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Absence of *AKT1* Mutations in Glioblastoma

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Abstract

Background: Oncogenic activation of the PI3K signalling pathway plays a pivotal role in the development of glioblastoma multiforme (GBM). A central node in PI3K downstream signalling is controlled by the serine-threonine kinase AKT1. A somatic mutation affecting residue E17 of the *AKT1* gene has recently been identified in breast and colon cancer. The E17K change results in constitutive AKT1 activation, induces leukaemia in mice, and accordingly, may be therapeutically exploited to target the PI3K pathway. Assessing whether AKT1 is activated by somatic mutations in GBM is relevant to establish its role in this aggressive disease.

Methodology/Principal Findings: We performed a systematic mutational analysis of the complete coding sequence of the *AKT1* gene in a panel of 109 tumor GBM samples and nine high grade astrocytoma cell lines. However, no somatic mutations were detected in the coding region of *AKT1*.

Conclusions/Significance: Our data indicate that in GBM oncogenic deregulation of the PI3K pathway does not involve somatic mutations in the coding region of *AKT1*.

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Introduction

A number of genetic and functional evidences have unequivocally established the importance of the PI3K pathway in human cancer [1,2]. For example oncogenic deregulation of the PI3K pathway plays a central role in the development of Glioblastoma Multiforme (GBM) as shown by the fact that many of its members are genetically altered [3,4]. Two main regulators, the lipid kinase PIK3CA and the lipid phosphatase PTEN, control this signalling pathway. We and other have shown that the *PIK3CA* gene is mutated in many tumour types, including GBM [4–7]. The corresponding mutations result in activation of the PI3K catalytic activity and constitutive downstream signalling. The tumour suppressor gene *PTEN* encodes for a lipid phosphatase which counteracts the effect of PI3K thus negatively controlling signalling. *PTEN* is mutationally and transcriptionally inactivated in many different tumour types, including GBM [8]. In most tumour lineages, including GBMs *PIK3CA* and *PTEN* mutations occur in a mutually exclusive manner [6,9]. This suggests that they exert overlapping cellular functions, and in fact, both control the cellular levels of phosphatidylinositol-3-phosphate (PIP3) [10,11]. Other mechanisms of activation of the PI3K pathway include

alterations in tyrosine kinase receptors acting upstream in the signalling cascade. This is the case for the receptor tyrosine kinase *EGFR* which can be activated by gene amplification and/or mutations [12]. Both missense point mutations and large extracellular domain deletions (*EGFRvIII*), which due to alternative splicing misses exon 2–7) affecting the *EGFR* gene have been reported at considerable frequency in GBM and result in constitutive activation of the receptor and of the underlying PI3K pathway [12].

Downstream in the signaling cascade, PI3K and PTEN control PIP3, which activates downstream effector molecules, such as the serine-threonine kinase AKT. A recent sequencing study led to the identification of oncogenic somatic mutations in the pleckstrin homology domain of *AKT1* in breast, colon and ovarian cancer [13]. Interestingly, in all cases examined the same mutation E17K was identified. This mutation alters the electrostatic interactions of the pocket and constitutively activates AKT1 in a PI3K-independent manner. By this mechanism, it transforms rodent cells in vitro and can induce leukaemia in mice [13]. Recently, we and others assessed the mutational status of the E17K mutation in different tumor types [14–17], confirming the mutations in breast and colon, and most interestingly revealing mutations in lung cancer

Table 1. Characteristics of 109 GBM tumor samples.

GBM subclasses	primary	94
	secondary	15
Patient sex	female	47
	male	62
Patient age	mean age (years)	53.6
	median age (years)	55

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[14,15]. On the contrary, we did not detect any mutations affecting the hotspot residue E17 in a panel of 128 GBMs [15]. We noted that, GBMs do exhibit a different mutation spectrum for some genes compared to other tumor types. For example, most *EGFR* and *ERBB2* mutations in lung cancer are found in the kinase domain [18,19], whereas these genes are predominantly mutated in the extracellular domain in GBM [12,20]. This led us to speculate that GBMs might bear mutations in other regions of the *AKT1* gene. To definitively assess this hypothesis, we successfully sequenced the complete coding sequence of *AKT1* in 109 GBM tumor samples and nine high grade astrocytoma cell lines.

Results and Discussion

We sequenced the complete coding sequence of the *AKT1* gene in a set of 109 GBM tumors and 9 high grade astrocytoma cell lines. Primers were designed to amplify and sequence the genomic region corresponding to all coding exons of *AKT1*, including exon 4, where the E17K residue is located. Amplicons included at least 15 intronic bases at both the 5' and 3' ends encompassing the splicing donor and acceptor sites. A total of 1535 PCR products, spanning 628 kb of tumour genomic DNA, were generated and subjected to direct sequencing. Sequencing was performed single stranded with either forward or reverse primer. Identified changes were independently confirmed by another round of PCR and sequencing.

Previous work focusing on the mutational analysis of exon 4 did not identify any E17K mutation in 128 GBM samples [15]. Importantly, we extended the mutational analysis for these tumors

to all other coding exons of *AKT1*, but no somatic mutations were found.

Our tumor set was validated by previous mutational profiling of common cancer genes, including the *IDH1* gene [21]. Furthermore, we found a number of previous reported SNPs in our samples (rs17846822, rs34664585, rs3730358, rs3730368, rs2494737, rs2494735, rs34670300, rs3730361, rs3730329, rs3730329, rs2494732). In addition, we identified three novel germline changes (IVS8-12C>T, R200H and W333*) at low frequency, they were found only once in different samples. Our work is focused on somatic mutations and therefore, we did not study these changes in further detail.

While previous studies have focused mainly on the hotspot mutation site *AKT1*^{E17} or the serine and threonine phosphorylation sites [3,15,17], this work is the first to show in a large panel of GBMs that the coding sequence of the AKT1 is not somatically mutated in this tumour lineage. Although the promoter region and/or the 5' and 3' UTR of *AKT1* may contain mutations, our data indicate that in GBM oncogenic deregulation of the PI3K pathway does not involve mutations in the coding region of *AKT1*.

Materials and Methods

Tumor sample, Ethics Statement and Isolation of Genomic DNA

All samples were collected from patients undergoing brain tumor surgery in the Academic Medical Center (Amsterdam, The Netherlands). Oral consent for removal of the tissue and its storage in the tumor bank for research purposes was obtained and documented in the patient's medical chart. Individual consent for this specific project was waived by our ethics committee because the research was performed on 'waste' material, stored in a coded fashion. Patient characteristics are displayed in Table 1. Tumor samples were included only if at least 80% of the sample consisted of cancer cells, as verified by H&E staining.

Nine astrocytoma cell lines were included: the cell lines U87MG, U118MG, U251MG, U373MG, T98G (ATCC, Middlesex, United Kingdom), SKMG-3 (a gift of Dr Christopher Y. Thomas, University of Virginia Division of Hematology/Oncology, Charlottesville, VA), SF763 (gift of Dr M.L. Lamfers,

Table 2. Primer details to sequence the coding sequence of AKT1.

Exon	Forward Primer Sequence	Reverse Primer Sequence	Sequencing Primer Sequence
3	GTCAGAGAGCTTAGAGGGATGG	GGCACAGGCACTCACAGA	AGTGGGTCTCTGGCTCACC
4	AGTCTGCCTCCCGTTGAC	CAGCCAGTGCTTGTGCTT	GTTTCTGCTGCTGGCCCTA
5	AGGCTTGGAGAGGAAGAGAT	GGAGTGAGGATGGCTACAGG	CTGGTGGGTGGTATGCAAG
6	AGGGCTGTCTCTGGGAACC	TGGAGTGCTGAGTGTCTCTG	GGGTGGGTGAAAGACGTG
7	GTTCCCTGTAAGCCTGGACTC	TAAAGCAGGGCTGGGTGA	GCTCTGCCTCCGACTCTG
8	CCGCTACTACGCCATGAAGA	ACATCGTCCCCTAGAGACAGC	CATGAAGATCCTCAAGAAGGAAG
9	AACACTCCTGGCACCTCAG	TAACCTCAGCAGGAACAAGTCACC	ATCAGGCGACGTGGTATCAA
10	AGTTAGGGCTTCTGAGACTTTCC	CTTGTCAGCATGAGGTTCTC	GTCCCTCCCTGTGCAATG
11	CTACCTGCACTCGGAGAAGAAC	CAGGACTCGGCATCAAGG	GCACAGAGAGGACACAGCATT
12	CTGAGCACACGCAATGCT	GACATCAAGCTTTGGCTATCAGT	AATGCTGTGCTCTCTGTGC
13	GTGAGCTGTGGTCTTTG	GCGTGAGTGTGGATATGTGG	GCCCTACATCACAGGAGGAA
14	GCTTGCTGCTCTGACATC	AGGCCTCTGAGTGTGGA	ATCCAGGTGCTTTGAAGGTCT
15	AGGTCCTGTGTCATCTGTG	CTCAAATGCACCCGAGAAATA	GAGGTTGGCTTCTACTGGAG

Primers in 5' to 3' direction.

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Department of Neurosurgery, Free University, Amsterdam, The Netherlands), SF126 (a gift of Dr C. Van Bree, University of Amsterdam, Laboratory for Experimental Oncology and Radiation Biology, Amsterdam, The Netherlands), Gli-6 was derived from our own laboratory. Genomic DNA was isolated as previously described [22].

PCR, Sequencing and Analysis

PCR primers were designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), and synthesized by Invitrogen/Life Technologies, Inc. (Paisley, England) (Table 2). PCR primers that amplify the selected exons and the flanking intronic sequences, including splicing donor and acceptor regions, were used and PCR products were on average 381 bps in length. PCRs were performed in both 384- and 96-well formats in 5- or 10- μ L reaction volumes, respectively, containing 0.25 mmol/L deoxynucleotide triphosphates, 1 μ mol/L each of the forward and reverse primers, 6% DMSO, 1 \times PCR buffer, 1 ng/ μ L DNA, and 0.05 unit/ μ L Platinum Taq (Invitrogen/Life Technologies). A touchdown PCR program was used for PCR amplification (Peltier Thermocycler, PTC-200, MJ Research, Bio-Rad Laboratories, Inc., Italy).

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Author Contributions

Conceived and designed the experiments: FB AAGvT AB. Performed the experiments: FB SL CZ. Analyzed the data: FB SL. Contributed reagents/materials/analysis tools: FB AAGvT SL DT TH WPV AB. Wrote the paper: FB WPV AB.