Mutational profiling of glioblastoma
Bleeker, F.E.

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Absence of AKT1 mutations in glioblastoma

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Abstract
Oncogenic activation of the PI3K signaling pathway plays a pivotal role in the development of glioblastoma. A central node in PI3K downstream signaling 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 leukemia in mice, and accordingly, may be therapeutically exploited to target the PI3K pathway. Assessing whether AKT1 is activated by somatic mutations in glioblastoma is relevant to establish its role in this aggressive disease. We performed a systematic mutational analysis of the complete coding sequence of the AKT1 gene in a panel of 109 tumor glioblastoma samples and nine high-grade astrocytoma cell lines. However, no somatic mutations were detected in the coding region of AKT1. Our data indicate that in glioblastoma oncogenic deregulation of the PI3K pathway does not involve somatic mutations in the coding region of AKT1.

Introduction
A number of genetic and functional evidences have unequivocally established the importance of the PI3K pathway in human cancer. For example oncogenic deregulation of the PI3K pathway plays a central role in the development of glioblastoma as shown by the fact that many of its members are genetically altered. Two main regulators, the lipid kinase PIK3CA and the lipid phosphatase PTEN, control this signaling pathway. We and others have shown that the PIK3CA gene is mutated in many tumor types, including glioblastoma. The corresponding mutations result in activation of the PI3K catalytic activity and constitutive downstream signaling. The tumor suppressor gene PTEN encodes for a lipid phosphatase which counteracts the effect of PI3K thus negatively controlling signaling. PTEN is mutationally and transcriptionally inactivated in many different tumor types, including glioblastoma. In most tumor lineages, including glioblastomas PIK3CA and PTEN mutations occur in a mutually exclusive manner. This suggests that they exert overlapping cellular functions, and in fact, both control the cellular levels of phosphatidylinositol-3-phosphate (PIP3). Other mechanisms of activation of the PI3K pathway include alterations in tyrosine kinase receptors acting upstream in the signaling cascade. This is the case for the receptor tyrosine kinase EGFR which can be activated by gene amplification and/or mutations. 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 glioblastoma and result in constitutive activation of the receptor and of the underlying PI3K pathway.

Downstream in the PI3K 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 plekstrin homology domain of AKT1 in breast, colon and ovarian cancer. 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 leukemia in mice. Recently, we and
others assessed the mutational status of the E17K mutation in different tumor types, confirming the mutations in breast and colon, and most interestingly revealing mutations in lung cancer. On the contrary, we did not detect any mutations affecting the hotspot residue E17 in a panel of 128 glioblastomas. We noted that glioblastomas 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, whereas these genes are predominantly mutated in the extracellular domain in glioblastoma. This led us to speculate that glioblastomas 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 glioblastoma tumor samples and nine high-grade astrocytoma cell lines.

Materials and methods

Tumor samples

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 C.Y. Thomas, University of Virginia Division of Hematology/Oncology, Charlottesville, VA), SF763 (gift of Dr M.L. Lamfers, Department of Neurosurgery, Free University, Amsterdam), SF126 (gift of Dr C. Van Bree, Department of Radiotherapy, Academic Medical Center), Gli-6 was derived from our own laboratory. Genomic DNA was isolated as previously described.

PCR and sequencing details

PCR primers were designed using Primer 3 and synthesized by Invitrogen (Life Technologies, Paisley, UK; 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 µl reaction volumes, respectively, containing 0.25 mM dNTPs, 1 mM each of forward and reverse primer, 6% DMSO, 1X PCR buffer, 0.05 U/µl Platinum Taq Invitrogen and 1 ng/µl DNA. A touchdown PCR program was used for PCR amplification (Peltier Thermocycler PTC-200; MJ Research, Bio-Rad Laboratories, Milan, Italy). PCR conditions were: 94°C for 2 min; 3 cycles of 94°C for 15 sec; 64°C for 30 sec, 70°C for 30 sec, 3 cycles of 94°C for 15 sec, 61°C for 30 sec, 70°C for 30 sec, 3 cycles of 94°C for 15 sec, 58°C for 30 sec, 70°C for 30 sec, and 35 cycles of 94°C for 15 sec, 57°C for 30 sec and 70°C for 30 sec, followed by 70°C for 5 min and 12°C forever.
PCR products were purified using AMPure® (Agencourt Bioscience Corporation, Beckman Coulter, Milan, Italy) and eluted in distilled water. Sequencing PCRs were carried out at 97°C for 3 min, and 29 cycles of 97°C for 10 sec, 50°C for 20 sec and 60°C for 2 min. Sequencing PCR products were purified using CleanSeq® (Agencourt Bioscience Corporation). Direct sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) with a 3730 DNA Analyzer, ABI capillary electrophoresis system (Applied Biosystems). Sequence traces were analyzed using Mutation Surveyor software (Version 2.02; SoftGenetics, State College, PA).

### Results and discussion

We sequenced the complete coding sequence of the **AKT1** gene in a set of 109 glioblastoma 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 1.535 PCR products, spanning 628 kb of tumor 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 of **AKT1** did not identify any E17K mutation in 128 glioblastoma samples. 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. 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.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Glioblastoma</td>
<td>Primary 94</td>
</tr>
<tr>
<td></td>
<td>Secondary 15</td>
</tr>
<tr>
<td>Sex</td>
<td>Female 47</td>
</tr>
<tr>
<td></td>
<td>Male 62</td>
</tr>
<tr>
<td>Age</td>
<td>Mean age (years) 53.6</td>
</tr>
<tr>
<td></td>
<td>Median age (years) 55</td>
</tr>
</tbody>
</table>

Table 1. Patient characteristics of 109 glioblastoma tumor 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 AKT1E17 or the serine and threonine phosphorylation sites, this work is the first to show in a large panel of glioblastomas that the coding sequence of the AKT1 is not somatically mutated in this tumor lineage. Although the promoter region and/or the 5' and 3' UTRs of AKT1 may contain mutations, our data indicate that in glioblastoma oncogenic deregulation of the PI3K pathway does not involve mutations in the coding region of AKT1.

**Table 2. Primer details for the coding part of AKT1**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Sequencing Primer</th>
</tr>
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<tbody>
<tr>
<td>3</td>
<td>5'-GTCAGAGAGCTTAGAGGATGG-3'</td>
<td>5'-GGCACAGGCACCTACAGA-3'</td>
<td>5'-AGTGTTCTCTGACTACC-3'</td>
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<tr>
<td>4</td>
<td>5'-AGTCTGCCCTCCGTTGAC-3'</td>
<td>5'-CACGCCAGCTCTGCTGTTG-3'</td>
<td>5'-GTTTTGCTCGCTGCCCCTA-3'</td>
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<tr>
<td>5</td>
<td>5'-AGGCCTGGAGGAGGAAGAGA-3'</td>
<td>5'-GGAGTGGAGGAGGCTACAG-3'</td>
<td>5'-CTGGTGGTTGTTATGCAAG-3'</td>
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<tr>
<td>6</td>
<td>5'-AGGCCCTGTCTCTGGAACC-3'</td>
<td>5'-TGAGTGAGATGGCTACAG-3'</td>
<td>5'-GGGTGGTTAGGAGGACCT-3'</td>
</tr>
<tr>
<td>7</td>
<td>5'-GTCCCCGTAAGGCGCTGACT-3'</td>
<td>5'-TAAAGCGGCTGGGCTGGA-3'</td>
<td>5'-GCCGCTGCTCGACTGTG-3'</td>
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<tr>
<td>8</td>
<td>5'-CCGTACCTGACCATGAGA-3'</td>
<td>5'-ACATCTCGCTCCAGAGCAG-3'</td>
<td>5'-CATGAAGATCTCCCTAGAGAG-3'</td>
</tr>
<tr>
<td>9</td>
<td>5'-AACACTCCCTGGACACCTCAG-3'</td>
<td>5'-TAACCTGACAGAAAGAGCT-3'</td>
<td>5'-ACAGCCGACCTGTATCAA-3'</td>
</tr>
<tr>
<td>10</td>
<td>5'-AGTTGGGCTCTGTGAGCACTTCC-3'</td>
<td>5'-CTTCTGCCGCTGACCTTCC-3'</td>
<td>5'-GTCCCCCTCTCTGCAAT-3'</td>
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<tr>
<td>11</td>
<td>5'-CTACCTGACCTGCAGGAGA-3'</td>
<td>5'-CGAGACTGCACCAGCAAGAG-3'</td>
<td>5'-GCACGAGAGGAGCAACAT-3'</td>
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<tr>
<td>12</td>
<td>5'-CTGACGACACCAATTGCT-3'</td>
<td>5'-GACATCAAGCTTTGGCTACAGT-3'</td>
<td>5'-AATGCTCGTCTGCTCAGTGC-3'</td>
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<tr>
<td>13</td>
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<td>5'-GCCGAGTTGAGGATATGGA-3'</td>
<td>5'-GTCCTAGCACAGAGGAAGAA-3'</td>
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<td>14</td>
<td>5'-GCTTGGCTGGCTCTGACTAC-3'</td>
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<td>15</td>
<td>5'-AGGTCCTGTGTAATGTTGCT-3'</td>
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<td>5'-GAGGTTGGCCTCTATTGGAG-3'</td>
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Chapter 7

References