RESEARCH ARTICLE

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KRAS Mutation in Pediatric Intracranial Germ Cell Tumors

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Abstract

Background: Intracranial germ cell tumors (IGCTs) are rare, highly curable neoplasms. KRAS is a gene in the KIT/RAS signaling pathway, and KRAS mutations have been reported in patients diagnosed with IGCTs. **Objectives:** To describe the clinicopathologic and molecular features of KRAS mutation and the treatment outcome of children diagnosed with IGCTs. **Methods:** Patients diagnosed with IGCTs at the Department of Pediatrics, King Chulalongkorn Memorial Hospital from 2007 to 2016 were retrospectively reviewed. DNA was extracted from formalin-fixed, paraffin-embedded tissue and used for molecular study. Mutations in codons 12, 13, and 61 of the KRAS gene were detected using the cobas® KRAS mutation test and pyrosequencing. **Results:** Eighteen patients were diagnosed with IGCTs (11 males and 7 females): nine with germinomas and nine with non-germinomatous GCTs (NGGCTs). The age range of the patients was 5–14 years (median 10.5 years). Elevated markers were revealed in approximately 25% of the patients. Four patients (two with germinomas and two with NGGCTs) had leptomeningeal involvement. All patients underwent tumor biopsy and received neoadjuvant chemotherapy. Radiotherapy was administered in 16 patients, and craniospinal radiation was administered only in patients with leptomeningeal metastasis. With a median follow-up of 26 months, overall survival was 88.9% in the patients with germinomas and 37% in the patients with NGGCTs. Mutation of the KRAS gene was detected using pyrosequencing in one patient. The mutation located at codon 61, with frequency 38.3% units, nucleotide substitution CAA > CTA, and amino acid substitution, was Q61L. The patient carrying the mutant gene was diagnosed with germinoma with cerebrospinal fluid metastasis and eventually died from treatment-related toxicity. **Conclusion:** Our study revealed the treatment outcomes of IGCTs in Thai children. The metastatic germinoma patient with KRAS codon 61 mutation had a poor outcome, supporting that Q61L has a clinical correlation with IGCTs.

Keywords: Intracranial germ cell tumors- KRAS- mutation- Thai- children

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Introduction

Intracranial germ cell tumors (IGCTs) are a group of heterogeneous brain tumors comprised of germinoma, embryonal carcinoma, yolk sac tumor, choriocarcinoma, teratoma, and mixed IGCTs (Echevarria et al., 2008), (Louis et al., 2016). The incidence of IGCTs is rare in Europe and America, with a rate of approximately 0.1–1:100,000 population/year, accounting for 3%–14% of brain tumor (Rickert and Paulus, 2001; McCarthy et al., 2012; Thakkar et al., 2013; Sun et al., 2014; Ostrom et al., 2015). In contrast to that in western countries, the incidence of IGCTs in Japan and other East Asia is higher (Kuratsu et al., 2001; Sun et al., 2014). In Thailand, the incidence of IGCTs was 3.5 per million between 2003 and 2005 (Wiangnon et al., 2011; Raiyawa et al., 2012).

Patients with IGCTs usually present in the second decade of life and occur predominantly in male, with a

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reported male-to-female ratio of 3–4 to 1 (Echevarria et al., 2008; McCarthy et al., 2012; Poynter et al., 2014; Sun et al., 2014). These tumors are commonly found in the suprasellar and pineal regions (Kreutz et al., 2010; McCarthy et al., 2012; Sun et al., 2014). Patients usually present symptoms of headache with increased intracranial pressure (ICP), weakness, visual loss, diabetes insipidus, and delayed growth and puberty (Echevarria et al., 2008; Kreutz et al., 2010; Sun et al., 2014). Diagnosis of IGCTs is based on the combination of clinical symptoms, tumor markers, neuroimaging characteristics, and cytological and histological confirmation. Differentiation entities can be broadly classified into germinoma and non-germinomatous germ cell tumor (NGGCTs) subtypes (Echevarria et al., 2008; Thakkar et al., 2013). Germinomas are more common than NGGCTs, accounting for 50%–70% of all cases (Echevarria et al., 2008; Jorsal and Rorth, 2012; McCarthy et al., 2012; Sun et al., 2014).

IGCTs are the most curable pediatric brain tumors; in particular, intracranial germinomas are very sensitive to radiotherapy (RT) and chemotherapy. The 10-year survival rates of germinomas are 80%–90% or higher in most studies (Kanamori et al., 2009; Kenjo et al., 2015; Cheng et al., 2016; Kakkar et al., 2016). However, approximately 10% of patients with germinomas and most patients with NGGCTs still have a poor outcome and refractory to treatment (Cheng et al., 2016). Overall survival (OS) of those with NGGCTs is approximately 30%–60% (Kanamori et al., 2009; Raiyawa et al., 2012; Kakkar et al., 2016).

To date, little is known about molecular biology of ICGTs underlying the oncogenesis and chemoresistance mechanism of ICGTs that contribute to disease severity and clinical outcomes. Few cytogenetic abnormalities, including chromosomal imbalance, p53, MERK, KIT/RAS, or AKT/mTOR signaling pathway mutations, have been reported in IGCT tissue. However, the specific gene mutations and clinical correlations in IGGTs have not been completely characterized (Rickert et al., 2000; Takeshima et al., 2004; Schneider et al., 2006; Fukushima et al., 2014; Wang et al., 2014; Ichimura et al., 2016; Schulte et al., 2016; Fukushima et al., 2017).

KRAS is a gene in the KIT/RAS signaling pathway that plays an important role in the pathogenesis of many cancers (Wang et al., 2013). KRAS mutations were reported in patients diagnosed with IGCTs (Takeshima et al., 2004; Fukushima et al., 2014; Wang et al., 2014; Ichimura et al., 2016; Schulte et al., 2016). A recent study has revealed that KRAS is the most statistically significant altered gene in gonadal and mediastinal GCT patients who are resistant to chemotherapy (Taylor-Weiner et al., 2016). Unfortunately, genetic studies about the KRAS gene in patients with IGCTs and their correlation are limited.

We therefore conducted a descriptive study to determine the clinicopathologic and molecular features of KRAS mutations and the treatment outcome of children diagnosed with IGCTs.

Materials and Methods

Upon approval by the Institutional Review Board (IRB number 545/60), medical records of patients diagnosed with primary ICGCTs between January 1, 2007 and June 30, 2019 at our institute were retrospectively reviewed. Clinical information, including age, gender, presenting signs and symptoms, tumor site and extent, tumor markers, imaging studies, pathological findings, treatment and outcome, was collected.

In this study, ICGCTs were diagnosed on the basis of histological findings and tumor markers and categorized into germinomas or NGGCTs. MRI spine and cerebrospinal fluid (CSF) cytopathology was performed for a metastatic workup.

For treatment information, surgery, radiation therapy, and chemotherapy protocols were reviewed in depth. Treatment outcomes were assessed in terms of OS and treatment complications. OS was defined as the time between diagnosis and death from any cause or last follow-up contact for patients who were alive. Survival was estimated using the Kaplan–Meier method.

Molecular study for KRAS mutation

KRAS mutation test was performed using PCR. DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) tissue. Codons 12,13, and 61 of the KRAS gene were identified using real-time PCR in the cobas® KRAS mutation test. For patients who showed invalid results from the cobas® KRAS mutation test, pyrosequencing was later performed using the KRAS Pyro Kit®.

DNA extraction

Genomic DNA was extracted from paraffin-embedded tumor specimens using a QIAamp DNA FFPE Tissue Kit (Qiagen GmbH, Hilden, Germany) in accordance with the manufacturer's protocols. The quality and concentration of DNA were assessed using a spectrophotometer (NanoDropTM 2000; Thermo Fisher Scientific, Waltham, MA, USA)

cobas® KRAS mutation test

The cobas® KRAS Mutation Test, which is performed using the cobas® 4800 System, is a real-time PCR test for the detection of seven somatic mutations in codons 12, 13, and 61 of the KRAS gene in DNA derived from FFPE tissue. A mutant control, a negative control, and a calibrator were included in each run to confirm the validity of the run. For target selection, primers that define an 85 base-pair sequence for exon 2 containing KRAS codons 12, 13, and 61 in human genomic DNA were used in the cobas® KRAS Mutation Test. Amplification occurred only in the regions of the KRAS gene between the primers; the entire KRAS gene was not amplified. A derivative of Thermus species Z05 DNA polymerase was utilized for target amplification. For automated Real-time Mutation Detection, the cobas z 480 analyzer was used to measure, in real-time, the amount of fluorescence generated by specific PCR products.

KRAS Pyro kit: Pyrosequencing analysis

The KRAS Pyro Kit was used for the quantitative measurements of mutations in codons 12, 13, and 61 of the human KRAS gene. The product consists of two

assays: one for detecting mutations in codons 12 and 13 and the other for detecting mutations in codon 61 (Figure 1). The two regions were amplified separately by PCR and sequenced through the defined region. Sequences surrounding the defined positions served as normalization and reference peaks for the quantification and quality assessment of the analysis.

Results

Clinical characteristics

Eighteen patients were diagnosed with IGCTs (11 males and 7 females): nine with germinomas and nine with non-germinomatous GCTs (NGGCTs). The age range of the patients was 5–14 years (median 10.5 years). Presenting symptoms included hydrocephalus (9 patients), weakness (2 patients), diabetes insipidus (2 patients), visual disturbances (3 patients), and increased ICP without hydrocephalus (2 patients). Tissue histopathology was obtained in all patients. The pathological result was germinoma in 11 patients (61%), yolk sac tumor in 2 patients (11%), and mixed germ cells in 5 patients (28%). Elevated markers were revealed in approximately 25% of the patients. Two patients whose pathology was germinoma showed elevated tumor markers and thus were diagnosed with NGGCTs. Tumor sites were mainly the pineal and suprasellar regions. Four patients (two patients with germinomas and two patients with NGGCTs) had leptomeningeal involvement. One patient was diagnosed with peritoneal carcinomatosis because malignancy cells were found in the peritoneal fluid during relapsed course. Clinical characteristic data of the patients are summarized in Table 1.

Treatment and outcome

All patients underwent surgical intervention and received adjuvant chemotherapy. Total removal was performed in three patients, partial removal in four patients, and biopsy in the remaining patients. Adjuvant chemotherapy was administered on the basis of the definite diagnosis considered by tumor markers and pathological results. Overall, 50% of the patients received cisplatin-based chemotherapy, and the other half received carboplatin-based protocols. Two of them were unable to complete chemotherapy because of early death. RT was administered to 16 patients, and craniospinal radiation was administered only to patients with leptomeningeal metastasis. RT protocols were considered individually for dose and types (whole ventricle or whole brain radiation) depending on the tumor location. Treatment information of the patients is shown in Table 1.

With the median follow-up of 26 months, six patients experienced relapse and five patients died of a disease. One patient died during treatment. OS was 88.9% in the patients with germinomas and 37% in the patients NGGCTs (the Kaplan–Meier curve is shown in Figure 2). Of the patients who relapsed or died, two had spinal metastatic germinomas, two had spinal metastatic NGGCTs, and the rest had non-metastatic NGGCTs.

Table 1. Characteristic and Treatment Data of the Patients

Age (year)	N=18 (%)
Range	5–14
Median	10.5
Gender	
Male	11 (61)
Female	7 (39)
Presenting symptoms	
Hydrocephalus	9 (50)
Weakness	2 (11)
Diabetes insipidus	2 (11)
Visual disturbances	3 (17)
Increased intracranial pressure without hydrocephalus	2 (11)
Tumor location	
Pineal gland	7 (39)
Suprasellar and sellar	5 (28)
Thalamic	1 (5.5)
Cerebellar	1 (5.5)
Multifocal	4 (22)
Histopathology	
Germinoma	11 (61)
Yolk sac tumor	2 (11)
Mixed germ cells	5 (28)
Metastasis	
No metastasis (M0)	14 (78)
Spinal metastasis	4 (22)
Tumor markers	
Normal	10 (56)
Increased serum/CSF b-HCG	5 (27)
Increased serum/CSF AFP	3 (17)
Final diagnosis	
Germinoma	9 (50)
Yolk sac tumor	2 (11)
Mixed germ cells	5 (28)
Germinoma with b-HCG secreting	2 (11)
Surgery type	
Gross tumor removal	3 (17)
Partial tumor removal	4 (22)
Tumor biopsy	11 (61)
Radiation	
No radiotherapy	2 (11)
Whole ventricle RT	11 (61)
Whole brain RT	5 (27)
CSI	5 (27)
Chemotherapy	
Cisplatin-based	9 (50)
Carboplatin-based	9 (50)

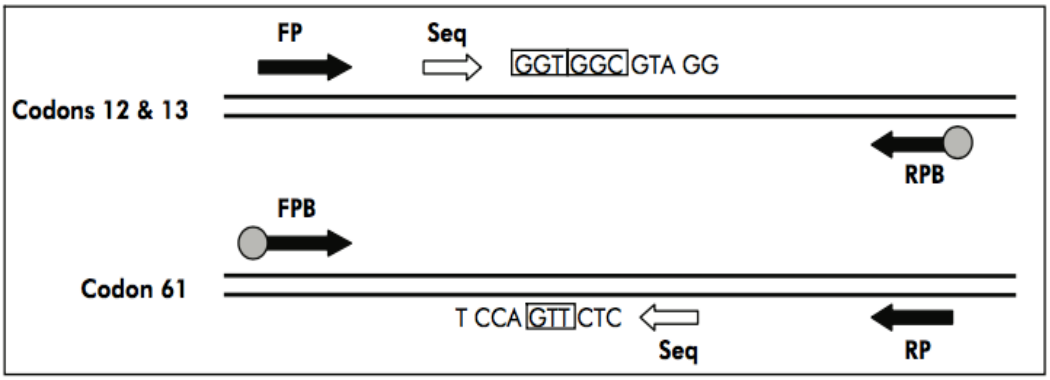


Figure1. Illustration of the KRAS Assay. The sequence indicated is the analyzed sequence for a normal sample. FP and FPB, Forward PCR primers (B indicates biotinylation); RP and RPB: Reverse PCR primers (B indicates biotinylation); Seq, Sequencing primers.

Molecular analysis

Molecular analysis was performed in 17 IGCT cases. The cobas® KRAS mutation test was performed to detect mutation at codons 12, 13, and 61 in all 17 cases. No mutation was detected in 10/17 patients. For seven patients whose results from cobas® were invalid, pyrosequencing was later performed. The clinical characteristics and KRAS mutation status of all 17 patients are described in Table 2.

Mutation of the KRAS gene was detected using pyrosequencing in one patient. The mutation located at codon 61, with frequency 38.3% units, nucleotide substitution CAA > CTA, and amino acid substitution, was Q61L. The mutant was compared with the wild type and/or positive control data by using Pyrogram, as shown in Figure 3. The patient carrying the mutant gene was diagnosed to have intracranial germinomas with CSF metastasis and eventually died during treatment.

Discussion

We describe our institutional experience with 18 patients diagnosed with IGCTs over a 10-year period. Clinical characteristics were similar to those in a previous report (Echevarria et al., 2008; McCarthy et al., 2012; Sun et al., 2014). Our patients were predominantly male, presented with neurology and endocrine symptoms. The most affected regions were the pineal, sellar, and suprasellar regions. Four patients had multilocal lesions. Interestingly, the incidence of germinoma and NGGCTs was equal in our study, and approximately one-fourth of the patients showed metastasis at the beginning. In most studies, the incidence of germinomas is higher than that of NGGCTs, and the metastasis rate is usually lower in the former than the latter (Jorsal and Rorth, 2012; Raiyawa et al., 2012).

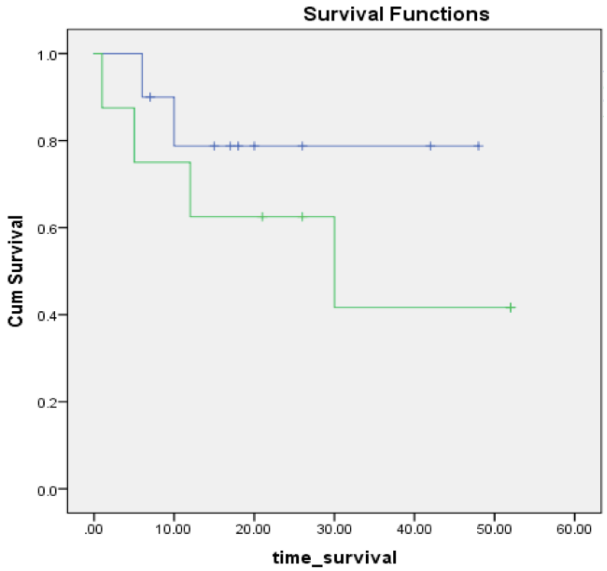


Figure 2. Kaplan–Meier Survival Analysis for Overall Survival (blue line, CNS germinomas; green line, NGGCTs)

The excellent treatment outcome of germinomas (OS nearly 90%) was correlated with outcomes from previous reports (Kenjo et al., 2015; Cheng et al., 2016). A study in Canada reported a 5-year PFS of 96% and an OS of 100% with a relapse rate of approximately 10% (Cheng et al., 2016). For eastern countries, studies reported a 3-year event-free survival (EFS) of 92.9% from China, a 10-year OS of 86% from Japan, and a 5-year EFS of 84% from India (Kanamori et al., 2009; Sun et al., 2014; Kakkar et al., 2016).

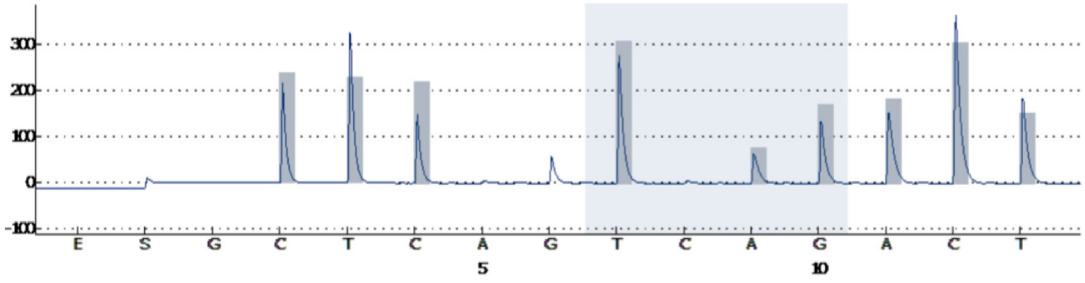
Our study reported a poor treatment outcome in patients with NGGCTs, with an OS of only 37%. This revealed a poorer outcome compared with most previous studies, which reported OS and EFS rates of 60%–79% (Robertson et al., 2014; Sun et al., 2014; Lai et al., 2015). Patricia et al. reported outcomes for CNS mixed malignant germ cell tumors in the US (Robertson et al., 2014). The 6-year relapse-free survival was 63%±10%, and the OS was 68±9%. However, they evaluated treatment outcome after aggressive treatment, including intensified neoadjuvant chemotherapy encouraging second-look surgery, and administered dose-intensive, stem cell-supported chemotherapy to patients with residual tumor, all prior to RT (Robertson et al., 2014). In a retrospective study from China, Sun et al., (2014) found a 3-year EFS of 64.8% for NGGCTs reviewed from 57 children and adolescents with primary IGCTs. Lai et al., (2015) conducted a study from Taipei in 1985–2010 to evaluate the outcomes of 39 patients with non-disseminated NGGCTs. After a median follow-up

Table 2. Clinical Characteristics and KRAS Mutation Test Results

Patient No./Sex/ Age (year)	Site	Histopathology	Metastasis	B-HCG (mIU/mL) serum/CSF	AFP (ng/ml) serum/CSF	Outcome	KRAS mutation (Cobas®)	KRAS mutation (Pyrosequencing)
1/M/11	Multifocal	Germinoma with syncytiotrophoblasts	M0	310/150	2.22/0.58	Alive	No mutation	
2/M/9	Pineal region	Germinoma, mature terratoma, choriocarcinoma	M0	175/411	4.8/<0.5	Alive	No mutation	
3/M/12	Pineal, suprasellar	Germinoma	M0	<5/<5	<0.5/<0.5	Alive	Invalid	No mutation
4/F/12	Sellar and suprasellar	Germinoma	M0	<5/<5	1.49/<0.5	Alive	No mutation	
5/M/5	Lt. cerebellar	Yolk sac tumor	M0	<5/<5	1989/0.1	Alive	NA	
6/M/12	Pineal region	Germinoma	M0	19/44	8/0.03	Alive	No mutation	
7/F/6	Sellar and suprasellar	Germinoma	Spinal metastasis	<5/<5	4.46/<0.5	Relapsed, alive	Invalid	No mutation
8/M/10	Thalamic and Rt basal ganglion	Germinoma	M0	<5/13	1.8/<0.5	Alive	Invalid	No mutation
9/M/14	Pineal region	Germinoma	M0	19/190	0.92/<0.5	Alive	No mutation	
10/M/7	Pineal region	Germinoma	M0	<5/5.7	5.4/<0.5	Alive	Invalid	No mutation
11/F/8	Rt thalamus, suprasellar	Germinoma	M0	<5/13	2.97/0.02	Alive	No mutation	
12/F/11	Sellar and suprasellar	Germinoma	M0	<5/<5	0.7/0.06	Alive	No mutation	
13/F/7	Suprasellar	Germinoma	Spinal metastasis	1159/<5	1.13/1.38	Dead	No mutation	
14/M/11	temporal lobe, suprasellar	temporal lobe: yolk sac, suprasellar: germinoma	Spinal metastasis	32/ NA	6634/ NA	Dead	Invalid	No mutation
15/F/13	suprasellar	Germinoma	Positive CSF	30/ NA	1.84/ NA	Dead	Invalid	Codon 61 mutation Q61L
16/M/12	Pineal region	Yolk sac tumor	M0	0.1/<5	1715/93	Dead	No mutation	
17/F/9	Pineal region	Mixed germinoma and yolk sac tumor	M0	<5/ NA	1.14/ NA	Dead	Invalid	No mutation
18/M/11	Pineal region	Mixed germinoma and mature terratoma	M0	519/1024	36/24	Dead	No mutation	

of 77.7 months (range 14–336), the 6-year OS and PFS rates were 74.4% and 79.5%, respectively. This study was performed in a non-metastasis group, which may explain the good outcome. However, the OS rate from our study correlated with those from some studies in Japan and India. Kanamori et al. reported a 3-year PFS rate of 29% for NGGCTs, and Kakkar et al., (2016) identified a 5-year EFS rate of 40% for NGGCTs (Kanamori et al., 2009). They followed-up for median times of 99 and 15 months after adjuvant chemotherapy and/or RT, respectively.

We observed all four patients who had a spinal metastasis event. Three of these patients had relapsed/



Result	Mutation
Frequency	38.3 % units (LOD: 3.1 % units)
Nucleotide Substitution	CAA>CTA
Amino Acid Substitution	Q61L

Figure 3. Representative Case of KRAS Mutations Detected by Pyrosequencing Analysis. Pyrograms are compared with wild type and/or positive control data. Significant peak changes located at codon 61, frequency 38.3% units, nucleotide substitution CAA > CTA, and amino acid substitution was Q61L.

progressive disease, and one had treatment-related mortality. Our dismal outcome in the metastasis group correlated with findings from some studies. Sun et al. from China reported a poorer survival outcome in patients with extensive disease, defined as positive CSF cytology, gross nodular seeding in the cerebellar-cerebral subarachnoid space and/ or spinal subarachnoid space, or extraneural metastasis (Sun et al., 2014). The 3-year EFS rates of patients with localized and extensive lesions were 82.9% and 38.9%, respectively.

Our results indicate that better modalities of treatment should be considered, especially in patients with NGGCTs, metastasis disease, or germinomas with treatment refractoriness.

KRAS mutation

KRAS is a common genetic mutation identified in various types of cancer (Wang et al., 2013). To date, the incidence of KRAS mutation is common in patients with extracranial germ cell tumors. A recent study from Amaro et al. has revealed that KRAS is the most statistically significant altered gene that plays a role in chemotherapy responsiveness and may impact clinical outcome (Taylor-Weiner et al., 2016). Few studies reported KRAS gene mutation in IGCTs, and its impact on clinical outcome has not been established.

Our study is among the first that reported KRAS mutation in IGGCTs. We detected KRAS mutation in 1/17 cases by using pyrosequencing. The mutation located at codon 61, with frequency 38.3% units, nucleotide substitution CAA > CTA, and amino acid substitution, was Q61L. No KRAS mutation was found in NGGCTs.

The incidence of KRAS mutation differed from those in previous studies. Wang et al. analyzed 62 cases by using next-generation sequencing, SNP array, and expression array. They found that KRAS and NRAS mutated in 19% of IGCTs cases. In the present study, novel mutation was identified on Exons 12, 59, 61, 63, and 146. For KRAS exon 61 mutation, amino acid change is Q61R (Wang et al., 2014). Fukushima et al. investigated genes involved in the KIT signaling pathway (Fukushima et al., 2014). A total of 65 IGCTs (30 pure germinomas, 14 teratomas, 18 mixed GCTs, 2 yolk sac tumors, and 1 choriocarcinoma) were screened for mutation of KIT, KRAS, NRAS, HRAS, BRAF, PDGFRA, and IDH1 by direct sequencing. Somatic mutations were detected only in KIT and RAS, which were frequently observed in pure germinomas (60.0%) but rare in NGGCTs (8.6%). Mutations in the RAS genes were identified in seven cases, which included KRAS in five, HRAS in one, and NRAS in one. All mutations were somatic missense. All KRAS mutations were found in four patients with germinomas and one patient with mixed germ cell tumors. Point mutations of the KRAS gene were located at G12V in two patients and E63K, Q61R, and Q61L in one each.

The lower rate of KRAS mutation compared with the others may be attributed to the small sample size. It may also be responsible for the low detection rate. Therefore, further studies aiming to enroll increased numbers of patients with ICGCTs are required.

Apart from KRAS mutation detection in one patient with intracranial germinoma, we interestingly observed that a patient carrying the mutant gene was diagnosed to have intracranial germinoma with CSF metastasis and eventually died during treatment. This result supports that KRAS codon 61 mutation (Q61L) has a clinical correlation in IGCTs.

Our study is the first to report a KRAS mutation-related poor outcome in IGGCTs, which is similar to the findings of extracranial germ cell tumor studies. For example, Amaro Taylor-Weiner et al. have recently performed the clinically integrated molecular analysis of 59 tumor samples from testicular and mediatinal germ cell tumors to identify genomic features associated with disease origin and progression. Mutational significance analysis identified KRAS as the most statistically significant altered gene in this cohort, where specific codons 12, 13, and 14 were found in six patients. One patient who had chemoresistance and died of disease showed an inframe indel of KRAS codons 13–14 with amino acid change 13-14 in sG. These findings indicate the specific site and type of KRAS mutation may have a clinical correlation in extracranial GCTs (Taylor-Weiner et al., 2016). However, our result was different those of from previous studies by Fukushima et al. Although KRAS mutation was found at the same site of codon 61, the patients with the mutation were diagnosed as mixed germ cell tumors and had long-term survival outcomes after treatment (Fukushima et al., 2014). Considering the discrepancy of KRAS mutation and various clinical outcomes, molecular studies in a large sample size of IGGCT tissue should be performed for further genetic information.

In summary, our study revealed the treatment outcomes of IGCTs in Thai children. We described the KRAS codon 61 mutation in a metastatic germinoma patient with a poor outcome, supporting that KRAS codon 61 mutation (Q61L) has a clinical correlation in IGCTs. Given the significant advances in treatment options for patients with CNS germinomas, the discovery of KRAS mutations underlying clinical outcomes will provide a great opportunity to develop a novel targeted therapy for patients' refractory to standard chemotherapeutic agents in the future.

Author Contribution Statement

CK, PT, CT and SS designed, collected the patient's data, and reviewed the results. EK and CT performed the laboratory studies. HP, KC, SL DS, KS, CN, KJ, JA and SS involved in patients' care and. CK and PT wrote and revised the manuscript. All authors read and approved the final version of the manuscript.

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This study was approved from the institute ethic

committee No. 545/60. The institutional review board chief committee is Emeritus Professor Tada Sueblinvong, MD.

Statement conflict of Interest

The authors disclose no potential conflicts of interest.

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