A Novel Splice Site Associated Polymorphism in the Tuberous Sclerosis 2 (TSC2) Gene May Predispose to the Development of Sporadic Gangliogliomas

MICHAEL PLATEN, BRIGIT MEYER-PUTTLITZ, INGMAR BLUMCKE, MD, ANDREAS WAHA, HELMUT K. WOLF, MD, MARKUS M. NÖTHEN, MD, DAVID N. LOUIS, MD, JULIAN R. SAMPSON, MD, AND ANDREAS VON DEIMLING, MD

Abstract. The tuberous sclerosis 2 (TSC2) gene is thought to function as a growth suppressor in sporadic and TSC-associated hamartomas and tumors. Clusters of dysplastic glial cells are a common feature of cortical tubers and subependymal nodules in tuberous sclerosis patients. In an effort to identify TSC2 gene alterations in sporadic gliomas, we detected a novel polymorphism adjacent to the 3′ splice site of intron 4. We evaluated the distribution of this variant allele in a series of 244 patients with glial tumors, including 55 gangliogliomas, 31 pilocytic astrocytomas (WHO grade I), 50 astrocytomas (WHO grades II and III), and 108 glioblastomas (WHO grade IV). The allele distribution in the general population was estimated by examining 381 healthy blood donors. This rare allele appeared in the control population and in the patients with astrocytomas with a virtually identical frequency (8.14%, and 8.20%, respectively). The frequency of the rare allele in gangliogliomas, however, was significantly higher (15.56%; p = 0.024). The fact that both gangliogliomas and cortical tubers in tuberous sclerosis contain neuronal and astrocytic elements and may resemble each other histologically suggests that the TSC2 gene may be involved in the development of these tumors. The rare allele of the TSC2 gene emerges as a candidate for a predisposing factor for the formation of sporadic gangliogliomas.

Key Words: Ganglioglioma; Polymorphism; Risk factor; TSC2 gene.

INTRODUCTION

The tuberous sclerosis complex (TSC) is an autosomal dominant tumor syndrome characterized by multiple tumor-like malformations (hamartomas) and tumors of the skin, viscera, and central nervous system. Common lesions occurring in TSC include cortical tubers, subependymal nodules or giant cell astrocytomas (SEGA), retinal astrocytomas, renal angiomyolipomas, cardiac rhabdomyomas, and facial angiofibromas (1). Brain involvement frequently results in intractable seizures and progressive mental retardation.

Tuberous sclerosis complex is a genetically heterogeneous syndrome that appears in an estimated 1 in 10,000 individuals, approximately 50% of the cases representing new mutations (2, 3). Two disease-determining genes, designated tuberous sclerosis 1 (TSC1) and 2 (TSC2), have been mapped to chromosomal regions 9q34 and 16p13.3, respectively (4, 5). While the TSC1 gene has not yet been identified, the TSC2 gene has recently been isolated (6). The TSC2 cDNA with an open reading frame of 5370 bp encodes a 5.5 kb mRNA transcript, which is abundantly expressed. The TSC2 gene encodes tuberin, a protein with a region of homology to the human GTPase-activating protein (GAP) GAP3 (6). GTPase-activating proteins are known to interact with human Ras proteins, a family of guanine nucleotide-binding proteins with oncogenic activity, and play an important role in the transduction of a variety of proliferation and differentiation signals. The function of tuberin, however, is not yet understood.

Allelic loss in the TSC2 region on chromosome 16p13 has been observed in hamartomas and tumors from tuberous sclerosis patients, indicating that the remaining copy of the TSC2 gene is altered and that the TSC2 gene acts as a growth suppressor in these lesions (7). Furthermore, transfection and expression of exogenous wild-type TSC2 demonstrated growth suppression of Eker Rat renal carcinoma cells (8). In addition, loss of heterozygosity in the TSC2 region has also been found in histologically identical, sporadic hamartomas (9). These findings further raise the possibility that the TSC2 gene is also altered in sporadic (non-TSC-associated) hamartomas.

The diagnostic hallmark of both cortical tubers and subependymal nodules in TSC patients is the admixture of dysplastic astrocytic and neuronal cells. Glial cells tend to occur in clusters and in some areas may be reminiscent of malignant gliomas (10). In some tuberous sclerosis patients, subependymal giant cell astrocytomas probably develop from subependymal nodules (11). These observations raise the hypothesis that the TSC2 gene may also be involved in the formation of glial tumors. Gangliogliomas represent a unique subset of brain tumors composed of neoplastic glial and neuronal elements. These tumors share many histopathological features with cortical tubers, and both entities may be difficult to distinguish, particularly in small and fragmented

From the Institut für Neuropathologie, Universitätsklinikum Bonn, Bonn (MP, BM-P, AW, AvD), Germany, the Institut für Pathologie, Universitätsklinikum Mainz, Mainz, Germany (HKW), the Institut für Humangenetik, Universitätsklinikum Bonn, Bonn, Germany (MMN), the Department of Pathology (Neuropathology) and Neurosurgical Service, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts (DNSL), and the Institute of Medical Genetics, University of Wales College of Medicine, Cardiff, Wales (JRS).

Correspondence to: Andreas von Deimling, MD, Institut für Neuropathologie, Universitätsklinikum Bonn, Sigmund-Freud-Straße 25, D-53105 Bonn, Germany.

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surgical specimens (12). This raises the question of the potential involvement of TSC genes in the formation of gangliogliomas.

In an ongoing effort to evaluate the role of the TSC2 gene in glial and glioneuronal tumors we systematically analyzed the TSC2 gene for structural alterations. In this process we have detected a polymorphism characterized by a single base pair substitution adjacent to the 3’ splice site of intron 4. Here we present data supporting the hypothesis that this polymorphism is a predisposing factor for the development of gangliogliomas.

MATERIALS AND METHODS

Materials

Tumor and blood samples were obtained from 244 patients with gliomas and gangliogliomas treated at the University of Bonn Medical Center (Bonn, Germany), the Hospital Köln-Merheim (Köln, Germany), and the University Hospital Zürich (Zürich, Switzerland). All tumors were reviewed by the same neuropathologists (HKW, AvD, DNL) and graded according to the guidelines of the World Health Organization (WHO) (13). Of the 244 tumors, 55 were gangliogliomas, 31 were pilocytic astrocytomas (WHO grade I), 28 were astrocytomas (WHO grade II), 22 were anaplastic astrocytomas (WHO grade III), and 108 were glioblastoma multiform (GBM). DNA samples from 381 healthy Caucasian blood donors served as controls.

PCR, RT-PCR Analysis and Northern Blotting

DNA was extracted from blood and histologically verified tumor tissue as detailed elsewhere (14). Total RNA was extracted from histologically verified tumor tissue using TRIzol Reagent (Life Technologies) according to the manufacturer’s protocol. cDNA was generated from 1-5 μg total RNA using 200 U SuperScript II reverse transcriptase (Life Technologies), 0.5 μg oligo(dT) (Life Technologies) in a total volume of 20 μl containing 2.5 mM of each dNTP, 0.1 M DTT, 25 mM MgCl₂, 20 mM Tris-HCl pH 8.4, and 500 mM KCl at 42°C for 50 minutes (min). For all reactions a control without reverse transcriptase was performed to exclude DNA contamination. A 222 bp fragment of genomic DNA containing the TSC2 variant was amplified using primers 5’-GGAGATGGTAGTCGCGGTGTC-3’ (located in intron 4) and 5’-CTCCGAGAGCTGAACTTGG-3’ (located in intron 5) for 30 cycles at 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds. A 246 bp cDNA fragment was amplified with primers 5’-GAACCTTCACGAAAAGGCTG-3’ (located in exon 4) and 5’-TTCTATGTCACAGGAGGCG-3’ (located in exon 6) for 32 cycles at 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 50+2 seconds. PCR was performed in an automated thermocycler (GeneAmp, Perking Elmer) in a total volume of 10 μl containing 10 ng DNA or cDNA, 50 mM KCl, 10 mM Tris-HCl pH 8.4, 200 mM of each dNTP, 1 U Taq polymerase, 1.5 mM MgCl₂, 0.1% gelatin and 20 μM of each primer.

DNA was isolated from glial cell lines U87MG and A172 and from 2 fresh frozen tumor specimens (one anaplastic astrocytoma WHO grade III and one GMB) using an Oligotex® direct mRNA Kit (Qiagen) protocol. Two μg of mRNA were separated on a 1% denaturing agarose gel and transferred to a nylon membrane by capillary action. A TSC2-specific cDNA probe spanning exons 4 to 8 was synthesized with primers 5’-GGAAGATGGTAGTCGCGGTGTC-3’ and 5’-GTCTATGTCACAGGAGGC-3’ using a PCR DIG Probe Synthesis Kit (Boehringer). Hybridization was performed under standard conditions (14). Membranes were visualized by enzyme-immunostaining using a DIG-Nucleotide Detection Kit (Boehringer).

Single Strand Conformational Polymorphism (SSCP) Analysis and Direct Sequencing

PCR products were electrophoretically separated (20 W and 15°C) on large nondenaturing gels containing 12% polyacrylamide (29 acrylamide/1 bisacrylamide), 5% glycerol, and 1× TBE buffer. The gels were visualized in a specialized device (15) using a silver stain protocol (16, 17). PCR products were analyzed by direct sequencing on a semi-automated sequencer (373, Applied Biosystems) using a Taq cycle sequencing kit (ABI, PRIMEd, Perkin Elmer) and the primer sets described above according to the manufacturer’s protocol.

Restriction Assay

Exon 5 of TSC2 was amplified and digested with 10 U of PvuII (Boehringer Mannheim). The frequent allele contains a PvuII restriction site in the intron 4/exon 5 boundary, creating 2 fragments of 174 bp and 48 bp. In the rare allele, the PvuII site is abolished by the CAG to TAG substitution in the 3′ splice site of intron 4. Following PvuII restriction, the DNA fragments were electrophoretically separated on 2% agarose gels and visualized with ethidium bromide.

RESULTS

Detection of a Polymorphism Adjacent to the Intron 4/Exon 5 Splice Site of TSC2

Initially, 60 glial tumor specimens were analyzed by SSCP analysis. An additional fragment with altered migration was observed in 16 samples. One sample exhibited the altered fragment in absence of the usual single strands, indicating homozygosity for this variant (Fig. 1).

Six of these 16 samples were sequenced, and all revealed...
a C to T substitution 3 base pairs 5' to the first coding nucleotide of exon 5. This sequence alteration deletes a PvuII site at the intron 4/exon 5 boundary present in the frequent allele (Fig. 1). All 16 samples of the initial series with altered SSCP migration patterns were analyzed by PvuII restriction and were shown to be missing this site in the variant alleles. Analysis of corresponding leukocyte DNAs was performed to determine the nature of the intron 4 variant. All blood specimens exhibited the same status as the corresponding tumor DNA samples, confirming a polymorphism. Exon 5 of the TSC2 gene contains a cryptic splice site, 15 bp downstream of the regular intron 4 splice site. To determine whether the alteration at the intron 4 splice site results in an alternative splicing of the TSC2 gene at the exon 5 cryptic splice site, we analyzed the exon 4 through exon 6 cDNA sequence in 2 samples homozygous for the TSC2 variant. Both samples revealed normal cDNA sequences indicating no alternative splicing at the intron 4/exon 5 boundary (data not shown). To determine whether the variant at the intron 4 splice site affects the TSC2 message at another site, Northern blotting was performed to analyze transcript size. We compared 2 glioblastoma specimens heterozygous for the intron 4 splice site associated polymorphism with 2 glioblastoma specimens homozygous for the frequent intron 4 splice site associated variant. All cell lines and tumor tissues showed identical TSC2 transcripts migrating as 5.5 kb products (data not shown). In order to establish the allelic distribution, leukocyte DNA from 381 healthy blood donors was analyzed for the presence/absence of the PvuII restriction site. The 762 alleles parted in 700 alleles containing the PvuII restriction site (allele A1; frequency = 0.919) and 62 without the PvuII restriction site (Allele A2; frequency = 0.081). Two individuals were homozygous for A2.

**TSC2 Polymorphism Adjacent to the Intron 4/Exon 5 Splice Site in Human Brain Tumors**

The brain tumor series was extended to 244 patients. Among 488 alleles the allele frequencies for A1/A2 were 0.902/0.098. Four patients were homozygous for A2. Among 16 patients of astrocytic gliomas, individual tumor types exhibited the following allele frequencies for A1/A2: Pilocytic astrocytomas (WHO grade I), fA1/A2 = 0.903/0.097; astrocytomas (WHO grade II), fA1/A2 = 0.911/0.089; anaplastic astrocytomas (WHO grade III), fA1/A2 = 0.932/0.068; GBM (WHO grade IV), fA1/A2 = 0.921/0.079; gangliogliomas, fA1/A2 = 0.845/0.155. In gangliogliomas, the frequency of allele A2 was significantly higher than in the donor group (p = 0.012 using a two tailed X² test, p = 0.024, corrected for multiple testing). All patients exhibited the identical alleles in tumor and leukocyte DNAs. In none of the tumor specimens from heterozygous patients was loss of heterozygosity observed within the TSC2 locus.

<table>
<thead>
<tr>
<th>Histology</th>
<th>Patients</th>
<th>Allele A1 (FA1)</th>
<th>Allele A2 (FA2)</th>
<th>p</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>Controls</td>
<td>381</td>
<td>700 (0.919)</td>
<td>62 (0.081)</td>
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<tr>
<td>Total astrocytomas</td>
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<tr>
<td>PA I</td>
<td>189</td>
<td>347 (0.918)</td>
<td>31 (0.082)</td>
<td>0.970</td>
</tr>
<tr>
<td>A II</td>
<td>31</td>
<td>56 (0.903)</td>
<td>6 (0.097)</td>
<td>0.672</td>
</tr>
<tr>
<td>A III</td>
<td>28</td>
<td>51 (0.911)</td>
<td>5 (0.089)</td>
<td>0.835</td>
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<tr>
<td>GBM</td>
<td>108</td>
<td>199 (0.921)</td>
<td>17 (0.079)</td>
<td>0.899</td>
</tr>
<tr>
<td>Gangliogliomas</td>
<td>55</td>
<td>93 (0.845)</td>
<td>17 (0.155)</td>
<td>0.012</td>
</tr>
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</table>

PA I = pilocytic astrocytoma (WHO grade I); A II = astrocytoma (WHO grade II); A III = anaplastic astrocytoma (WHO grade III); GBM = glioblastoma multiform (WHO grade IV); fA1 = frequency of allele A1; fA2 = frequency of allele A2. Results from statistical calculations (X² test, two-tailed) are given for comparison of allele frequencies between patients and controls.

The distribution of the A1/A2 alleles was virtually identical for the patients recruited in Switzerland at the University Hospital Zürich (astrocytic tumors, n = 72, fA1/A2 = 0.910/0.090; gangliogliomas, n = 6, fA1/A2 = 0.833/0.167) and for patients recruited in Germany at the Hospital Kölner-Heim and the University Hospital Bonn (astrocytic tumors, n = 117, fA1/A2 = 0.923/0.077; gangliogliomas, n = 49, fA1/A2 = 0.843/0.153). This data is compiled in Table 1.

**DISCUSSION**

The TSC2 gene product tuberin is believed to function as a growth suppressor in TSC-associated and sporadic hamartomas. Allelic losses in the TSC2 region on the short arm of chromosome 16 have been observed in different hamartomas from tuberous sclerosis patients (7) and in sporadic angiomylipomas (9). On the other hand, a lower frequency of allelic losses in brain lesions than in kidney lesions has been reported in TSC patients (18), and a recent study failed to detect such allelic losses in isolated TSC-like cortical hamartomas of patients without the additional stigma of TSC (19). These findings suggest the presence of additional genetic mechanisms in the genesis of cortical tubers and TSC-like lesions.

Cortical tubers and subependymal nodules of TSC patients often contain clusters of dysplastic neurons and/or astrocytes (10, 11). However, this morphological aspect is not unique to TSC-associated lesions, but is frequently encountered in gangliogliomas, a central nervous system tumor with neuronal and glial components. We therefore hypothesized that the TSC2 gene may also be altered in gangliogliomas. Furthermore, TSC patients often develop subependymal giant cell astrocytomas, pointing toward the possibility that this gene is involved in the formation
of gliomas. In an effort to identify alterations in the TSC2 gene in a series of patients with glial tumors including gangliogliomas, we detected a novel polymorphism in the TSC2 gene created by a C to T substitution in the 3′ splice site of intron 4. The allelic distribution was examined on the basis of 762 alleles from healthy blood donors and determined as A1/A2 = 0.919/0.081.

To our surprise, this polymorphism had a different distribution within our patient series. The frequency of the A2 allele in a population of 55 patients with gangliogliomas was significantly greater (fA2/ganglioglioma = 0.155; p = 0.012; p = 0.024, corrected for multiple testing) than in the control population. The frequencies of A1/A2 in purely astrocytic gliomas and the blood donor group, however, were virtually identical. The accumulation of the A2 allele in the ganglioglioma population may indicate that this variant is a risk factor for the development of gangliogliomas. This hypothesis may find support in some clinical and pathological features shared by patients with ganglioglioma and TSC patients. Gangliogliomas frequently occur in patients with focal seizures, a clinical trait observed in most patients with TSC (20). Both TSC-associated tubers and gangliogliomas are believed to arise from a single stem cell differentiating along both glial and neuronal cell lines (21–23). Furthermore, some gangliogliomas are associated with glioneuronal malformations (12), suggesting that gangliogliomas may originate from these malformative lesions through neoplastic transformation.

The highly conserved nucleotide sequence AG at the 3′ end of introns is essential for the splicing process. Nucleotides just upstream of this sequence are also important in this process. The TSC2 A2 allele is characterized by an exchange of C to T nucleotide just adjacent to the highly conserved 3′ end sequence of intron 4. An alteration of this splice site may therefore influence mRNA processing. Alternative splicing of the TSC2 gene has been observed in other regions of the gene (24–26). In order to evaluate whether the alteration of the intron 4 splice acceptor site of the TSC2 gene results in the use of a cryptic splice site in the coding sequence of exon 5, we have amplified and sequenced cDNAs containing this region from brain and leukocytes. The cDNA from patients homozygous for the A2 allele exhibited the identical nucleotide sequence as cDNA from patients carrying two A1 alleles. Northern blotting of RNA from 2 glial tumor specimens heterozygous for A1/A2 and 2 glial cell lines homozygous for the A1 allele revealed identically migrating TSC2 transcripts. This indicates that the intron 4 polymorphism is not associated with alternatively spliced TSC2 transcripts in brain tissue. However, an altered splice site may also influence the efficiency of mRNA splicing. We are currently investigating whether the presence of one or two A2 alleles affects mRNA levels. On the other hand, we cannot exclude the possibility that the polymorphism is not involved directly in the etiology of tumor development but is in linkage disequilibrium with the true functional variant either within the TSC2 gene itself or within an adjacent gene.

In summary, we describe a novel DNA polymorphism associated with the intron 4/exon 5 splice site of the TSC2 gene. The present data may suggest that one allele of this polymorphism constitutes a risk factor for the development of ganglioglioma, a lesion morphologically reminiscent of cortical tubers in patients with tuberous sclerosis. While this genetic association may connect morphological and molecular data, further studies are required to confirm the role of the TSC2 gene in sporadic brain tumors.

**REFERENCES**


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