Detection of RAF fusion transcripts in FFPE samples of Medullablastoma and Ependymom in Iraqi children with RT-RQPCR assays

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Received 10, August, 2014
Accepted 14, September, 2014

Abstract:

Medulloblastomas and ependymomas are the most common malignant brain tumors in children. However genetic abnormalities associated with their development and prognosis remain unclear. Recently two gene fusions, KIAA1549–BRAF and SRGAP3–RAF1 have been detected in a number of brain tumours. We report here our development and validation of RT-RQPCR assays to detect various isoforms of these two fusion genes in formalin fixed paraffin embedded (FFPE) tissues of medulloblastoma and ependymoma. We examined these fusion genes in 44 paediatric brain tumours, 33 medulloblastomas and 11 ependymomas. We detected both fusion transcripts in 8/33, 5/33 SRGAP3 ex10/RAF1 ex10, and 3/33 KIAA1549 ex16/BRAF ex9, meduloblastomas but none in the 11 ependymomas examined. This investigation provided evidence to the value of RT-RQPCR assays for the detection of these fusion genes in large-scale studies on FFPE tissues. The study also reports the first detection of RAF fusion genes in meduloblstomas.

Key words :Medulloblastoma, ependymoma, RAF Gene, RT-RQPCR technique.

Introduction:

Tumors of the central nervous system (CNS) are the second most common malignancy of childhood and are generally associated with a worse prognosis [1]. Medulloblastomas are the most common malignant brain tumors in children and constitute 20% of all pediatric brain tumors [2]. They arise in the infratentorial posterior fossa and have a tendency to metastasize within the CNS. These tumours encompass a collection of clinically and molecularly diverse tumour subtypes [3-6]. Medullablastomas classified as WHO grade IV and divided into two risk-stratification groups, namely: standard risk or high risk, depending on clinical factors such as age, extent of resection, and presence of metastases [7,8].

Ependymoma (WHO grades I–III) is the third most common type of pediatric brain tumor [9,10]. That comprise approximately 9% of childhood brain tumors [11]. These tumours arise in the spinal cord, the supratentorial brain, and, most often in children, the posterior fossa. Even though that ependymomas of various anatomic origins are histologically indistinguishable, they are in fact made up of a heterogeneous group of clinically distinct diseases. Our understanding of the molecular pathology of medulloblastomas and ependymomas is limited. [9-11].

Genetic rearrangements are widely detected in cancers. One possible consequence of genomic rearrangements is the creation of in-

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frame gene fusions. Fusion genes are detected in leukemias, lymphomas, and sarcomas.

The RAF family genes (includes three highly homology ARAF, BRAF and CRFA (or RAF1) play essential role in the Ras-Raf_mitogen-activated protein kinase (MAPK)-extracellular signal regulated kinase (MEK)-MAPK signaling pathway. These proto-oncogenes have been found rearranged in various carcinomas, such as melanomas, prostate, gastric cancer and small-cell lung cancer [12, 13].

Two gene fusions, KIAA1549–BRAF at tandem duplication at 7q34 and SRGAP3–RAF1 at 3p25, have been detected in low-grade astrocytoma, which produces a novel oncogenic fusion gene incorporating a constitutively active BRAF and RAF1 kinase domain. The frequency and specificity of this change underline its potential both as a therapeutic target and a diagnostic marker [14-18].

The aims of this project are to develop and validate RT-RQPCR assays for the detection of these fusion transcripts in FFPE samples, and to assess the incidence of these fusion transcripts in pediatric medullablastoma and ependymoma.

Materials and Methods:

Samples:

Formalin fixed paraffin embedded (FFPE) samples from 44 paediatric brain tumour patients were examined for the presence of RAF fusion genes. Those samples obtained from patients treated at Neurosurgery Hospital (Baghdad, Iraq) during 2006-2010. Histological diagnosis was established according to WHO classification criteria. All of the samples were primary untreated tumours. Patients age at diagnosis time ranging (0.5 -17) years, the mean patients age was 8.75 years. Thirty three samples examined were posterior fossa medulloblastomas grade IV, 29 (19 males and 10 females) of whom were classic type, and 3 desmoplastic medulloblastomas 2 males and one female.

Eleven ependymoma samples also investigated for the RAF fusion, composed of 2 mixopapillary ependymomas grade I (2 males) and 9 ependymoma grade II (5 males and 4 females). Only one sample (a female aged 6 month) with no pathogenic singes was investigated.

Synthesis of RAF fusion positive constructs:

RAF fusion constructs were synthesised for all the fusion transcripts investigated, as positive controls for the RQPCR quantification, based on the overlap extension principles.

RT-RQPCR:

RNA was extracted from formalin fixed PE sections using QIAGEN RNeasy FFPE kit, according to manufacturer's protocol.

cDNA was synthesised with random hexamers primer and Invitrogen's Thermoscript kit. 4µl of cDNA were subjected to RQPCR quantification of KIAA145/BRAF (KIAA1549 ex15/BRAF ex9; KIAA1549 ex15/BRAF ex11; KIAA1549 ex16/BRAF ex9; KIAA145 ex16/BRAF ex11) and SRGAP3/RAF1 (SRGAP3 ex10/RAF1 ex10; SRGAP3 ex12/RAF1 ex10) fusion transcripts. PGK1 transcript was used as an internal control to assess the quality of RNA/cDNA from each sample. RQPCR primers were designed to amplify short amplicons approximately 100bp, to circumvent the effect of RNA degradation in FFPE samples. Primers sequences are shown in Table 1.

RQPCR quantification was performed on a Light Cycler LC480 (Roche) in a 20µl reaction containing 1.4µM $MgCl_2$, 1µM dNTPs, 1µM primer mixes (1/3, 2/4, 5/9, 5/10, 6/8,

6/10, 7/9, 7/10, and 11/12), 0.2U Taq DNA polymerase, and 0.8μM Syto9. PCR parameters were 95°C for 10 minutes, followed by 35 cycles of 95°C 10 seconds, 62°C for 10 seconds, 72°C for 1 minute. No-template, negative and positive controls were used in each reaction for each transcript.

Table 1: RQPCR primers

1	SRGAP3 ex10-F	GCACGATTTACTCAAGCAGACCC
2	SRGAP3 ex12-F	AACGGCAGTATGGAAGCATT
3	RAF1 ex10-R1	GTGGACAGCATCACTTCACTGGC
4	RAF1 ex10-R2	CAAAAGAGCCTGACCCAATC
5	KIAA1549-ex15-F	ACAGCGATGGCACCTACAG
6	KIAA1549-ex16-F1	CAGTGGGGGTCCTTCTACAG
7	KIAA-ex16-F2	TCACTCGAGTCCCGTCTACC
8	BRAF ex9-R	CTCCATCACCACGAAATCCT
9	BRAF ex9 R2	CACCACGAAATCCTTGGTCT
10	BRAF ex11 R	CCCACTGTAATCTGCCCATC
11	PGK1-F	GGGAAAAGATGCTTCTGGGAA
12	PGK1-R	TTGGAAAGTGAAGCTCGGAAA

RQPCR products were electrophoresed to confirm the size of the amplicons amplified, and selected positive products were sequenced.

Results:

Suitable RNA was extracted from all 44 samples examined, 33 Grade IV medullablastomas and 11grade I-II Ependymomas, as demonstrated by the RT-RQPCR quantification of PGKI transcript. The mechanism of RAF fusion rearrangements is elucidated in figure (1-3).

Of the 6 isoforms of the two RAF fusion transcripts investigated, only 2 transcripts were detected (SRGAP3 ex10/RAF1 ex10, and KIAA1549 ex16/BRAF ex9). RAF fusion transcripts were detected in 8/33 (24.24%) medulloblastoma samples examined, 5/33 (15.15%) had SRGAP3 ex10/RAF1 ex10 transcript, and 3/33(9.09%) had KIAA1549 ex16/BRAF ex9 fusion transcript. No RAF fusion transcript detected in any of the (11) Ependymoma samples examined. The RQPCR results were confirmed by agarose gel electrophoresis (figure 4). One RQPCR positive amplicon for KIAA1549 ex16/BRAF and one for SRGAP3 ex 10/RAF1 ex10 were purified and sequenced to confirm the fusion transcript.

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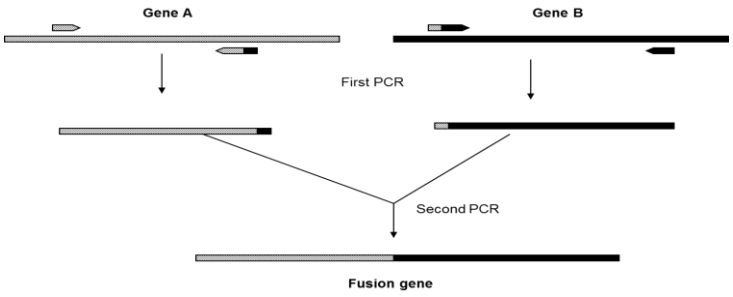

Fig. 1 : RAF fusion Positive constructs synthesis by 'Splicing by overlap extension' principle

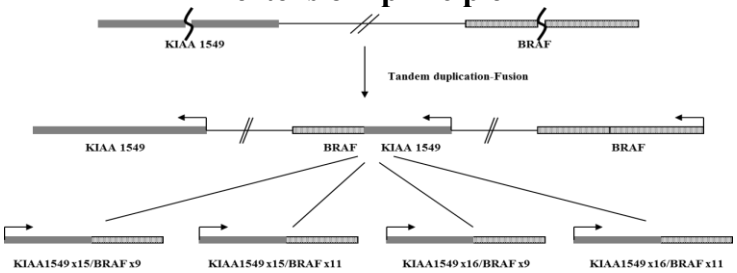

Fig. 2: Genetic rearrangements at 7q34 leading to a fusion between KIAA1549 and BRAF, producing various isoforms of the KIAA1549/BRAF fusion gene

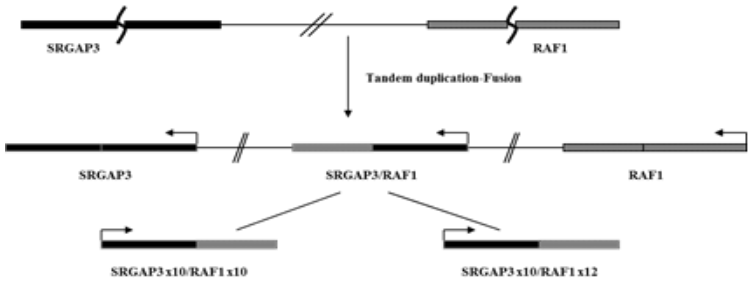

Fig.3: Genetic rearrangements at 3p25 leading to fusion between *SRGAP3*and *RAF1*, producing various isoforms of the *SRGAP3/RAF1* fusion gene

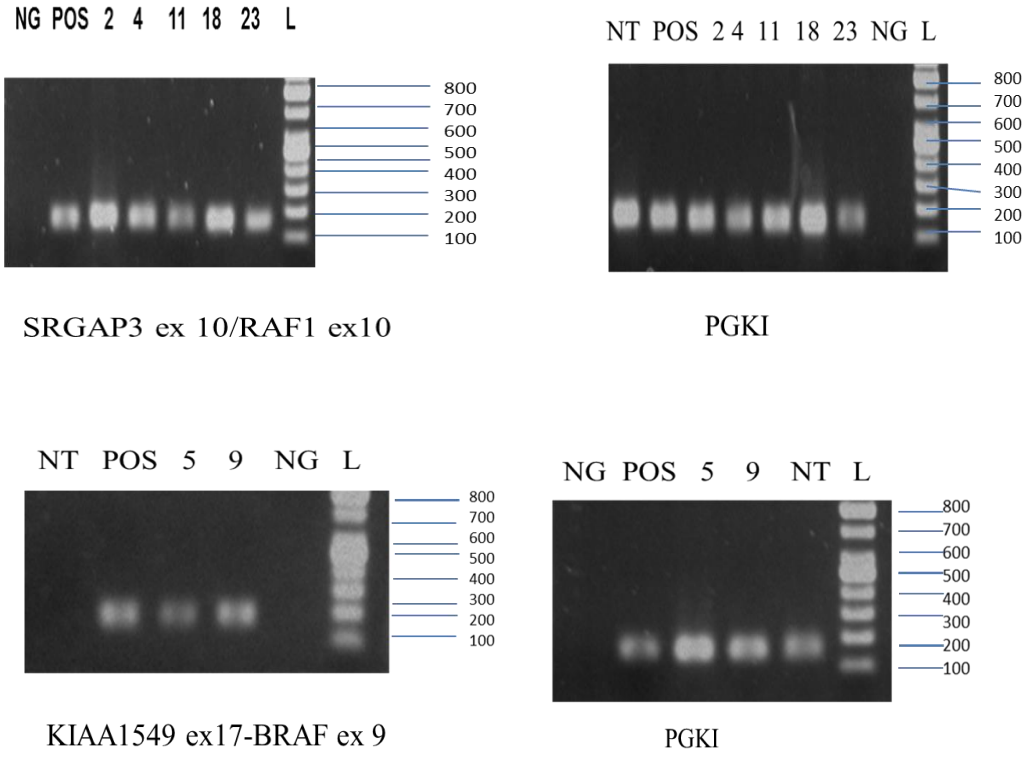

Fig. 4: Agarose gel electrophoresis of RAF fusion genes and PGKI gene RT-RQPCR products as SRGAP3/RAF1 fusion and PGKI, KIAA1549/BRAF and PGKI fusion

Discussion:

The Ras/Raf/MAPK pathway plays a vital role in the regulation of cellular proliferation, differentiation and apoptosis. Three isoforms of the RAF gene have been identified (RAF1, BRAF and ARAF). All the isoforms of the RAF gene that have been identified (RAF1, BRAF and ARAF) share Ras as a common upstream activator and MEK as the only commonly accepted downstream substrate [19, 20].

BRAF activation, mainly through point mutations, with a hotspot at residue 600 (BRAFV600E) has been reported in many tumors. Several studies have confirmed *BRAF* tandem duplications in PA I and grade II-IV pediatric astrocytoma [21-32], with some of these reports showing that tandem duplication at 7q34 leads to a fusion between *KIAA1549* and *BRAF* in approximately 70% of these tumors [29-32]. This fusion protein

incorporate the BRAF kinase domain, but lacking the amino-localized auto-inhibitory domain. The truncated *BRAF*, produced by this fusion is constitutively active. Such rearrangements have been shown to be more common in cerebellar versus non-cerebellar tumours [33]. Genomic sequencing has revealed a number of breakpoint/fusion variants for the KIAA1549/BRAF fusion gene. However, the prevalence of *KIAA1549/BRAF* fusions in other brain tumor subtypes remains unknown.

Another mechanism of MAPK pathway activation in PA I results from the tandem duplication at 3p25 leading to an in-frame oncogenic fusion between *SRGAP3*and *RAF1* [19, 23, 24]. The genes encoding the three Slit-RoboGTPase activating proteins (SRGAP) are expressed during embryonic and early postnatal development in the murine nervous system. [34]

Large scale studies of the prevalence and clinical relevance of these fusion genes are essential to improve understanding of their biological roles in tumours and in particular brain tumours.

To date, no investigation was reported on the occurrence and clinical relevance of these fusion genes in medulloblastomas and ependymomas. To investigate these objectives large scale investigations on FFPE samples are required. However, FFPE tissues contain high level of RNA degradation, which prevents the amplification and detection of large amplicons of targeted transcripts.

In this investigation, we developed new RT-RQPCR assays to detect the various isoforms of the KIAA1549/BRAF and SRGAP3/RAF1 fusion transcripts in FFPE samples from paediatric medulloblastoma and ependymoma. To circumvent RNA degradation in FFPE samples and enable such investigation, the RT-RQPCR assays were designed to amplify short fusion amplicons. In vitro fusion constructs were synthesised and mixed with fusion negative FFPE cDNA to assist in this study as positive controls.

This investigation detected both KIAA1549/BRAF (KIAA1549 ex16/BRAF) and SRGAP3/RAF1 (SRGAP3 ex 10/RAF1 ex10) fusion transcripts.

It is interesting to note that these fusion transcripts were only detected in grade IV medulloblastoma, but not in desmoplastic medulloblastoma and ependymoma samples.

The clinical value of these fusion genes in these tumours remains unclear, and would require larger series of patients. However, this study provided the first report of rearrangements that produced transcribed KIAA1549/BRAF and SRGAP3/RAF1 fusion genes in medulloblastoma. These fusion genes may also provide new targets for treatment in these tumours.

Our investigation also shows that RQ-RQPCR designed to amplify and quantify short amplicons could be used for large scale investigation of RAF fusion genes in cancer.

The assays developed and reported here; lend themselves for use in further investigations to ascertain the prevalence and clinical relevance of the various RAF rearrangements in these and other paediatric brain tumours as well as other cancers.

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التحري عن نسخ ملتحمات مورث *RAF* في عينات مثبتة بشمع البرافينين لأورام الارومة النخاعية واورام ارومة االبطانة العصبية في الاطفال العراقيين باختبار RT-RQPCR

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الخلاصة :

اورام الارومة النخاعية واورام ارومة البطانة العصبية من اكثر الاورام الدماغ الخبيثة شيوعا في الاطفال. والتغيرات الوراثية المرتبطة بتطورهما والتكهن بهما مازالت غير واضحة. تم الشكف عن وجود ملتحمات *لمورثي KIAA1549–BRAF و SRGAP3–RAF1* في عدد من اورام الدماغ . لذلك هدف البحث الى التحري عن هذه الملتحمات في اورام الارومة النخاعية واورام ارومة البطانة العصبية في الاطفال العراقيين، فضلا عن تطوير وتقييم اختبار RT-RQPCR للتحري عن الانماط المختلفة لملتحمات هذين المورثين في عينات مثبتة بشمع البرافين لاورام الارومة النخاعية واورام ارومة البطانة العصبية . تم التحري عن هذه الملتحمات في 44 عينة من اورام الدماغ في الاطفال (33 عينة لاورام الارومة النخاعية و11 عينة لاورام ارومة البطانة العصبية) . وجد كلا النوعين من الالتحامات في 8/ 33 عينة ،كانت 33/5 هي *SRGAP*3 ex10/*RAF*1 ex10 و 33/3 هي *KIAA*1549 ex16/*BRAF* ex9 في اورام الارومة النخاعية ولم تظهر في اي من 11 عينة من اورام البطانة العصبية . اثبتت هذه الطريقة عن قيمة تقنية RT-RQPCR في التحري عن هذا النوع من الالتحامات الوراثية على مدى واسع من الدراسات على انسجة محفوظة بالبرافين . كذلك اشارت هذه الدراسة الى الكشف لاول مرة عن ملتحمات مورث *RAF* في اورام الارومة النخاعية.