**Abstract.** Background: Fusion genes driving tumourigenesis have drawn the attention of researchers and oncologists. Despite the importance of such molecular alterations, there are no comprehensive reproducible methods for detecting fusion genes. Materials and Methods: Nineteen paediatric brain tumours of five types, namely pilocytic astrocytoma, oligodendroglioma, anaplastic astrocytoma, glioblastoma and, ganglioglioma, were examined to detect fusion genes using a pyrosequencing-based method following RNA isolation, cDNA synthesis and real-time polymerase chain reaction. Results: Our method successfully detected KIAA1549–v-raf murine sarcoma viral oncogene homolog B1 (BRAF) fusion in 14 out of 19 patients suffering from five types of paediatric brain tumours providing information on fusion breakpoints within 2 h. Conclusion: A comprehensive method for detecting fusion genes in paediatric brain tumours was evaluated. This method identified KIAA1549–BRAF fusion variants quickly. Our results may help researchers interested in the role of fusion genes in tumourigenesis.

In recent years, molecular biology techniques have modified the classification of tumours (1). Although the majority of tumour classifications have been based on histopathological findings, oncologists are noticing that the characteristics revealed by newer techniques may be more helpful than histopathological features (2) However, the pathogenesis of cancer harbouring fewer genetic mutations such as paediatric cancer remains unclear (3). One hypothesis explaining the aetiology of these tumours is the presence of chromosomal rearrangements which lead to gene fusions (4). Fusion genes are thought to cause abnormal activation of adjacent genes (5). Sometimes, even when dealing with the same tumour type, the presence of a fusion gene is an independent prognostic factor (6). Moreover, oncologists usually fine-tune the treatment strength for tumours in the same pathological subdivision based on the presence or absence of fusion genes (6). Therefore, focussing on fusion genes is important both for treatment and advancing research on this mechanism of tumourigenesis. However, detection of these genes is challenging due to the quality of the samples, which may not provide adequate DNA or RNA purity (3). In addition, fusion variants created by different breakpoints may cause different phenotypes (7, 8). Moreover, previous studies have revealed correlations between fusion variants and histopathological diagnoses/prognoses (9).

Despite the established importance of fusion genes and their breakpoint variants, no comprehensive method exists for their identification. Moreover, some identification techniques are restricted to molecular biology laboratories and not widely utilized.

The most accurate identification method involves direct sequencing followed by real-time polymerase chain reaction (RT-PCR). However, this method requires a good specimen quality for analysis and is costly, despite developments in DNA and RNA purification techniques (10). Therefore, the most common method for detecting the presence of fusion genes is dual-probed fluorescence in situ hybridization (FISH) (11), although this method does not allow for identification of the fusion breakpoints and has a relatively high rate of false-positive results (11). It is fair to say that no comprehensive, accurate and cost-effective method exists for detection of fusion genes.

The Pyromark system (Qiagen, Hilden, Germany) has been developed as a method to obtain the genetic sequence of a particular site. This method can be used for shorter sequences than direct sequencing methods, but the technique has been shown to be accurate and reproducible enough to detect fusion genes (12).
Chromosomal rearrangements caused by fusion genes are common in brain tumours (13-15); the KIAA1549-exon15–v-raf murine sarcoma viral oncogene homolog B1 (BRAF) exon-9 (KIAA1549–BRAF) fusion, is considered a driver of genetic events and a diagnostic marker (13), particularly in pilocytic astrocytoma, a major type of paediatric brain tumour (15). Studies have revealed that specific breakpoints of KIAA1549–BRAF correlate with tumour characteristics of different types of brain tumour (14). Therefore, the detection of a fusion gene by FISH alone is insufficient to reveal its clinical relevance.

In this study, we focused on evaluating the Pyromark system to identify fusion genes and their breakpoints in paediatric brain tumour samples. The time requirements for this method were also assessed and the meaning of our findings for paediatric brain tumours was discussed.

Materials and Methods

Study design. KIAA1549–BRAF fusions including their fusion breakpoints in paediatric brain tumour samples were identified using a pyrosequencing-based method.

Tumour samples. Tumour samples were obtained from tumour bulks removed from paediatric patients suffering from brain tumours those were under the age of 15 at the time of diagnosis. Tumour specimens removed from patients during surgery were immediately placed in an RNA-stabilising agent (RNA later; ThermoFisher, Waltham, MA, USA) after collection. The Juntendo University Ethics Committee approved the study (IRB #2010-014), and written informed consent was obtained from all the patients or their legal guardians. No any exclusion criteria were used for this study.

RNA isolation and cDNA synthesis. Total RNA was prepared from 20 mg of tumour tissue and isolated using All Prep DNA/RNA Mini Kit (Qiagen, Manchester, UK), according to the manufacturer’s instructions. The RNA concentration was measured using QuantiFluor RNA System (Promega Corp., Madison, WI, USA) on instructions. The RNA concentration was measured using Quantis™ Fluorometer (Promega Corp.). A total of 250 ng RNA was reverse transcribed to obtain cDNA using SuperScript™ IV VILO™ Master Mix (ThermoFisher), following the manufacturer’s instructions. RNA extraction required 15 min and cDNA synthesis 25 min.

KIAA1549–BRAF transcripts by RT-PCR. Three well-documented fusion genes: KIAA1549-exon-15–BRAF exon-9, KIAA1549-exon-16–BRAF exon-9 and KIAA1549 exon-16–BRAF exon-11. Three PCRs were performed to detect the three different fusion products of KIAA1549–BRAF using 1 μl (25 pmol/μl) of cDNA primers (Table I) and Fast Cycling PCR Kit (Qiagen), following the manufacturer’s instructions. PCR was performed with 30 amplification cycles at an annealing temperature of 60˚C. A total of 25 μl PCR products were obtained in approximately 30 min. The PCR products were then analysed using electrophoresis on 2% agarose gels.

Pyrosequencing reaction and assay design. Pyrosequencing was performed using 10 μl of the PCR product, sequencing primer (Table I) and PyroGold Reagents (Qiagen), according to the manufacturer’s instructions, on a PyroMarkQ24 (Qiagen). Pyrogram outputs were analysed using PyroMark Q2.0 software (Qiagen) using the short quantification assay. It took approximately 45 min for this process.

Table I. Primer list for detecting three variants of the KIAA1549–v-raf murine sarcoma viral oncogene homolog B1 (BRAF) fusion.

| Assay | Fusion gene | Primers | Sequence (5’ to 3’) | PCR Product (bp) |
|-------|-------------|---------|---------------------|-----------------|
| 15-9  | KIAA1549 exon 15–BRAF exon 9 | Forward | cgtggtgtggtgtgtggt | 106 |
|       |             | Reverse (biotinylated) | acagtcagagcag | |
| 16-9  | KIAA1549 exon 16–BRAF exon 9 | Dispensation order | GATGACCAGATCTGATGAGAGTAC | 89 |
|       |             | Forward | caggaatctcccaatcatc | |
| 16-11 | KIAA1549 exon 16–BRAF exon 11 | Dispensation order | GCTGCTAGTGTACTGATCAGA | 97 |
|       |             | Forward | caggaatctccacactca | |

BRAF: v-Raf murine sarcoma viral oncogene homolog B1.

Table II. Summary data of the tumour types and fusion positivity.

| Tumour type       | Number (%) | Rate of KIAA1549–BRAF fusion |
|-------------------|------------|-----------------------------|
| Pilocytic astrocytoma | 10 (52.6) | 90% (9/10)                  |
| Glioblastoma      | 3 (15.8)  | 33% (1/3)                   |
| Oligodendroglioma | 2 (10.5)  | 100% (2/2)                  |
| Anaplastic astrocytoma | 2 (10.5) | 50% (1/2)                  |
| Ganglioglioma     | 2 (10.5)  | 50% (1/2)                   |
| Total             | 19         | 14/19 (74%)                 |

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The entire procedure from RNA extraction to obtaining mutation data was performed in 2 h or less. The details of the procedure are summarised in Figure 2.

Discussion

Detection of the KIAA1549–BRAF fusion variant. The detection of KIAA1549–BRAF fusions with their specific breakpoints is important in clinical settings. The identification of the type of fusion present is important for prognosis; therefore, any diagnostic method implemented should provide this information. We achieved this using the Pyromark system. Not all previously reported studies on pilocytic astrocytoma mention the fusion gene variant. We found studies related to KIAA1549–BRAF fusions in pilocytic astrocytoma by searching PubMed using the search terms ‘KIAA1549’, ‘BRAF’, ‘fusion gene’ and ‘pilocytic astrocytoma’. Searches were limited to human studies published in English from 2014 and 2017. We found 10 studies that demonstrated detection methods for KIAA1549–BRAF fusion (10, 14-22). Five of these studies identified the fusion gene variant using direct sequencing, and three studies used only FISH. The results of FISH are often difficult to interpret because it is difficult to distinguish a fusion signal from a normal one (23). As mentioned earlier, FISH cannot identify the fusion gene breakpoint; hence a phenotype cannot be assigned to a particular variant, and the information obtained from these studies is limited. Therefore, a method like the one we used here is superior because it helps assign clinical features to specific fusion genes.
We successfully detected KIAA1549–BRAF fusion and its breakpoints in 14 out of 19 patients presenting five types of paediatric brain tumours. Few studies have investigated the relevance of KIAA1549–BRAF fusion in paediatric brain tumours other than those focusing on pilocytic astrocytomas. Especially in cases of high-grade glioma, the significance of KIAA1549–BRAF fusion has not been well investigated. Studies on 15 adult glioblastoma cases found no KIAA1549–BRAF fusions (1, 23). In another study, two out of 157 patients with high-grade gliomas under the age of 18 years had KIAA1549–BRAF fusions, and both had the same fusion breakpoint between exons 15 and 9 (24). Our study showed that two out of five patients with high-grade glioma, anaplastic astrocytoma and glioblastoma had KIAA1549–BRAF fusions; one of them with a fusion between exons 16 and 9. To the best of our knowledge, this fusion breakpoint has not been previously reported in a glioblastoma. These important findings in our small-scale study show the potential of further application of our method that may be helpful even as an alternative to next-generation sequencing, which is considered accurate and sensitive for detecting different genetic alterations (25).

**Time required for analysis.** The commonly used methods for detecting fusion breakpoints involve direct sequencing following RT-PCR, and take at least 4 h to obtain results (60 min for the PCR run and 3-4 h for the cyclic amplification). Our method, on the other hand, required just 2 h, indicating the diagnostic results can be made available to surgeons during the operation itself. In addition, the histopathological distinction between pilocytic astrocytoma and malignant glioma can be challenging because pilocytic astrocytomas are occasionally accompanied by necrosis and microvascular proliferation and may look like malignant gliomas, particularly in frozen sections examined during surgery. In cases in which the results of intraoperative histopathological diagnosis are ambiguous, intraoperative molecular diagnosis using our method may allow the surgeon to decide on the best surgical approach for the patient.
Limitations of this method and study. Our method clearly showed the presence of fusion gene variants. However, we detected only the three most common fusion variants, and studies have reported that KIAA1549–BRAF fusions can involve different exons and fusions such as KIAA1549 exon-15–BRAF exon-11 and KIAA1549 exon-17–BRAF exon-10 (14). Increasing the number of variants examined may increase the time needed to obtain results, and thus our method may not be suitable for detecting fusion genes with a large number of variants.

In addition, we studied only detection of variants of KIAA1549–BRAF fusion; therefore, we cannot account for the impact of different fusion genes. We found two cases of paediatric oligodendroglioma with KIAA1549–BRAF fusion. The presence of this type of oligodendroglioma may represent a subset of oligodendrogliomas sharing characteristics of pilocytic astrocytomas, particularly in children (14). Of note, activation of the mitogen-activated protein kinase pathway by KIAA1549–BRAF fusion in pilocytic astrocytomas has drawn the attention of paediatric neuro-oncologists because of its potential as a therapeutic target (26). Our study involved only a small number of samples, and we did not compare our methods with other detecting methods; in order to confirm the clinical implications using this method and generalise our findings to other tumours, further studies are necessary.

Conclusion

In this study, we used a comprehensive, quick method for the detection of KIAA1549–BRAF fusion from tumour tissues of paediatric patients with brain tumours. This method detected three KIAA1549–BRAF fusion variants within 2 h of collection of the tumour specimen. Our results will be useful for elucidating tumourigenesis of paediatric brain tumours and studies on intraoperative diagnosis and classification of tumours for improving surgical decisions.

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