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Mutational analysis of PDGFR–RAS/MAPK pathway activation in childhood medulloblastoma

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ABSTRACT

Aberrant signalling via platelet derived growth factor receptors (PDGFRs) and the RAS/MAPK pathway has been implicated in the development of medulloblastoma, the most common malignant brain tumour in childhood. To determine whether genetic mechanisms play a role in the activation of PDGFR–RAS/MAPK signalling in medulloblastoma, we performed a direct sequence analysis of the established mutational "hotspots" of known targets of activating mutations within the pathway (*PDGFRA*, *NRAS*, *KRAS*, *HRAS* and *BRAF*) and *PDFRFB*, in a cohort of 28 primary tumours. A synonymous sequence variation in *PDGFRA* (CCG to CCA; PRO 567 PRO) was detected in two cases (~7%), but not in 150 normal chromosomes assessed, suggesting that the *PDGFRA* locus may be associated with medulloblastoma development in certain cases. No evidence for oncogenic mutations affecting *NRAS*, *KRAS*, *HRAS*, *BRAF* or *PDFRFB* was found in any case. These data demonstrate that activating mutations in established mutational hotspots within the PDGFR–RAS/MAPK pathway are rare events in medulloblastoma development, and suggest that alternative mechanisms are responsible for RAS/MAPK pathway activation in this disease.

1. Introduction

Mutational activation of developmental cell signalling pathways plays a significant role in the pathogenesis of medulloblastoma, the most common malignant brain tumour of childhood. Aberrant activation of the Sonic Hedgehog (SHH) pathway occurs in ~25% of cases and the Wnt/Wingless (Wnt/Wg) pathway is activated in a further ~15%. Disruption of both pathways arises through mutations affecting multiple alternative pathway components in a mutually exclusive fashion; *PTCH1* (~10% of total cases), *SUFU* (~10%) or *SMO* (~5%) in the SHH pathway, and *APC* (~3%), *AXIN1* (~5%) or β-*catenin* (~6%) in the Wnt/Wg pathway (reviewed in Refs. 1 and 2). However, few other specific genetic defects have been identified, and the genetic basis of medulloblastoma development is otherwise poorly understood. Recent expression microarray-led studies have demonstrated upregulation of Platelet Derived Growth Factor receptors (PDGFRs) and members of the downstream RAS/mitogen activated protein kinase (MAPK) signalling cassette in metastatic medulloblastoma, and have demonstrated a potential biological role for the pathway in disease development[3,4], however the mechanisms underlying this upregulation remain unclear.

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Aberrant cell signalling via PDGFR and the RAS/MAPK pathway plays a major role in the development of human cancers[5,6], and specific activating mutations have been identified in a number of alternative pathway components, providing a genetic basis for pathway activation. Mutations generally fall within established regions of these genes (i.e. mutational "hotspots"). One fifth of all human cancers possess mutations in codon 12, 16 or 61 of *KRAS*, *HRAS* or *NRAS*, that cause RAS to accumulate in its constitutively active GTP-bound state[5]. More recently, oncogenic mutations in *BRAF*, which encodes a serine/threonine kinase downstream of RAS, have been described in two thirds of malignant melanomas and a range of other human cancers. Mutations of *BRAF* have elevated kinase and transforming activity and are located in its kinase domain (exons 11 and 15), with a single substitution (V599E) accounting for 80% of mutations[7]. Similarly, activating mutations in the *PDGFRA* receptor tyrosine kinase (affecting exons 12 and 18) have recently been reported in gastrointestinal stromal tumours (GIST; Ref. 8). However, the genetic status of the PDGFR–RAS/MAPK pathway has not been previously investigated comprehensively in medulloblastoma.

Conventional therapies fail to cure up to 40% of children with medulloblastoma and inflict severe long-term toxicity on survivors[2]. Consequently, there is a great need for the development of more effective and less toxic treatments. An increased understanding of oncogenic cell signalling has led to the development of highly effective, relatively non-toxic therapies for human cancer[9], a number of which target the PDGFR–RAS/MAPK pathway[5,6]. These treatments include Imatinib, a small molecule inhibitor of the PDGFR and KIT kinases that possesses single agent activity against GIST[10], and inhibitors of various RAS/MAPK pathway members are under development and have shown activity in early clinical trials[5]. Thus, a more detailed understanding of the basis of PDGFR–RAS/MAPK pathway activation in medulloblastomas may provide opportunities for novel therapeutic interventions. We therefore investigated mutational "hotspots" in the PDGFR–RAS/MAPK pathway for evidence of genetic activation in medulloblastomas.

2. Patients and methods

Following Institutional Review Board approval, we obtained 28 fresh frozen primary medulloblastomas from a representative cohort of children diagnosed between 1984 and 2002 at St. Jude Children's Research Hospital, Memphis, TN (11 female, 17 male, average age 7.0 years, range 0.7–19.0 years). Metastatic stage was assessed in all patients according to Chang's criteria[11]; 46% ($n = 13$) of patients had metastatic disease (M1, $n = 5$; M3, $n = 8$), and 54% ($n = 15$) had no evidence of metastases (M0) by a combination of brain and spine MRI imaging and lumbar CSF sampling. The tumour was totally resected (<1.5 cm^2 residuum on post-operative scan) in 79% ($n = 22$) of patients. Samples underwent central histopathological review (by C.F.), and were classified as either classic (CLA; 47% [$n = 13$]), nodular/desmoplastic (ND; 32% [$n = 9$]) or large cell/anaplastic (LCA; 21% [$n = 6$])[12]. Light microscopy confirmed that samples contained $\geqslant$80% tumour cells, and genomic DNA was extracted using standard techniques[13]. For mutational analysis, specific exonic regions of genomic DNA (including intron–exon junctions) were isolated by polymerase chain reaction (PCR) amplification using specific primers (sequences available from authors on request) and standard reaction conditions. Direct DNA sequence analysis was carried out (in forward and reverse directions) on purified PCR products (QiaQuick™ gel extraction kit, Qiagen), using the CEQ2000 Dye Terminator Cycle Sequencing with Quick Start Kit, and analysed on the CEQ™8000 Genetic Analysis System (both Beckman Coulter™).

3. Results

Multiple oncogenic mutations have been reported in exons 12 (juxtamembrane region) and 18 (activation loop) of the *PDGFRA* gene, which result in constitutive PDGFRA activation[8]. *PDGFRA* and *PDGFRB* share 85% and 86% protein sequence identity in exons 12 and 18, which are also identical in size and position in both gene transcripts. We reasoned that mutations of *PDGFRB* in these regions may also play a role in oncogenesis, and therefore assessed exons 12 and 18 of both *PDGFRA* and *PDGFRB* for evidence of mutations in our panel of medulloblastomas.

In *PDGFRA* exon 12, a synonymous sequence variation was identified at the third position of codon 567 (CCG → CCA; Genbank Accession No. NM_006206) in two primary medulloblastomas (see Fig. 1). Further analysis of the *MspI* restriction fragment length polymorphism created by this variation demonstrated that it was absent in a panel of 150 normal chromosomes (constitutional (newborn cord blood) DNA samples from 75 individuals; obtained from the North Cumbria Community Genetics Project) and is thus likely to represent a tumour-specific variation (Fig. 1). This variation was not associated with any specific clinical feature; one sample had ND pathology with no evidence of metastasis, while the other was a CLA tumour with metastatic (M3) disease. The significance of this tumour-specific non-coding variant detected is unclear and suggests that either: (i) this variant; or (ii) the *PDGFRA* locus may be associated with medulloblastoma development in certain cases, and this now requires further investigation in extended cohorts and at a functional level. No further sequence variations were detected. This evidence indicates that exon 12 or 18 coding mutations leading to direct activation of PDGFRA or PDGFRB are rare in medulloblastomas, although mutations lying outside these regions cannot be excluded at this stage.

Downstream members of the RAS/MAPK pathway might also represent the targets of oncogenic mutations in medulloblastoma. We therefore directly sequenced the mutational hotspots of *BRAF* (exons 11 and 15), alongside exons encoding codons 12, 13 and 61 of *HRAS*, *KRAS* and *NRAS*[5] in our cohort. No sequence variations were identified for any of these genes in any of the tumour samples.

4. Discussion

Together, these data demonstrate that activating mutations in established mutational hotspots within the PDGFR–RAS/MAPK pathway (*PDGFRA*, *HRAS*, *KRAS*, *NRAS* or *B-RAF*) are rare events

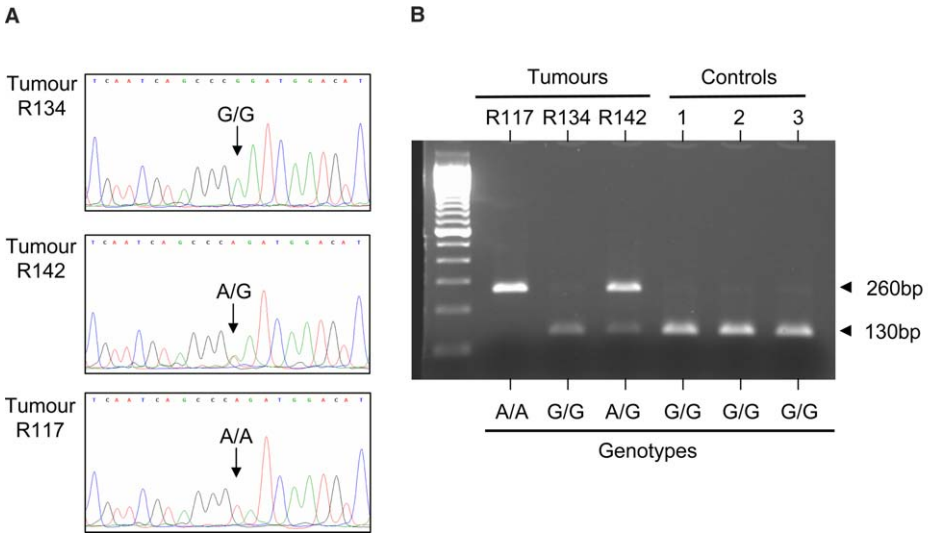

Fig. 1 – *Identification of a PDGFRA sequence variant in medulloblastoma*. (A) Direct DNA sequence analysis of *PDGFRA* exon 12 revealed a synonymous missense variation (CCG to CCA; PRO 567 PRO) in two samples (R142, heterozygous; R117, homozygous or hemizygous). Wild-type sequence was observed in the remaining 26 tumour samples (see sample R134). Variations were confirmed by sequencing of the reverse strand (not shown). (B) This variation creates an *MspI* restriction fragment length polymorphism (RFLP). Further analysis of this RFLP confirmed our sequence analysis results for all 28 tumours (R117, R134 and R142 are illustrated). Analysis of a panel of 75 control constitutional DNA samples (150 chromosomes; samples 1, 2 and 3 are illustrated) detected the G/G genotype in all cases, indicating that the 'A' genotype represents a tumour-specific variant. Samples are shown following PCR amplification and *MspI* restriction digestion, relative to a 100 bp size marker (left hand lane). Presence of the 'G' allele creates an *MspI* site, which allows digestion of the 260 bp PCR product into two 130 bp fragments. No PCR product was observed in negative control reactions (i.e. DNA omitted; not shown).

in medulloblastoma development. This further underlines the distinctive molecular pathogenesis of medulloblastomas, exemplified by the low rates of both RAS/MAPK pathway and P53 mutations detected in these tumours, despite the widespread roles of these genes in other major tumour types (including other brain tumours; see Refs. 14 and 15; http://www.sanger.ac.uk/genetics/CGP/cosmic/). Furthermore, it highlights requirements for the identification of further genetic events involved in medulloblastoma development.

However, these data do not exclude a significant role for the PDGFR–RAS/MAPK pathway in medulloblastoma pathogenesis. First, the analysis reported herein was restricted to the established mutational hotspots of the genes assessed. Although the overwhelming majority of mutations reported in these genes to date fall within these regions (see http://www.sanger.ac.uk/genetics/CGP/cosmic/), we cannot exclude a role for mutations affecting alternative regions of these genes in medulloblastoma. Second, components of the PDGFR–RAS/MAPK pathway are overexpressed in medulloblastomas[3,4], and the possibility therefore remains that the pathway is activated via alternative mechanisms in this disease. Alternative upstream effectors may engage the RAS/MAPK pathway, and recent reports have begun to identify further candidate mechanisms of constitutive pathway activation. Mutational analyses of the tyrosine kinome have identified additional mutational targets that could potentially underlie genetic activation of the RAS/MAPK pathway[16]. Moreover, epigenetic mechanisms affecting genes within the RAS/MAPK pathway may also impact. In particular, epigenetic inactivation of the RAS association domain family 1A (*RASSF1A*) tumour suppressor gene, by promoter hypermethylation, has been reported to be mutually exclusive with mutational activation of *RAS* and *BRAF* in certain adult cancers[17–20], supporting co-dependent function within a common biological pathway, although these events have been reported to occur independently in other tumour types[21,22]. We have recently demonstrated epigenetic inactivation of *RASSF1A* in 96% (27/28) of the tumours investigated in the present study[23,24]. Since we have not identified any coding mutations in the mutational hotspots of *HRAS, KRAS, NRAS* or *BRAF* in any of these tumours, our findings suggest that a mutually exclusive relationship may also exist in medulloblastoma. This observation is of interest, as it may provide: (i) a potential basis for the absence of *RAS* and *BRAF* mutations observed, and (ii) a candidate alternative mechanism for pathway activation in this disease, for further investigation.

In summary, our data exclude a major role for mutations affecting the mutational hotspots of *PDGFRA, HRAS, KRAS, NRAS* and *BRAF*, and *PDGFRB* (exons 12, 18) in medulloblastoma. Further investigations are now required to establish the biological and therapeutic significance of the PDGFR–RAS/MAPK pathway in this disease, and the mechanisms that underlie its activation.

Conflict of interest statement

None declared.

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