

Aim of the study: Examination of copy number changes in a group of intracranial germ cell tumors (GCTs) with particular focus on putative aberrations of the main genes coding SHh pathway proteins.

Material and methods: The study was performed on DNA isolated from fresh-frozen tumor tissue samples from eight GCTs, including six intracranial GCTs. The intracranial group consisted of three germinomas, two mature teratomas and one mixed germ cell tumor. Comparative genomic profiling analysis was carried out using microarray-CGH method (Cytosure ISCA UPD 4×180k, OGT). The results were analyzed with Feature Extraction (Agilent Technologies) and Nexus Copy Number (BioDiscovery) softwares.

Results and conclusions: Chromosomal aberrations were found in two intracranial germinomas. These tumors were characterized by complex genomic profiles encompassing chromosomes 7, 8, 9, 10, 11, 12, 16, 17 and 19. Common findings were gain at 12p13.33p11.1 of 35 Mbp and gain at 17q11.1q25.3 of 55 Mbp. In one tumor, also *SHh* (7q36.3), *SMO* (7q32.1) and *GLI3* (7p14.1) copy gains occurred together with 9q21.11q34.3 loss, including *PTCH1*, all being elements of SHh signaling pathway. Moreover, both tumors showed various copy gain of genes being ligands, regulators, receptors or target genes of SHh (*MTSS1*; *PRKACA* and *FKBP8*) as well as gain of genes of SHh coopting WNT pathway (*WNT3*, *WNT5B*, *WNT9B* in both tumors; *WNT16*, *WNT2* in pineal lesion). Further studies on larger group are needed to characterize SHh-related gene alterations in intracranial GCTs and for searching genotype-phenotype relations.

Key words: intracranial germ cell tumors, germinoma, array-CGH, Sonic Hedgehog signaling.

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Comparative genomic analysis of intracranial germ cell tumors – the preliminary study focused on Sonic Hedgehog signaling pathway

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Introduction

Germ cell tumors (GCTs) constitute a complex heterogeneous group of gonadal and extragonadal (cranial and extracranial) neoplasms, occurring in patients of different sex and age. As a group, GCTs present a broad spectrum of histological features with possible ways of differentiation into the embryonic and extraembryonic lineage components. Biologically, they can be benign or malignant tumors with infiltrative and metastatic phenotype, depending mainly on their histology. Morphologically, GCTs are divided into five main histological subtypes: germinoma (dysgerminoma, seminoma-site specific names), embryonal carcinoma, teratoma (mature and immature), yolk sac tumor, choriocarcinoma, as well as mixed germ cell tumors. GCTs account for up to 8% of all solid tumors diagnosed in patients < 18 years old. Intracranial GCTs affect mainly adolescent boys and young men, grow in the pineal and suprasellar regions. The geographic and ethnic differences exist between Asian and Western countries, accounting respectively for 14 and 3% of all central nervous system tumors [1, 2]. Therapy and prognosis of GCTs is individualized based on the established clinico-pathological factors. In malignant tumors, multimodal therapy is often necessary, with successful cure rate of 80%. New therapeutic tools and targets are, however, needed for incurable advanced tumors and to minimize long-term therapy side effects in the survivors.

Several pathogenetic theories of GCTs are under debate, such as disturbed migration of embryonic cells, inhibition of apoptosis, disturbances of cell division or meiosis errors, signaling pathways aberrations, and gene expression changes. It is postulated that GCTs derive from embryonic primordial germ cells (PGCs) at various stages of differentiation. During embryogenesis PGCs have to migrate through the embryo to reach the gonadal buds [3, 4]. Disruptions of the PGCs migration process result in ectopic retention of some of the germ cells at the body midline, predisposing to development of GCTs [5]. The molecular changes in gonadal GCTs, especially testicular cancer, have been examined extensively in the adults. However, the pathogenesis of pediatric GCTs, including intracranial tumors, remains unclear. In a few molecular studies, frequent imbalances of chromosomes 1, 8, 12, 13, 18, and X have been described in intracranial GCTs [6–10]. Moreover,

alterations in genes coding for *p14* and c-kit receptors were reported, however, their importance needs still to be elucidated [11, 12]. A recent study showed that germinomas are characterized by global low DNA methylation, making them distinct from all other GCTs subtypes. The pattern of methylation resembles that of PGCs at the migration phase, indicating the cell of origin for these tumors [10]. Migration and differentiation of PGCs are coordinated by a range of interacting signals from several pathways, such as WNT, Notch, FGF, PI3K/Akt or TGF β /E-cadherin [13]. Also various signaling morphogens, such as sonic hedgehog (SHh) influence PGCs migration [14, 15].

Sonic Hedgehog signaling pathway

Hedgehog signaling pathway plays a crucial role in vertebrate embryonic development, including formation of midline structures in the brain, neural crest development, limb axis formation and nasofrontal bud development. After birth, the activity of this pathway diminishes with possible constitutional reactivation during tissue regeneration and repair. Malfunctioning of Sonic Hedgehog (SHh) signaling, caused by hereditary mutations in genes coding proteins of the SHh pathway, leads to severe congenital malformations, such as holoprosencephaly or polysyndactyly. Impaired pathway signaling, resulting from somatic mutations, occurs in many malignant tumors, including hematological, gastro-intestinal, lung, breast, prostate and skin cancers [16, 17].

Sonic Hedgehog signaling transduction begins with an interaction between ligand SHh protein and receptor complex, built up by transmembrane proteins: PTCH (PTC; patched) and SMO (smoothened). As SHh binds to PTCH, the latter undergoes internalization and degradation and hence no longer inhibits SMO. Activated SMO transfers and accumulates in the cilium cell membrane. It also triggers GLI transcription factors, including activators GLI1 and GLI2, and an inhibitor GLI3 that regulate transcription of target genes, such as *HOX* and *WNT* in the nucleus [17, 18]. Modifiers of SHh signaling pathway include, among other, *SUFU*, *HHIP*, *GPC3* and *RAB23* proteins [19].

In cancer, Hedgehog signaling pathway can be either ligand-dependent (autocrine or paracrine) or ligand-independent [20]. The latter is caused by mutations in the key genes of the pathway (i.e. *PTCH*, *SMO*, *SUFU*) or their amplification (i.e. *GLI* family) [21, 22]. The resulting activation of target genes, such as *CCND*, *ABCG2*, *BCL2*, *SNAIL1*, *WNT2*, *VEGF*, and *TGF β 1* leads to development and/or progression of cancer [23]. The iconic malfunctioning of Hedgehog signaling pathway leading to cancer occurs in nevoid basal cell carcinoma syndrome (NBCCS; also Gorlin syndrome, MIM#109400). It is caused by loss of function mutations in tumor suppressor gene *PTCH1* [24]. Aberrant SHh signaling pathway is also crucial in the pathogenesis of medulloblastoma, its syndromic and sporadic subtypes. Here, not only mutations in *PTCH1*, *PTCH2* and *SUFU* as in Gorlin syndrome, but also in the *SMO* gene have been reported in 10% of cases. Similarly, chromosomal gains encompassing *GLI1* gene locus are present in ~20% of rhabdomyosarcomas and glioblastomas. Constitutive activation of the SHh

signaling pathway through activating mutations in the *GLI2* or *GLI3* genes, deletions in *HHIP* and *SUFU* genes was reported in various human tumors [25].

Sonic Hedgehog signaling affects gonocyte migration, gonadal development, sexual differentiation and viability of germ cells, thus it might be also involved in pathogenesis and progression of GCTs. The aim of this study was to determine copy number changes in pediatric intracranial germ cell tumors, with special attention to SHh-related genes. Accordingly, we have applied an array-CGH technique to evaluate the incidence and to characterize genomic imbalances in the pilot group of tumors.

Material and methods

Tumor specimens

This preliminary study was performed on eight germ cell tumors from the children diagnosed and treated in the Department of Pediatric Oncology at the Children's Memorial Health Institute in Warsaw. Six tumors were intracranial, operated in Department of Pediatric Neurosurgery. None of the patients was previously treated oncologically. Histologically, the analyzed group consisted of three germinomas, four mature teratomas and one mixed germinal tumor (Table 1). Each of the studied tumors underwent intraoperative exam. Samples for deep freezing for molecular analysis were also secured. Tissue material was evaluated microscopically by two independent pathologists to determine its histology and alive neoplastic tissue content. Samples with cellularity exceeding 70% were used for the further molecular studies. In each case the second pathological opinion, based on routinely processed tumor sections, was performed in the external center.

Genomic profiling (array-CGH analysis)

The salting-out protocol [26] was used to extract genomic DNA from fresh-frozen tumor tissue samples. Based on the obtained DNA quality, 7/8 tumors were eligible for genomic analysis using array comparative genomic hybridization (array-CGH) at the average resolution of 150 kbp (Cytosure ISCA UPD 4x180k, Oxford Gene Technology). Sex-mismatched Human Genomic DNA (Promega) was used as the reference control DNA. Arrays were scanned at 2 μ m with MS200 Microarray Scanner (Roche) and analyzed using Feature Extraction (Agilent Technologies) and Nexus Copy Number 8.0 (BioDiscovery) softwares. A minimum of five consecutive probes were required to define a region as a copy number aberration (CNA). All identified genomic imbalances were verified against the in-house database of > 1000 benign copy number variations (CNVs) identified in local population and in the online Database of Genomic Variants (DGV; last accessed on June 2017) [27]. Numbering of map positions was based on hg19/GRCh37 reference sequence.

The study was approved by the Ethical Committee of the Medical University of Gdansk, Poland. Written informed consent was obtained from the relevant guardians of the children.

Results

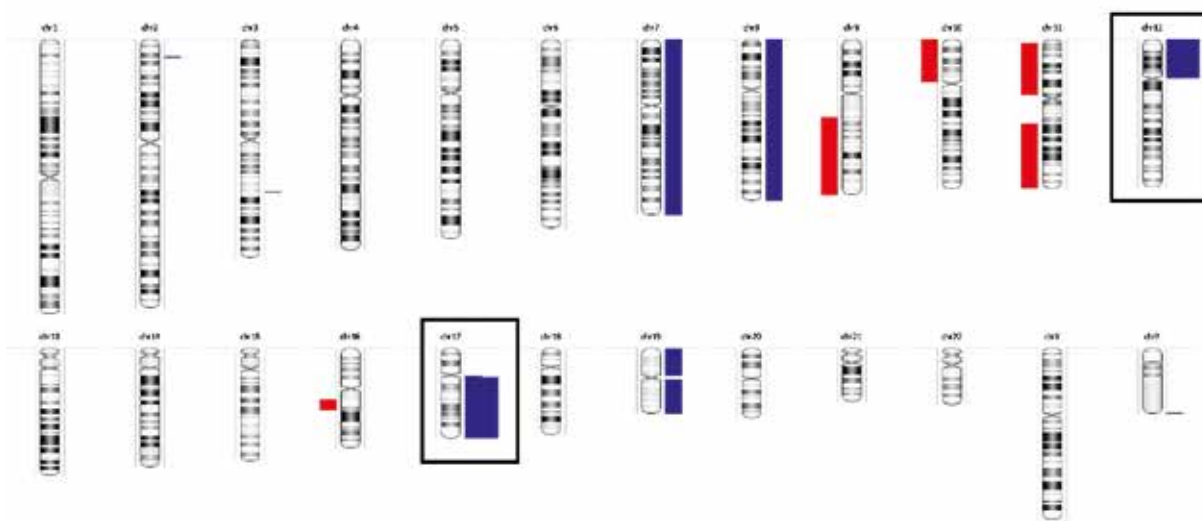
Several copy number alterations (CNAs) were identified in the studied cohort of GCTs. No large genomic imbalances were found in pineal region germinoma, all teratomas and the mixed GCT, where only a few benign recurrent copy number variations (CNVs) were detected.

Complex genomic profiles, involving chromosomes 7, 8, 9, 10, 11, 12, 16, 17 and 19 were observed in two germinomas (#4 and #5), located in the pineal gland region and midbrain, respectively (Fig. 1A). The two CNAs identified as common in both tumors were gains of the short arm of chromosome 12 and of the long arm of chromosome 17

with the minimal overlapping regions at 12p13.33p11.1 and 17q11.1q25.3, respectively (Fig. 1B). Within the identified rearrangements, 26 genes classified in COSMIC database as involved in cancer pathogenesis were located: *KDM5A*, *CCND2*, *ZNF384*, *ETV6*, *KRAS*, *NF1*, *SUZ12*, *TAF15*, *MLL2*, *LASP1*, *CDK12*, *ERBB2*, *RARA*, *BRCA1*, *ETV4*, *COL1A1*, *HLF*, *MSI2*, *CLTC*, *BRIP1*, *CD79B*, *DDX5*, *PRKAR1A*, *SRSF2*, *CANT1*, *ASPSCR1* [28].

The in-depth analysis of the genomic *loci* associated with genes coding for members of SHh signaling pathway revealed a number of aberrations in the regions encompassing genes coding for ligands, regulators, receptors or

A



B

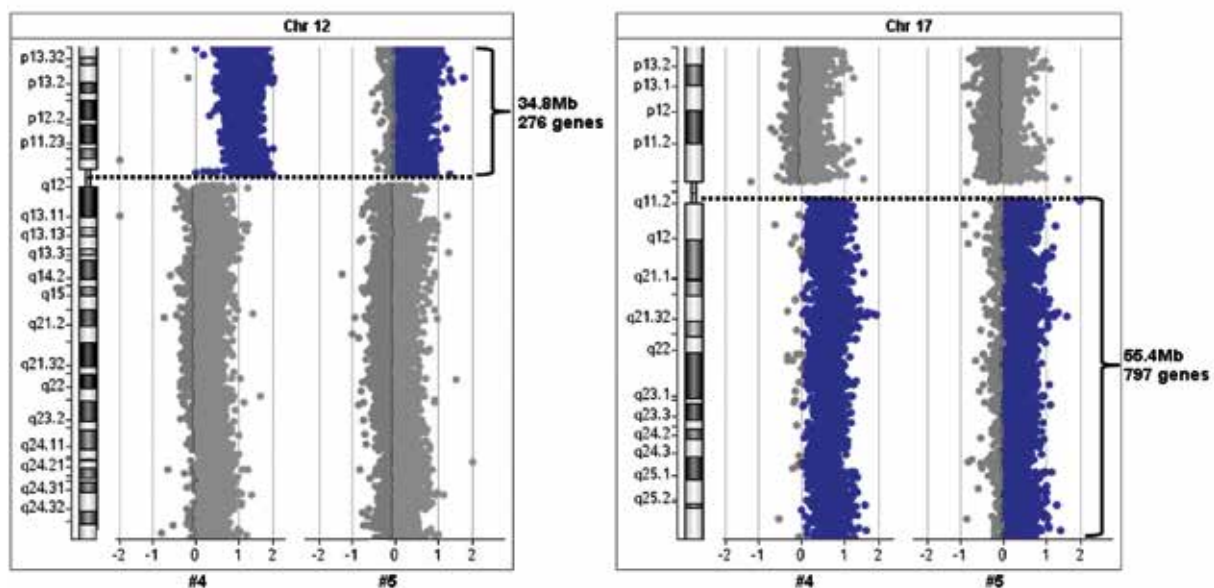


Fig. 1. Genomic profiles of germinomas located in the pineal gland region (#4) and midbrain (#5). Genomic localization of the imbalances detected in the two germinomas is shown on panel A. The two common changes at chromosome 12 and chromosome 17 are marked in boxes and further deciphered on panel B. Red and blue colors represent losses and gains, respectively. X-axis: the \log_2 ratio, Y-axis: chromosomal coordinates. The minimal overlapping regions of the aberrations at chromosomes 12 and 17 with the size and a total number of genes, located within these regions were depicted by black arrows on panel B

that are target genes of the pathway. In particular, pineal gland germinoma (#4) had an additional copy of *MTSS1* gene (MIM*608486; 8q24.13), while the midbrain germinoma (#5) carried additional copies of *PRKACA* (MIM*601639; 19p13.12) and *FKBP8* (MIM*604840; 19p13.11). Also, gains of *WNT3* (17q21.31-q21.32), *WNT5B* (12p13.33) and *WNT9B* (17q21.32) genes of the Wnt signaling pathway were noted in both of tumors. Additional gains of *WNT16* (7q31.31) and *WNT2* (7q31.2) genes in pineal gland germinoma (#4) were observed. Furthermore, in pineal gland germinoma (#4) gains at *loci* of *SHH* (7q36.3), *SMO* (7q32.1), and *GLI3* genes (7p14.1) were reported, together with the loss of the long arm fragment of chromosome 9q21.11q34.3 covering the *PTCH1* gene.

Discussion

In the present preliminary study, we performed an array-CGH analysis of eight germ cell tumors. This molecular method is precise and gives an opportunity to examine all the imbalanced aberrations in the genome at once. It was deliberately used here in place of low-resolution conventional cytogenetic procedures and/or targeted FISH which allows to analyze selected regions only. It also has much better resolution than the previously used in research of GCTs conventional CGH technique [8].

To the best of knowledge, only two genomic studies of intracranial GCTs have so far been performed, but none analyzed *loci* related to SHh pathway [7, 9]. Terashima *et al.* (2014) analyzed 62 GCTs using SNP-array and quantitative real-time PCR reporting gains of chromosomes 1q, 2p, 7q, 8q, 12p, 14, 20q, 21, 22, Xq and losses on chromosomes 1p, 4q, 5q, 9q, 10q, 11q and 13. Two frequent aberrations, encompassing *loci* of *CCND2* (12p13) and *RB1* (13q14) genes were identified in 52% and 48% tumors, respectively, suggesting the involvement of cyclin/CDK-RB-E2F pathway in their pathogenesis. Additionally, gains of chromosome 8q13 encompassing *PRDM14* locus were noted, highlighting the role of transcriptional regulation in GCTs specification as an important factor in cancerogenesis [9]. Another study by Okada *et al.* (2002), describing the partial genomic profiles of 25 intracranial GCTs established in a series of FISH experiments, reported the presence of additional copy of X chromosome in 92% tumors. Conversely, genomic imbalances, involving chromosomes 12p and 13q were reported only anecdotally, in 20% and 12%, respectively [7]. In the current study, aberrations neither of X chromosome nor of 1, 2, 4, 5, 13, 14, 21, 22 chromosomes were observed.

The involvement of improper activation of signaling pathways in human cancer has been widely investigated. Recent studies focus mainly on the identification of key genes and pathways as a promising approach in the diagnosis and development of more attractive therapies in GCTs patients. Evaluation of somatic copy number aberrations (CNAs), as one of the most prominent markers of genetic heterogeneity associated with human cancer, can provide a new insight on the molecular profiling of GCTs. Knowledge of specific aberrations of genome could result in development of a useful diagnostic and/or prognostic

tool based on corresponding oncogenes or tumor suppressor genes identification. Inhibitors of the SHH pathway that act as SHH proteins blockers i.e. *SMO* (*vismodegib*, *itraconazole*) and GLI1 inhibitors are currently evaluated in numerous clinical trials as a promising treatment option in individuals with skin basal cell carcinoma, subtypes of medulloblastoma, and pancreatic cancer [29, 30]. It has been reported that tumors harboring pathway activating mutations or presenting over-expression of the pathway proteins better respond to targeted therapy compared to the wild-type neoplasms [31].

Herein, we report complex genomic profiles in 2/3 intracranial germinomas sharing two common chromosomal gains of 12p and 17q regions. Similarly, another subgroup – extracranial postpubertal GCTs but not teratomas, are highly aneuploid, with large-scale copy number alterations frequently observed [32]. The detected in our study gain of the short arm of the chromosome 12 most likely stands for the presence of a 12p isochromosome i(12p), a hallmark of postpubertal extracranial GCTs. In testicular postpubertal type tumors, i(12p) is formed in late phase of germ cell neoplasia *in situ*, being required for invasive growth phase [33]. However, in pediatric seminomas and teratomas i(12p) is not observed, moreover pathogenesis and genetic landscape of intracranial tumors differs from gonadal neoplasia [7]. A gain of the long arm of chromosome 17 was reported only anecdotally in extracranial GCTs or other human cancers [34]. Among the genes located in the minimal overlapping region at chromosome 17q, a high level of expression and/or gain of the *ERBB2* oncogene (17q12) has been reported in various cancers, including breast and ovarian tumors [35, 36].

In our study, we report a gain of two genes crucial for Shh signaling pathway functioning, namely *SHH* and *SMO*, together with a deletion of the *PTCH1* gene in pineal gland germinoma. Additional copies of *GLI3* transcription factor were also found in this particular tumor. Small interstitial deletion of 9q, covering the *PTCH1* gene has previously been reported in patients with NBCCS. The *PTCH1* gene is highly intolerant to loss of function mutations and is likely to exhibit haploinsufficiency (pLI of 1.0 and HI of 0.46%, respectively) in cases harboring either germline or somatic deletion of this gene resulting from genomic imbalances at chromosome 9q [37, 38]. Additionally, gains of the *MTSS1* and *PRKACA* genes were noted in pineal germinoma and in midbrain germinoma, respectively; these *loci* have already been associated with the SHh signaling pathway [37, 39]. Studies of epidermal proliferation and invasion in regenerated human skin showed that the transcriptional activity of *GLI1* and *GLI2* can be potentiated by the SHH-responsive gene – *MTSS1*. Further studies indicated that *MTSS1* protein is a part of GLI/SUFU complex, since SUFU interacted with *MTSS1* in the absence of GLI. In turn, the data presented by Callahan *et al.* (2004) described *MTSS1* as a new member of Hh signaling pathway able to modulate Gli response [39]. CAMP-dependant protein kinase A (PKA), a known down regulator of Hh signaling pathway, is built up by two subunits α and β encoded by the *PRKACA* and *PRKACB* genes, respectively. Jia *et al.* (2004) pro-

vided the evidence that PKA along with CKI phosphorylate Smo, that is crucial in Hh signal transduction [40].

Few available studies of the SHh pathway concern on adult onset GCTs; therefore, there is a need to analyze also pediatric tumors. Our study is among the first to evaluate this particular pathway in such cases. In the current research, we demonstrate that germinomas are characterized by a complex genomic profile, while the other tested GCTs cases present normal molecular karyotypes. Germinomas are undifferentiated GCTs closely related with PGCS phenotypically. The potential paucity of the characteristic genomic pattern may undermine the importance of the implementation of the molecular testing into routine diagnostic practice; however, further studies on a larger dataset of GCTs samples are required to confirm these initial findings. Nevertheless, the Shh signaling pathway seems to play a role in the GCTs biology, thus it might be worthwhile to evaluate genotype-phenotype correlations and its prognostic significance in a more comprehensive manner.

In conclusion, performed pilot study revealed new data on SHh related genes in intracranial pediatric germ cell tumors.

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The authors declare no conflict of interest.

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