Subependymal giant cell astrocytomas in Tuberous Sclerosis Complex have consistent TSC1/TSC2 biallelic inactivation, and no BRAF mutations

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ABSTRACT

Subependymal giant cell astrocytomas (SEGAs) are rare, low-grade glioneuronal brain tumors that occur almost exclusively in patients with tuberous sclerosis complex (TSC). Though histologically benign, SEGAs can lead to serious
neurological complications, including hydrocephalus, intractable seizures and death. Previous studies in a limited number of SEGAs have provided evidence for a biallelic two-hit inactivation of either TSC1 or TSC2, resulting in constitutive activation of the mechanistic target of rapamycin complex 1 pathway. The activating BRAF V600E mutation is a common genetic alteration in low grade gliomas and glioneuronal tumors, and has been reported in SEGAs as well. In the present study, we assessed the prevalence of the BRAF V600E mutation in a large cohort of TSC related SEGAs (n=58 patients including 56 with clinical TSC) and found no evidence of either BRAF V600E or other mutations in BRAF. To confirm that these SEGAs fit the classic model of two hit TSC1 or TSC2 inactivation, we also performed massively parallel sequencing of these loci. Nineteen (19) of 34 (56%) samples had mutations in TSC2, 10 (29%) had mutations in TSC1, while 5 (15%) had no mutation identified in TSC1/TSC2. The majority of these samples had loss of heterozygosity in the same gene in which the mutation was identified. These results significantly extend previous studies, and in agreement with the Knudson two hit mechanism indicate that biallelic alterations in TSC2 and less commonly, TSC1 are consistently seen in SEGAs.

INTRODUCTION

Subependymal giant cell astrocytomas (SEGAs) are rare, low-grade brain tumors that generally develop during the first two decades of life in 10-20% of patients with tuberous sclerosis complex (TSC) [1–3]. TSC is an autosomal dominant neurocutaneous disorder caused by mutations in either TSC1 encoding hamartin, or TSC2 encoding tuberin. Together these two proteins form the TSC protein complex that regulates mechanistic target of rapamycin complex 1 (mTORC1) [4–6]. In the central nervous system, TSC is characterized by the development of SEGAs, subependymal nodules (SEN), cortical tubers and cortical migration tracts [7]. SEGAs represent 1%-2% of all pediatric brain tumors and usually arise near the foramen of Monro [8–10]. They are a potential cause of major morbidity and mortality in TSC [11]. Extended growth of the tumor can cause obstruction of cerebrospinal fluid tract resulting in hydrocephalus and increased intracranial pressure with subsequent death if neglected. SEGAs are treated with either surgical resection or mTORC1 inhibitors including everolimus.

Histopathologically, SEGAs consist of spindle cells, gemistocytic-like cells and giant cells. According to the present world health organization (WHO) classification of brain tumors, SEGAs belong to the group of astrocytic neoplasms, even though they have both glial and neuronal expression patterns [12, 13]. SEGAs likely develop from SEN, but the molecular mechanisms underlying their progressive growth, in contrast to SEN, are unknown so far [14, 15]. There is evidence of second-hit inactivation of TSC1 or TSC2 in SEGAs, suggesting that one contributor to SEGAs development is the complete loss of a functional tuberin-hamartin complex and the subsequent mTORC1 activation [16–18]. However, it is likely that second-hit mutations in TSC1 and TSC2 also contribute to SEN formation, suggesting that additional genetic events may contribute to the progressive growth of SEGAs.

BRAF is a kinase that activates the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway which regulates cell proliferation, survival and cell-cycle arrest [19]. The BRAF c.1799T>A (p.V600E) mutation (BRAFV600E) results in constitutive activation of MAPK/ERK signaling and is well known in both pediatric and adult low-grade gliomas, including pilocytic astrocytoma (PA), pleomorphic xanthoastrocytoma (PXA), ganglioglioma (GG), desmoplastic infantile gangliogliomas (DIG), and dysembryoplastic neuroepithelial tumor (DNET) [20–26]. Although the prevalence of BRAF mutations in low grade gliomas is relatively low [22], BRAFV600E mutations have been consistently reported as genetic driver in gangliogliomas (18-56%), and have been associated with mTORC1 activation [20, 25].

Both protein kinase B (AKT) and MAPK/ERK pathways have been reported to be activated in SEGAs [27–31]. However, the genetic basis for MAPK/ERK and AKT activation in SEGAs is unknown. The BRAFV600E mutation was reported in a small set (6 of 14 cases) of SEGAs [23] suggesting that it could explain MAPK/ERK and AKT activation in SEGAs. However, subsequent studies have produced contradictory results, failing to confirm the presence of the BRAFV600E mutation in SEGAs [18, 21, 23, 26, 32].

In the present study, we examined the possibility that BRAF mutations occur in SEGAs using a large international cohort of fifty-eight SEGAs from both pediatric and adult TSC patients.
RESULTS

Samples and clinical features

Fifty-eight SEGAs and one SEN from 58 patients were analyzed (62% male, 36% female; Table 1). Fifty-six patients had a definite clinical diagnosis of TSC, whereas two patients did not show other signs of TSC apart from the tumor. TSC1/TSC2 mutation analysis was performed as part of routine clinical care on blood or tumor DNA for 19 subjects, such that 7 had TSC1 and 12 had TSC2 mutations. For 34 samples we performed TSC1/TSC2 mutation analysis using massively parallel sequencing (MPS); for the remaining 5 samples there was insufficient DNA for this analysis.

Ages ranged from 1 to 53 years at the time of surgery. The large majority of patients had a lesion located in the lateral ventricle near the foramen of Monro and five patients had bilateral tumors. Histological diagnosis was confirmed following the current WHO classification guidelines by two independent neuropathologists [33]. All cases had classical histological features of SEGA, showing mainly giant cells with eosinophilic cytoplasm (Figure 1A). Smaller gemistocytic cells, fibrillary astrocytes and a variable number of multinucleated cells were also noted in all cases. Calcifications were observed in 13/44 FFPE cases (30%). As previously reported [13, 34–36], immunohistochemical analysis revealed variable expression of glial and neuronal markers (Figures 1B-1C). We also observed prominent presence of microglial cells intratumoral T-lymphocytes (Figures 1D-1E) and evidence of activation of mTORC1 pathway with phospho-S6 ribosomal protein immunoreactivity (Figure 1F). The differential diagnosis of SEGA takes into account other tumors arising in the region of the basal ganglia and in the lateral and third ventricles (diffuse astrocytoma, ependymoma, central neurocytoma, choroid plexus papilloma). SEGA outside the setting of TSC are rare [37, 38], as well as SEGA within cortical tubers [39]. In our cohort, nearly all patients (n=56) had other central nervous system TSC-associated lesions (SEN and cortical tubers) associated with refractory epilepsy, making the diagnosis reasonably certain before resection.

BRAF mutational analysis

Sanger sequencing analysis for the BRAFV600E mutation in all 58 SEGA samples tested and the SEN was negative (Figure 2). Furthermore, no other variants were found in exon 15 of BRAF in any sample. We also performed RT-PCR to screen for five different types of gene fusions between KIAA1549 and BRAF on 6 SEGAs from which RNA was available (Table 2; data not shown). There was no evidence for the presence of KIAA1549-BRAF fusions in the six SEGA cases analyzed. BRAF mutational analysis was also performed by MPS for all SEGA samples for which there was sufficient DNA to permit this method of analysis, n=31 (Table 3B). None of the samples showed the BRAFV600E mutation, even at an allele frequency of 5-10%. Five intronic variants were identified, all known single nucleotide polymorphisms (SNPs; data not shown). Two coding variants in exon 1 were identified, c.82G>T (p.G28C) at allele frequency 100% in one sample, and c.31G>Ap (p.G11S) at allele frequency 56% in a second sample (Table 3B). These are not known germline variants (per Exac). The p.G11S variant has been reported in a single hepatocellular carcinoma, while the p.G28C variant has not been seen previously in cancer (per cBio). Furthermore, these two variants showed no evidence of pathogenicity based on three different in silico prediction tools.

Table 1: Summary of clinicopathological features in TSC patients with subependymal giant cell astrocytoma

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤18</td>
<td>37</td>
<td>64</td>
</tr>
<tr>
<td>&gt;18</td>
<td>21</td>
<td>36</td>
</tr>
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<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>36</td>
<td>62</td>
</tr>
<tr>
<td>Female</td>
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<td>37</td>
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<tr>
<td>Tumor location</td>
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<td></td>
</tr>
<tr>
<td>Lateral ventricle</td>
<td>49</td>
<td>84</td>
</tr>
<tr>
<td>Foramen of Monro</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Third ventricle</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>TSC-lesions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEN/Tubers</td>
<td>56</td>
<td>96</td>
</tr>
<tr>
<td>Tuberous Sclerosis Complex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Definite</td>
<td>56</td>
<td>97</td>
</tr>
<tr>
<td>Possible</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

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TSC1/TSC2 mutational analysis

TSC1/TSC2 mutational analysis was performed by MPS for 34 SEGA samples (Table 3A, Figures 3 and 4). In 19 (56%) samples TSC2 mutation was identified, 10 (29%) had mutations in TSC1, and 5 (15%) had no mutation identified (NMI) in either TSC1 or TSC2. Of the 5 NMI samples 3 showed copy neutral loss of heterozygosity (CN-LOH) for TSC2 and another sample had a possible TSC1 mutation. Nine of 10 (89%) samples with a TSC1 mutation also showed evidence of CN-LOH for TSC1, 14 of 19 (74%) samples with a TSC2 mutation also showed evidence of CN-LOH for TSC2, while in 1 sample two small TSC2 mutations were identified.

DISCUSSION

SEGAs are low-grade brain tumors associated with TSC and represent 1%-2% of all pediatric brain
Table 2: Primer sequences for detection of *KIAA1549:BRAF* fusion genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Exon variant (KIAA1549:BRAF)</th>
<th>Forward Primer (5’-&gt;3’)</th>
<th>Reverse primer (5’-&gt;3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>KIAA1549-BRAF</em> fusion</td>
<td>Ex16:Ex9</td>
<td>CTACAGCCCAGCCACAGAC</td>
<td>GTGAGCCAGGAATGAGGCAG</td>
</tr>
<tr>
<td><em>KIAA1549-BRAF</em> fusion</td>
<td>Ex15:Ex9</td>
<td>CCACACTCAGCCTACATCGG</td>
<td>GTGAGCCAGGAATGAGGCAG</td>
</tr>
<tr>
<td><em>KIAA1549-BRAF</em> fusion</td>
<td>Ex16:Ex11</td>
<td>AGACGCGCAATACTCCCTGC</td>
<td>GTCCCACTGTAATCTGCCC</td>
</tr>
<tr>
<td><em>KIAA1549-BRAF</em> fusion</td>
<td>Ex18:Ex10</td>
<td>GAGGGATCTAATCGAGGAG</td>
<td>GTGAGCCAGGAATGAGGCAG</td>
</tr>
<tr>
<td><em>KIAA1549-BRAF</em> fusion</td>
<td>Ex19:Ex9</td>
<td>GAAGCGGGCGAGAAGAG</td>
<td>GTGAGCCAGGAATGAGGCAG</td>
</tr>
<tr>
<td><em>PBGD</em></td>
<td>-</td>
<td>CTGGTAACGGCAATCGGCT</td>
<td>GCAATGCGCTCCGATGGTGAG</td>
</tr>
<tr>
<td><em>B2M</em></td>
<td>-</td>
<td>AGCATTCAGACTTTTTCAG</td>
<td>GATGCTGCTTAGATGTC</td>
</tr>
</tbody>
</table>

Figure 2: Direct sequencing of exon 15 of *BRAF* for detection of the V600E mutation. (A) Schematic overview showing the forward and reverse sequence of *BRAF* exon 15 at codon 598 through codon 602 for both wild-type and the c.1799T>A (p.V600E) mutation. (B) Positive control. Pilocytic astrocytoma with the *BRAF*V600E mutation shows c.1799T>A in the forward sequence (left) and reverse sequence (right), resulting in the p.V600E amino acid substitution (arrow). (C) SEG showing the wild-type GTG forward sequence (left) and CAC reverse sequence (right). (D) SEN with the V600 codon showing the wild-type GTG forward sequence (left) and CAC reverse sequence (right). Arrows indicate codon 600 of *BRAF*. 
Table 3A: Summary of results for TSC1/TSC2 mutational analysis in 34 SEGA samples by MPS. NMI = No Mutation Identified, MAF = mutant allele frequency, CN-LOH = Copy neutral loss of heterozygosity, point = point mutation or small insertion or deletion

<table>
<thead>
<tr>
<th>Case (№)</th>
<th>Gene</th>
<th>Nucleotide change</th>
<th>MAF (%)</th>
<th>Mutation type</th>
<th>Protein change</th>
<th>CN-LOH</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NMI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>NMI</td>
<td>TSC1 c.1-7G&gt;A</td>
<td>50</td>
<td>Possible initiator</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>TSC1</td>
<td>chr9:135700060-135799506del</td>
<td>78</td>
<td>Genomic deletion</td>
<td>deletion of exons 6-23</td>
<td>Yes</td>
<td>large del+ CN-LOH</td>
</tr>
<tr>
<td>4</td>
<td>TSC1</td>
<td>c.1498C&gt;T</td>
<td>71</td>
<td>Nonsense</td>
<td>p.R500*</td>
<td>Yes</td>
<td>point+CN-LOH</td>
</tr>
<tr>
<td>5</td>
<td>TSC1</td>
<td>c.641_644dupAGAC</td>
<td>93</td>
<td>Insertion</td>
<td>p.F216Dfs3'</td>
<td>Yes</td>
<td>point+CN-LOH</td>
</tr>
<tr>
<td>6</td>
<td>TSC1</td>
<td>c.2074C&gt;T</td>
<td>39</td>
<td>Nonsense</td>
<td>p.R692*</td>
<td>Yes</td>
<td>point+CN-LOH</td>
</tr>
<tr>
<td>7</td>
<td>TSC1</td>
<td>c.1525C&gt;T</td>
<td>12</td>
<td>Nonsense</td>
<td>p.R509*</td>
<td>No</td>
<td>Point-no LOH</td>
</tr>
<tr>
<td>8</td>
<td>TSC1</td>
<td>c.2699dupA</td>
<td>65</td>
<td>Insertion</td>
<td>p.Q901Efs3'</td>
<td>Yes</td>
<td>point+CN-LOH</td>
</tr>
<tr>
<td>9</td>
<td>TSC1</td>
<td>c.1802dupC</td>
<td>79</td>
<td>Insertion</td>
<td>p.P602Sfs4'</td>
<td>Yes</td>
<td>point+CN-LOH</td>
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<tr>
<td>10</td>
<td>TSC1</td>
<td>c.935dupA</td>
<td>29</td>
<td>Nonsense</td>
<td>p.Y312*</td>
<td>Yes</td>
<td>point+CN-LOH</td>
</tr>
<tr>
<td>11</td>
<td>TSC1</td>
<td>c.1525C&gt;T</td>
<td>76</td>
<td>Nonsense</td>
<td>p.R509*</td>
<td>Yes</td>
<td>point+CN-LOH</td>
</tr>
<tr>
<td>12</td>
<td>TSC1</td>
<td>c.2695C&gt;T</td>
<td>70</td>
<td>Nonsense</td>
<td>p.Q899*</td>
<td>Yes</td>
<td>point+CN-LOH</td>
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<tr>
<td>13</td>
<td>TSC2</td>
<td>c.4375C&gt;T</td>
<td>50</td>
<td>Nonsense</td>
<td>p.R1459*</td>
<td>No</td>
<td>point no LOH (sporadic SEGA; no other signs of TSC)</td>
</tr>
<tr>
<td>14</td>
<td>TSC2</td>
<td>c.3412C&gt;T</td>
<td>68</td>
<td>Nonsense</td>
<td>p.R1138*</td>
<td>Yes</td>
<td>point+CN-LOH</td>
</tr>
<tr>
<td>15</td>
<td>TSC2</td>
<td>c.2353C&gt;T</td>
<td>12</td>
<td>Nonsense</td>
<td>p.Q785*</td>
<td>Yes</td>
<td>point+CN-LOH</td>
</tr>
<tr>
<td>16</td>
<td>TSC2</td>
<td>c.2221-1G&gt;C</td>
<td>55</td>
<td>Splice</td>
<td>p.L741_splice</td>
<td>Yes</td>
<td>point+CN-LOH</td>
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<tr>
<td>17</td>
<td>TSC2</td>
<td>c.790_791delCT</td>
<td>30</td>
<td>Deletion</td>
<td>p.L264Wfs73'</td>
<td>No</td>
<td>Point, no LOH</td>
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<tr>
<td>18</td>
<td>TSC2</td>
<td>c.903_922delGGCTCTCTGGGGAGGCCCAAC</td>
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<td>Deletion</td>
<td>p.W304Ffs27'</td>
<td>Yes</td>
<td>point+CN-LOH</td>
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<tr>
<td>19</td>
<td>TSC2</td>
<td>c.5227_5244delCGGCTCCGCCACATCAAG</td>
<td>72</td>
<td>In-frame deletion</td>
<td>p.R1743_-K1748del</td>
<td>Yes</td>
<td>point+CN-LOH</td>
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<tr>
<td>20</td>
<td>TSC2</td>
<td>c.1832G&gt;A</td>
<td>65</td>
<td>Missense</td>
<td>p.R611Q</td>
<td>Yes</td>
<td>point+CN-LOH</td>
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<tr>
<td>21</td>
<td>TSC2</td>
<td>c.3526_3527insT</td>
<td>38</td>
<td>Insertion</td>
<td>p.P1176fs</td>
<td>Yes</td>
<td>point+CN-LOH</td>
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<tr>
<td>22</td>
<td>TSC2</td>
<td>c.1513C&gt;T</td>
<td>47</td>
<td>Nonsense</td>
<td>p.R505*</td>
<td>Yes</td>
<td>point+CN-LOH</td>
</tr>
<tr>
<td>23</td>
<td>TSC2</td>
<td>c.3171_3172insA</td>
<td>17</td>
<td>Insertion</td>
<td>p.T1059Nfs109</td>
<td>No</td>
<td>point -no LOH</td>
</tr>
<tr>
<td>24</td>
<td>TSC2</td>
<td>c.268C&gt;T</td>
<td>75</td>
<td>Nonsense</td>
<td>p.Q90*</td>
<td>Yes</td>
<td>point+CN-LOH</td>
</tr>
<tr>
<td>25</td>
<td>TSC2</td>
<td>c.2251C&gt;T</td>
<td>63</td>
<td>Nonsense</td>
<td>p.R751*</td>
<td>Yes</td>
<td>point+CN-LOH</td>
</tr>
<tr>
<td>26</td>
<td>TSC2</td>
<td>c.5227_5244delCGGCTCCGCCACATCAAG</td>
<td>80</td>
<td>In-frame deletion</td>
<td>p.R1743_-K1748del</td>
<td>Yes</td>
<td>point+CN-LOH (sporadic SEGA; no other signs of TSC)</td>
</tr>
<tr>
<td>27</td>
<td>TSC2</td>
<td>c.5168C&gt;A</td>
<td>34</td>
<td>Nonsense</td>
<td>p.S1723'</td>
<td>Yes</td>
<td>point+CN-LOH</td>
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<tr>
<td>28</td>
<td>TSC2</td>
<td>c.3599G&gt;C</td>
<td>57</td>
<td>Missense</td>
<td>p.R1200P</td>
<td>Yes</td>
<td>point+CN-LOH</td>
</tr>
</tbody>
</table>

(Continued)
Due to the scarcity of resected SEGAs, studies to investigate the genetic profile of this tumor type have been restricted to a small number of samples/cases. More specifically, investigation of the presence of a \textit{BRAF} \textsuperscript{V600E} mutation in SEGAs has been limited to four individual studies with controversial results [18, 23, 26, 32].

In the present study, we analyzed the largest SEGA cohort to date, consisting of fifty-eight SEGAs. Amongst the cohort the vast majority of cases (97%) were clinically diagnosed as definite TSC meeting the required criteria [40, 41]. We did not detect the cancer-actionable \textit{BRAF} \textsuperscript{V600E} mutation by direct sequencing or in the MPS analysis in any of the samples tested. Furthermore, there was no evidence for the presence of \textit{KIAA1549-BRAF} fusions in the 6 SEGAs that were analyzed. However, no significant conclusions on \textit{BRAF} fusion mutations in SEGAs could be drawn based on this small sample size (N=6). In the studies that have reported SEGA cases with \textit{BRAF} \textsuperscript{V600E} mutations, only two were diagnosed with definite TSC, while the remaining \textit{BRAF} \textsuperscript{V600E} positive samples were either TSC negative or defined as possible TSC [23, 26]. Altogether, these results suggest that SEGAs derived from patients with TSC, are negative for the \textit{BRAF} \textsuperscript{V600E} mutation [18, 21, 23, 26, 32].

### Table 3B: Summary of results for \textit{BRAF} mutational analysis by MPS in 31 SEGA samples

<table>
<thead>
<tr>
<th>Case (＃)</th>
<th>Gene</th>
<th>Nucleotide change</th>
<th>MAF (%)</th>
<th>Mutation type</th>
<th>Protein change</th>
<th>CN-LOH</th>
<th>Summary</th>
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</thead>
<tbody>
<tr>
<td>25</td>
<td>\textit{BRAF}</td>
<td>c.82G&gt;T</td>
<td>100</td>
<td>Missense</td>
<td>p.G28C</td>
<td></td>
<td>Novel per cBio, not seen in ExAC</td>
</tr>
<tr>
<td>8</td>
<td>\textit{BRAF}</td>
<td>c.31G&gt;A</td>
<td>56</td>
<td>Missense</td>
<td>p.G11S</td>
<td></td>
<td>Seen once in an hepatobiliary cancer (cBio), not seen in ExAC</td>
</tr>
</tbody>
</table>

Additionally, our results indicate that \textit{TSC1}/\textit{TSC2} alterations, including CN-LOH, are nearly universally present in SEGAs, consistent with \textit{TSC1}/\textit{TSC2} molecular findings seen in other TSC-related tumors e.g. renal angiomyolipomas (AMLs) and lymphangioleiomymatosis (LAM) [42]. \textit{TSC2} LOH has also been reported in sporadic renal and hepatic AMLs as well as sporadic perivascular epithelioid cell tumors [42,43]. Conversely, TSC1 mutation and LOH is rare in angiomyolipoma and perivascular epithelioid cell tumors [42-45]. In contrast TSC1 mutations and LOH were relatively common in this series, seen in 10 of 34 (29%) and 9 of 34 (26%), respectively. Regarding the 5 SEGA cases in which no definite small mutation was identified, there are several possible causes. First the DNA quality of many SEGA samples was poor, limiting the sensitivity of the MPS analysis. In particular large genomic deletions may have been missed in this analysis, and are relatively common in \textit{TSC2} [42].

Consequently, the mechanism of MAPK/ERK and AKT pathway activation in SEGAs [27–31] is uncertain, and further investigation is required.
Figure 3: Map of TSC1 and TSC2 mutations identified in 10 and 19 SEGA tumors, respectively. Novel variants (n = 9) are in blue font whereas variants previously reported (n = 16) are in black font. Circle colors present different mutation types, as indicated. (A) Map of TSC1 mutations. One TSC1 mutation (p.R509*) was seen in two different tumor samples; a large genomic deletion (deletion of exons 6-23) and a possible TSC1 mutation (c.1-7G>A) are not shown. (B) Map of TSC2 mutations. Two TSC2 mutations differ by a single nucleotide position in the same amino acid (p.R611Q/p.R611W), and hence their circles overlap; one TSC2 mutation (p.R1743_K1748del) was seen in two different tumor samples.

Figure 4: Pie charts demonstrating the TSC1/TSC2 variant types and mutant allele frequencies in the SEGA tumors analyzed. (A) Percentage of subjects with TSC1/TSC2 mutations identified vs. NMI. (B) Different mutation types in the SEGA cohort studied.
MATERIALS AND METHODS

SEGA tumor specimens

SEGA specimens were obtained from the following sites: the Academic Medical Center of Amsterdam, the University Medical Center Utrecht, University Medical Center Groningen, University Hospital Erlangen, University Hospital Münster, Medical University of Vienna, Children’s Memorial Health Institute in Warsaw, Meyer Children’s Hospital in Florence, Hacettepe University in Ankara, and the University Hospital de Santa Maria (CHLN) University Hospital de Santa Maria (CHLN) in Lisbon. Fifty-eight SEGAs and one SEN were available from 58 patients of which 56 met standard diagnostic criteria for TSC (Table 1) [40, 41]. Specimens were obtained and used in accordance with the Declaration of Helsinki and this study was approved by the Medical Ethics Committee of each institution.

Histopathological evaluation

Tissue was fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded tissue was sectioned at 6 μm, mounted on organosilane-coated slides (Sigma, St. Louis, MO, USA) and stained with hematoxylin-eosin (HE) for the morphological evaluation. Histological diagnosis was performed according to the 2016 WHO classification of the central nervous system [33]. Sections of the most representative paraffin-embedded specimen of each case were used for additional immunocytochemical staining, as previously reported [34, 35]. The following antibodies have been used: glial fibrillary acidic protein (GFAP; polyclonal rabbit, DAKO, Glostrup, Denmark; 1:4000; monoclonal mouse; DAKO; 1:50), microtubule-associated protein (MAP2; mouse clone HM2; Sigma 1:100), anti-human leukocyte antigen (HLA)-DP, DQ, DR (mouse clone CR3/43; DAKO; 1:100), CD3 (mouse monoclonal, clone F7.2.38; DAKO; 1:200; T-lymphocytes), phospho-S6 ribosomal protein (Ser235/236; pS6, rabbit polyclonal, Cell Signaling Technology, Beverly, MA, USA; 1:50) and Ki67 (mouse clone MIB-1, DAKO, Glostrup, Denmark. 1:20) were used in the routine immunocytochemical analysis of tumor specimens to document the presence of a heterogeneous population of cells and the activation of the mTORC1 pathway. After washing in PBS, sections were stained with a polymer based peroxidase immunocytochemistry detection kit (BrightVision Peroxidase system, ImmunoVision, Brisbane, CA, USA). Signal was detected using the chromogen 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich, St. Louis, MO, USA).

DNA extraction and BRAF^{V600E} mutation analysis

DNA was extracted from both FFPE (n=44) and frozen (n=14) SEGA tumor samples. Since SEGA often display intratumoral hemorrhages, areas of representative tumor (identified on hematoxylin & eosin stained sections) were selected for cases in which hemorrhages, were observed within the FFPE SEGA tissue samples (n=44). Tumor DNA was extracted from 10-μm-thick paraffin sections using BiOstic FFPE Tissue DNA Isolation kit (MO BIO) according to the manufacturer’s instructions. From frozen tissue samples (N=14) DNA was recovered from the organic phase following QIAzol (Qiagen) extraction of RNA and was further purified using QIAamp DNA mini Kit (Qiagen). PCR amplification for the entire extent of exon 15 of BRAF including codon 600 was performed as previously described using primers TCATAATGCTTGCTCTGATAGGA and GGCCAAAAATTTATACGTTGGA [26]. Purified PCR products were sequenced by the Sanger method using the Big Dye Terminator Cycle Sequencing Kit (PerkinElmer Biosystems, Foster City, CA, USA).

KIAA1549–BRAF gene fusion

Six SEGA tissue samples were tested for KIAA1549–BRAF fusions in a diagnostic setting. Total RNA was extracted from frozen tissue samples using miRNeasy mini kit (Qiagen) according to the manufacturer’s instructions. One microgram of total RNA was reverse-transcribed into cDNA, followed by PCR using primer sets corresponding to different KIAA1549–BRAF fusion genes and the PBGD and B2M reference genes (Table 2). PCR products were analyzed on a 2% agarose gel. Pilocytic astrocytoma tissue containing the KIAA1549–BRAF fusions was used as a positive control. Additionally, tonsil tissue known to lack the KIAA1549–BRAF fusion genes was used as a negative control.

TSC1/TSC2 mutation and LOH analysis of SEGAs

In 3 cases (fresh frozen samples), targeted MPS was performed using a HaloPlex custom capture array as described previously [46]. In the other 31 cases (24 FFPE and 7 fresh frozen samples), targeted MPS was performed using a customized gene bait set (Agilent platform) designed in the Kwiatkowski lab that covers the entire TSC1 and TSC2 genes including 10 kb upstream and downstream and all coding exons and introns. This bait set also covered all coding exons and adjacent introns of BRAF. MPS was performed according to the following methods. Briefly, DNA was subjected to fragmentation using Covaris sonication to an average size of 250bp. The
fragmented DNA was purified using Agencourt AMPure XP beads and ligated to the dual indexed adaptors for Illumina sequencing. A MiSeq run was performed to quantify each library. Libraries were then pooled in equal mass and captured using the custom baitset using Agilent SureSelect hybrid capture kit. The captured libraries were then sequenced on the either the HiSeq2500 or the HiSeq 3000 instrument.

The sequencing output was de-convoluted into individual sample reads and sorted using Picard tools [47]. Reads were aligned to the reference sequence hg19 from the Human Genome Reference Consortium using bwa [42, 48–50], and duplicate reads were identified and marked using the Picard tools. The alignments were further refined using the GATK tool for localized realignment around indel sites and recalibration of the quality scores was also performed using GATK tools [42, 49, 51]. Mutation analysis for single nucleotide variants (SNV) was performed using MuTect v1.1.4 and annotated by Variant Effect Predictor (VEP) [52, 53]. Insertions and deletions were called using Indel Locator and SomaticIndelDetector tool [42, 54]. MuTect was run in paired mode using a CEPH sample as a normal since normal DNA samples were not available, and a germline variant filter was then applied. Variants were filtered against the 6,500 exome release of the Exome Sequencing Project (ESP) database ExAC (exclude variants seen in more than 3 normal subjects; http://exac.broadinstitute.org), 1000G and GnomAD [55, 56]. Variants represented at >1% in either the African-American or European-American subsets of these reference databases and not in COSMIC > 2x were considered to be germline. Variants found in BRAF were analysed using cBio (http://www.cbioportal.org) and were further assessed for functionality using 3 different in silico prediction tools: PROVEAN (http://provean.jcvi.org), SIFT (http://sift.jcvi.org) and MutationAccess (http://mutationassessor.org) [57–61].

A second approach was used in parallel to analyze the sequence data, with capture of read calls at all positions using SAMtools Pileup, followed by custom processing in Python and Matlab to determine base call frequency at each position in each read orientation. These data were then filtered to eliminate variant calls observed in only a single read orientation, or seen in multiple samples to exclude artifacts derived from the sequencing process. All variants observed at a frequency of >1% were directly reviewed using the Integrative Genomics Viewer, to identify bona fide variant calls and exclude sequencing or alignment artifacts [21, 23, 26]. Potential pathogenic variants seen at frequency > 1% were also examined in the GnomAD database and the TSC LOVD database.

A minimal median read depth of 20x coverage for the coding exons of TSC1 and TSC2 was required for the samples reported here. The median read depth for coding exons of TSC1 and TSC2 was a median of 107 (range 20 – 1120) among the 31 samples.

LOH was assessed using two allele frequencies: 1) at the site of mutation, using Unix grep to precisely quantify mutant vs. wild-type reads for indel mutations; and 2) at all SNPs identified in the TSC1 and TSC2 genes that had a population allele frequency of > 0.05% in the GnomAD database. If either the mutant allele frequency for the mutation was > 55%, or the median SNP minor allele frequency for TSC1/TSC2 was < 40%, this was considered evidence of CN-LOH. LOH was assessed only in the tumor samples; normal brain tissue adjacent to the tumor, was not available.

Abbreviations


Author contributions

EA and AM conceived the study and participated in its design and coordination together with DK. FJ, WS, WD, RC, IB, WP, TS, MF, KK, SJ, JJ, AMB, FG, FS, JP, AM and EA contributed to the collection and selection of tissue samples and/or clinical data. KG, MN and DK performed the DNA analyses including MPS. AB, KG conducted and analyzed most of the experiments supported by RR, JA and DK. AB, KG, JM, AM, EA and DK wrote the paper. All authors read and approved the final manuscript.

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CONFLICTS OF INTEREST

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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