, and have been, applied for the identification of downstream targets for miRNAs [44-46]. However, proof of direct binding between those miRNAs and target mRNAs, as well as the direct translational repression of target mRNAs, is still needed. Such confirmation will require more wetlab experiments such as immunoblotting and reporter assays.

When compared with NGMGCTs, the germinomas largely recapitulate the features of self-renewing pluripotent human embryonic stem (hES) cells, such as involvement of POU5F1 (OCT4), NANOG and KLF4 (\(q < 0.01\)). Both seminomas and embryonal carcinomas are known to express stem cell markers, such as POU5F1 and NANOG [47,48]. In addition, in an attempt to find coordinated overexpressed gene clusters in GCTs, Korkola et al. found NANOG at chromosome 12p13.31 is overexpressed in undifferentiated (embryonal carcinomas and seminomas) versus differentiated (teratoma, yolk sac tumor, and choriocarcinoma) tumors [16]. By overexpressing POU5F1, NANOG and KLF4, it is now possible to reprogram the transcriptomes of somatic primary cells, which results in their dedifferentiation from matured cells to ES cell-like iPS (induced pluripotent stem) cells [49]. The abundant expression of these dedifferentiation factors in germinomas therefore mirrors the more undifferentiated histopathological characteristics of these tumors. Whereas
such similarities have previously been described for adult and pediatric seminomas [16,17,47,48], we now know that this also applies to Asian pediatric CNS germinomas.

Although germinomas abundantly express the above three stemness factors, it is NGMGCTs (IPG/PPG) who show a closer gene expression pattern to ESCs (Figure 2C). This observation is consistent with pervious global gene expression reports whereby the gene expression patterns of human ES cell lines are similar to those of the human embryonic carcinoma cell samples but are more distantly related to those of seminoma samples [12]. The close relationship between NGMGCTs and ES cells supports the hypothesis that germinomas are closely related to primordial germ cells (PGCs), and EC cells/NGMGCTs represent a reversion to a more ICM- or primitive ectoderm-like cell type [12]. Whether germinomas and zygotes/blastosomes share similar mRNA or microRNA profiles is under investigation at present. The close relationship between NGMGCTs and ES cells may additionally be reflected in the worse prognosis for these tumors. Recently, via novel genomic approaches, it has been shown that aggressive and poor prognostic tumors, such as glioblastomas, inherit preferential ES cell gene expression profiles [36]. The similarity between pediatric NGMGCTs and human ES cells may therefore reflect the clinical observation that CNS NGMGCTs are more malignant and show a higher fatality rate than germinomas.

The close relationship in genetic makeup between NGMGCTs and ESCs also suggest that factors other than POU5F1 (OCT4), NANOG or KLF4 are responsible for ESC gene expression. In this study, we found that two key epithelial-mesenchymal transition (EMT) regulators, SNAI2 (SLUG) and TWIST2, are abundantly expressed in the NGMGCT group (IPG/PPG) (Table 2 and Figure 2D). It has been reported that EMT transcription factors, SNAI1 (alias SNAIL) and TWIST, can independently dedifferentiate mammalian cancer cells and induces the generation of cancer stem-like cells, which then form mammospheres [50]. It is possible that SNAI2 (SLUG) and TWIST2 behaves like Snail and TWIST and can introduce malignancy and stemness in pediatric GCTs. Targeting oncogenic stemness genes or EMT-related embryonic signaling pathways (such as the Wnt/β-catenin pathway) may differentiatate a highly malignant NGMGCT into a more matured transcriptome type, thereby increasing the sensitivity of these tumors to the classical therapeutic regimen of radical resection, irradiation and chemotherapy, which would produce a better prognosis for the patients.

In addition to stemness genes (such as genes involved in reproduction and male gonad development), the germinomas were found to overexpress genes involved in the DNA damage checkpoint, which indicates active DNA integrity checking in the germinomas and thereby reflects why the clinical phenotype of germinomas has a better prognosis (Figure 3A). Among the other genes that were found to be expressed abundantly in germomatous tissues were genes associated with the immune system process and this correlates with the abundant lymphocytic infiltration of germinomas found during histological observation. Relative to germinomas, we observed a significant enrichment of overexpression of differentiation and morphogenesis (especially neurogenesis) genes in NGMGCTs, which correlates with the differentiated state of these tumor cells (Figure 3B). There is also evidence of overexpression of genes in the Wnt/β-catenin pathway in our dataset (Figures 3B-C), which is consistent with previous studies of nonseminomatous malignant GCTs [15,51]. In concordance with the higher recurrence and disseminating clinical behaviors of NGMGCTs, a significant enrichment for overexpression of motility, tight junction, focal adhesion, and adherent junction genes in NGMGCTs was observed (Figures 3B-C). Our results thereby integrate molecular profiles with clinical observations and provide a better understanding of the underlying molecular mechanisms. The combined targeting of hub genes involved in all these biological modules by a cocktail therapy-like regimen may eventually lead to an alleviation of these malignant CNS tumors.

During the submission of this manuscript, a very recent reference based on testis GCTs identified gene expression signatures that predicted outcomes in patients with extra-cranial adult GCTs [52]. We compared the age and tumor characteristics between our series against the genomic study group of CNS GCTs in children and the reported study of extra CNS GCTs in adult men (Additional file 1-C) [52]. We included, whereas Korkola’s study involved adult men with nonseminomatous GCTs (NSGCTs) [52]. In our series, 118 tumors were pure germinomas or tumors with a germinoma component, 49 tumors were pure teratoma or tumors with a teratoma component, and 27 cases were classified as YSTs including 10 pure YSTs, 11 tumors with a YST component, and 6 cases with serum AFP elevation (pure immature teratomas excluded). Among the 21 cases with genomic studies, 9 tumors were pure germinomas, 2 tumors were pure mature teratomas, and 9 tumors were mixed GCTs, including one mature teratoma with serum AFP elevation and one germinoma with serum AFP elevation. The correlation of tumor characteristics between the studies of Korkola et al. and ours in Additional file 1-C constituted the basis for the comparison of genomic molecular
findings across the different therapeutic prognostic groups and histology between these two studies.

Korkola et al. concluded that using a 140-gene signature, they could predict 5-year overall survival (OS) \( p < 0.001 \) \[52\]. Both our study and that of Korkola et al. identified good outcome GCTs express gene sets involved in immune function and the repression of differentiation (such as POU5F1/OCT4), while poor outcome GCTs express genes involved in active differentiation (in particular, neuron differentiation) (Fig. 3) \[52\]. A 10-gene prognosis model was also built using a univariate Cox model. When the samples were dichotomized by median score, there was significant separation of the survival curves \( p < 0.002 \) \[52\]. These 10 genes were STX6, CFLAR, FNBP1, ITSN2, SYNE1, MAP3K5, PTGDS, PXMP2, IRAK4, and RABGAP1L \[52\]. Among these 10 genes STX6 (syntaxin 6) and CFLAR (CASP8 and FADD-like apoptosis regulator) are over-expressed in our germinoma group \( p < 0.01 \). It will be interesting to fit their prognosis signatures onto our dataset to see whether GCTs of different anatomic locations, ages and ethnic populations express similar prognosis genes. However, since all the tissues used in our study were freshly collected over the last 2 years, only one death has been recorded so far (Additional file 1). As a result, this work needs to be carried out at a later stage.

The variation in chromosome copy number variation (CNV) regions between germinomas and NGMGCTs were mapped to cytobands 4q13.3-4q28.3 and 9p11.2-9q13 (Figure 4). Chromosome abnormality analysis of adult testicular germ cell tumors (tGCTs) revealed that all GCTs show 12p gain \[25,26\]. In 2007, Palmer et al. used metaphase-based comparative genomic hybridization (CGH) to analyze genomic imbalance in 34 pediatric GCTs (22 yolk sac tumors (YSTs), 11 germinomatous tumors and 1 metastatic embryonal carcinoma). The YSTs showed an increased frequency of 1p loss \( p = 0.003 \), 3p gain \( p = 0.02 \), 4q loss \( p = 0.07 \) and 6q loss \( p = 0.004 \) compared to germinomas \[30\]. Most of their cases were from the testis, the ovary or the sacrococcygeal region and only 2 germinomas and 1 YST brain GCTs were included \[30\]; this is a possible explanation of the discrepancies between their results and ours. We also observed 4q loss in the NGMGCTs (including YSTs), suggesting that genomic imbalance in this region, and the genes/miRNAs encoded by this chromosomal region, may play a crucial tumor suppressing role during NGMGCT pathogenesis and affect clinical performance (Table 4). Six genes (BANK1, CXCL9, CXCL11, DDIT4L, ELOVL6 and HERC5) within 4q13.3-4q28.3 showed higher expression levels in the germinomas (Table 4). DDIT4L, ELOVL6 and HERC5 are among the top 50 highly expressed genes in germinomas (Table 1). A putative GCT tumor suppressor gene SYNPO2 (Synaptopodin 2), also known as myopodin, is also within the 4q13.3-4q28.3 deletion region (Table 4). SYNPO2 has recently been shown to have the highest predictive value when assessing 5-year overall survival \[52\], which is consistent with a possible role as a tumor suppressor. However, we do not observe differential SYNPO2 expression between NGMGCTs and germinomas (Table 4). It is unclear whether SYNPO2 expression is also downregulated in Taiwanese germinomas compared to normal brains. In addition, whether survival predictors derived from Western cases can be applied to Asian patients still awaits elucidation.

Recently two independent genome-wide association studies (GWAS) have reported on susceptibility loci associated with tGCT: Kanetsky et al. mapped seven markers at 12p22 near KITLG (c-KIT ligand) and two markers at 5q31.3 near SPRY4 (sprotty 4) \[53\]; furthermore Rapley et al. identified loci on chromosome 5, 6 and 12 \[54\]. A third locus, in an intron of BAK1, a gene that promotes apoptosis, was also identified by Rapley et al. \[54\]. Similarly, the CGH profiles in childhood GCTs have been reported to resemble those in adults \[55,56\]. In terms of cytogenetics differences between the different histological entities, loss of chromosome 19 and 22 material and gain of 5q14-q23, 6q21-q24 and 13q material were found to occur at a significantly lower frequency in seminoma adult tGCTs compared to non-seminoma adult tGCTs \[25\]. Among Taiwanese pediatric GCTs, no common copy number variation (CNV) could be found in either the germinomas or the mature teratomas (Figure 4). The divergence between our results and published Caucasian ones may be partly due to the different ethnic samples used, the application of different bioinformatics algorithms and the fact that we compared the differences between germinomas and NGMGCTs but not common aberrations across all GCTs.

In summary we have identified miRNome, mRNA signatures and CNV regions that are associated with two pediatric GCT histological entities (germinoma and NGMGCTs) and two prognostic groups (GPG and IPG/PPG). The clinical discrepancies between the two histological entities (germinomas of GPG and NGMGCTs of IPG/PPG) are therefore mirrored by their differences in global transcriptome patterns and their unique stem cell traits. One of the interesting questions that remain is whether pediatric GCTs from other ethnic background also express similar transcriptome traits and CNV regions. If Caucasian and Taiwanese GCTs possess unique transcriptome traits, therapeutic and diagnostic experience from Western countries may not be applicable directly to Asian or Taiwanese patients. Therefore, the genes and miRNAs identified here hold the potential of being novel therapeutic targets and may be used for...
further differentiation therapy. The Wnt pathway, for example, is activated in NGMGCTs (Figure 3C), and drugs targeting this specific pathway may hold potential as a treatment approach to NGMGCTs. Transdifferentiating ESC-like NGMGCTs into a benign status may also be a novel and useful tactic against these fatal pediatric tumors.

Methods

Patient details and microarray expression data

All procedures were approved by the Institutional Review Board of the Taipei Veteran General Hospital, Taiwan and informed consent was obtained from each subject or the subject’s guardian according to the Helsinki Declaration. In this study, we reviewed a clinical database containing 176 cases of primary pediatric CNS GCTs involving patients less than 18 years old; the database was collected from 1970 to 2007 at Taipei Veterans General Hospital (Taipei VGH). Among them, RNA samples from the hospital tissue bank were obtained in 13 cases, and mRNA and miRNA microarray analysis were performed in 13 cases and 12 cases respectively. The histological types of this series of 176 primary CNS GCTs and other selected clinical data are summarized in Additional file 1-A. Excluding operative mortality, the overall survival rates of the 95 germinoma cases and 59 NGMGCT cases that form this series were studied to support the difference in malignancy and outcome between these two groups of CNS GCTs. Overall survival was analyzed by the Kaplan-Meier method, and the log-rank test was applied to compare the cumulative survival durations in the different patient groups and this was done using SPSS statistics software (SPSS Inc., Chicago, Illinois, USA).

The clinical features of the 22 CNS GCT cases used in microarray studies are listed in Additional file 1-B in order to help correlation with the results of the genomic analysis. In the transcriptome analysis, 13 cases had both mRNA and miRNA analyzed, except that case 7 had only mRNA analyzed (Additional file 1-B); the latter was due to insufficient RNA being available. The histological subtypes in the dataset are germinoma (6), mixed GCT of germinoma and mature teratoma (1), immature teratoma (1), mixed GCTs of NGMGCTs category (4), YST (1). Caucasian embryonic stem cell (ESC) array data that had been previously published [57], and the array data of Taiwanese ESC line hES-T3 (T3ES) were downloaded from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) database (accession number GSE9440) [58]. All ESC and GCT mRNA array data were implemented using the Affymetrix Human Genome U133 Plus 2.0 chips. The ESC array dataset was downloaded from GEO datasets GSE7234, GSE7896, GSE9440 (for Taiwanese ESC lines) together with GSE9832 and GSE13828. All GCT raw array data (including gene expression array, microRNA array and SNP array) are available from the GEO database (accession number GSE19350).

MicroRNA microarray and data analysis

The Agilent Human miRNA Microarray Kit V2 (Agilent, Foster City, CA, USA) containing probes for 723 human microRNAs from the Sanger database v10.1 was used. GeneSpring GX 9 software (Agilent, USA) was used for value extraction. A 2-tailed Student’s t-test was then used for the calculation of the p value for each miRNA probe. Principal component analysis (PCA) was performed using the Partek Genomics Suite software http://www.partek.com to provide a visual impression of how the various sample groups are related. To predict the downstream mRNA targets of the miRNAs, the TargetScan web tool http://genes.mit.edu/targetscan/index.html was used. The miRNA-target pairs were then mapped by examining whether there were any candidate mRNA target genes whose expressions became diminished in a given group of tumors while there was overexpression of the correlated miRNAs. A Fisher’s exact test was used to examine whether the associations obtained were by chance or not.

Copy number variation (CNV)

The materials used in the CNV study were fresh frozen tumor tissues, and the genomic DNA from each sample was isolated using a DNeasy Blood & Tissue Kit according to the manufacturer’s instructions (Qiagen, GmbH, Germany). The Human610-Quad Beadchip (Illumina Technologies, USA) with 550,000 selected tag SNPs and 60,000 genetic markers covering 4.7 KB mean probe spatial resolution was used for the analysis. Normalized bead intensity data obtained for each sample were loaded into the Illumina BeadStudio™ software version 3.1.3.0, which calculated CNV data from intensity and B allele frequency. The calculated results were then exported to Partek Genomics Suite software. Chromosome abnormalities were identified by the cnvPartition algorithm using the default threshold provided by the BeadStudio software and finally visualized by the Partek Genomics Suite v6.4 http://www.partek.com/. A copy number baseline dataset containing 125 Human 1 M HapMap samples (generated by the Partek Inc.) was used to identify aberrant chromosomal regions in GCTs.

Gene expression microarray probe preparation and data analysis

Total RNA collection, cRNA probe preparation, array hybridization and data analysis were done as described previously [59]. In brief, fresh tissues were immersed in
Trizol™ solution (Invitrogen Inc., Carlsbad, CA, USA) and total RNA, including the small RNA fraction, were extracted and precipitated according to the manufacture’s instructions. RMA log expression units were calculated from Affymetrix™ HG-U133 Plus 2.0 whole genome array data using the ‘affy’ package included in the Bioconductor http://www.bioconductor.org suite of software for the R statistical programming language http://www.r-project.org. The default RMA settings were used to background correct, normalize and summarize all expression values. Significant differences between the sample groups was identified using the ‘limma’ (Linear Models for Microarray Analysis) package of the Bioconductor suite, and an empirical Bayesian moderated t-statistic hypothesis test between the two specified phenotypic groups was performed [60]. To control for multiple testing errors, we then applied a false discovery rate algorithm to these values in order to calculate a set of q values, thresholds of the expected proportion of false positives, or false rejections of the null hypothesis [61].

Heat maps were created by the dChip software http://www.dchip.org/. Classical multidimensional scaling (MDS) was performed using the standard function of the R program to provide a visual impression of how the various sample groups are related. Gene annotation was performed by the ArrayFusion web tool http://microarray.ym.edu.tw/tools/arrayfusion[62]. Gene enrichment analysis was performed by the Gene Ontology (GO) and KEGG databases using the WebGestalt http://bioinfo.vanderbilt.edu/webgestalt/41 and DAVID Bioinformatics Resources 2008 http://david.abcc.ncifcrf.gov[63] interfaces, respectively. The Euclidean distance between two groups of samples is calculated by the average linkage measure (the mean of all pair-wise distances (linkages) between members of the two groups concerned) [59]. The standard error of the average linkage distance between two groups (the standard deviation of pair-wise linkages divided by the square root of the number of linkages) is quoted when inter-group distances are compared in the text.

Real-time quantitative polymerase chain reaction
Between 100 ng to 1 μg of total RNA was used to perform reverse transcription (RT) using the RevertAid™ Reverse transcriptase kit (Cat. K1622; Fermentas, Glen Burnie, Maryland, USA) as directed by the manufacturer. Real-time PCR reactions were performed using Maxima™ SYBR Green qPCR Master Mix (Cat. K0222; Fermentas, Glen Burnie, Maryland, USA), and the specific products were detected and analyzed using the StepOne™ sequence detector (Applied Biosystems, USA). The expression level of each microRNA was normalized to GAPDH expression. For has-miR-142-5p, the forward primer was 5'-CGCCGGCATAAAGTGAAAGC-3' and the reverse transcription primer was 5'-GTCGTATCATGCGAGGTCGAGGTATTCGGACTGATACGACGATGT-3'. For has-miR-335, the forward primer was 5'-GGCGTCAGAGGCAATACGAA-3' and the reverse transcription primer was 5'-GTCGTATCAGTGCGAGGTCGAGGTATTCGGACTGATACGACCATTT-3'. For has-miR-654-3p, the forward primer was 5'-GGCGCTAGTTCTGCTGACCAT-3' and the reverse transcription primer was 5'-GTCGTATCATGCGAGGTCGAGGTATTCGGACTGATACGACGAC-3'. For hsa-miR-142-5p, the forward primer was 5'-AACGCTTCAGAATTTGCCGAGATCAGGAGTG-3'. For U6, the forward primer was 5'-CTCGTCCTCGCCAGAC-3' and the reverse primer was 5'-AAGCCTTCAGAATTTGCCGAGATCAGGAGTG-3'. For NANO1, the forward primer was 5'-AGAATCCTGCGACACCCTGCTC-3' and the reverse primer was 5'-TGCCACCTCAGGTTATCGTCTC-3'. For SNAI2 (alias SLUG), the forward primer was 5'-TGACAGGCTAGGAGTACTCTCA-3' and the reverse primer was 5'-AATGCTGGGAATGAGAAG-3'. For POU5F1 (alias OCT4), the forward primer was 5'-CCAGAAGTCCGACGAC-3' and the reverse primer was 5'-CCACATCGCCCTGTGTATAT. For GAPDH, the forward primer was 5'-CCAGCACCAGCACATCGCTC-3' and the reverse primer was 5'-ATGAGCCCCAGCCTTCTCCAT-3'.

Additional file 1: Summary of patient details and microarray data
(A) Classification, age distribution, gender ratio, and percentage of specific types of primary pediatric intracranial germ cell tumors from Taiwan. (B) Clinical data for the 21 cases of primary pediatric CNS GCTs used for genomics studies at Taipei VGH. (C) Characteristics of the patients (Korkola et al) and CNS GCTs used in this report. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2164-11-132-S1.PDF]

Additional file 2: Enumeration of differentially expressed probe sets in germinoma and NGMGCT, respectively. 13 cases were used to analyze the probe sets specifically enriched in the germinoma group or NGMGCT group (q < 0.001). Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2164-11-132-S2.XLS]

Additional file 3: Distribution of TW NGMGCT genes in the Wnt signaling pathway. Schematic representation of Wnt signaling pathway is obtained from KEGG pathway database http://www.genome.jp/kegg/. The locations of the signature genes are labeled by asterisks. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2164-11-132-S3.PDF]

Additional file 4: Distribution of TW NGMGCT genes in the focal adhesion pathway. Schematic representation of focal adhesion pathway is obtained from KEGG pathway database http://www.genome.jp/kegg/. The locations of the signature genes are labeled by asterisks. Click here for file [http://www.biomedcentral.com/content/supplementary/1471-2164-11-132-S4.PDF]
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Authors’ contributions

HWW, YHW, and TTW conceived the study and identified its value to pediatric tumor research. HWW and YHW designed the analysis approach. MILL, MEC, DIL, and TTW collected the tumor samples. HWW, YHW, and JYH carried out the implementation of data analysis. HWW, MTH, and TTW provided biological guidance during the analysis process. The manuscript was written by HWW and YHW, and all authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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