A novel gene, *LGII*, from 10q24 is rearranged and downregulated in malignant brain tumors

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Loss of heterozygosity for 10q23–26 is seen in over 80% of glioblastoma multiforme tumors. We have used a positional cloning strategy to isolate a novel gene, *LGII* (Leucline-rich gene–Glioma Inactivated), which is rearranged as a result of the t(10;19)(q24;q13) balanced translocation in the T98G glioblastoma cell line lacking any normal chromosome 10. Rearrangement of the *LGII* gene was also detected in the A172 glioblastoma cell line and several glioblastoma tumors. These rearrangements lead to a complete absence of *LGII* expression in glioblastoma cells. The *LGII* gene encodes a protein with a calculated molecular mass of 60 kD and contains 3.5 leucine-rich repeats (LRR) with conserved flanking sequences. In the LRR domain, *LGII* has the highest homology with a number of transmembrane and extracellular proteins which function as receptors and adhesion proteins. *LGII* is predominantly expressed in neural tissues, especially in brain; its expression is reduced in low grade brain tumors and it is significantly reduced or absent in malignant gliomas. Its localization to the 10q24 region, and rearrangements or inactivation in malignant brain tumors, suggest that *LGII* is a candidate tumor suppressor gene involved in progression of glial tumors.

Keywords: tumor suppressor gene; human chromosome 10; gene rearrangements; chromosome translocation; glioma; leucine-rich repeat

Introduction

Multiple genetic alterations are involved in the development and progression of malignant brain tumors. Frequently these events involve the functional loss of a tumor suppressor gene(s) which can occur as a result of either mutation, deletion or as a result of structural chromosome rearrangement. Loss of heterozygosity (LOH) studies, and the identification of consistent chromosome rearrangements, frequently serve to implicate a particular region of the genome in tumorigenesis (Cavenee *et al.*, 1983; Ponder, 1988). Cytogenetic and molecular studies indicate that loss of one complete copy of chromosome 10 is the most common event in high-grade gliomas, presumably exposing a recessive mutation(s) in the remaining allele(s) of a critical tumor suppressor gene(s).

Rearrangement and loss of at least some parts of the second copy of chromosome 10, especially in the 10q23–26 region, has been demonstrated in approximately 80% of glioblastoma multiforme (GBM) tumors (Bigner and Vogelstein, 1990; James *et al.*, 1988; Karlbom *et al.*, 1993; Rasheed *et al.*, 1992). These changes are rarely seen in low-grade gliomas. Extensive analysis performed by several groups has identified at least three distinct regions of LOH on chromosome 10 (Albarosa *et al.*, 1996; Karlbom *et al.*, 1993; Rasheed *et al.*, 1995). This data indicates that multiple genes on chromosome 10 are associated with the genesis and progression of malignant brain tumors. Recently, two candidate tumor suppressor genes, PTEN/MMAC (Li *et al.*, 1997; Steck *et al.*, 1997) and DMBT (Mollenhauer *et al.*, 1997), have been isolated from the 10q23 and 10q25–26 regions, respectively. Mutations and deletions in each of the genes were found in approximately 25% of GBMs (Li *et al.*, 1997; Rasheed *et al.*, 1997; Steck *et al.*, 1997). These observations suggest that there may still be other genes in 10q which are important for tumorigenesis.

Cloning genes interrupted by chromosome translocations has proved to be a useful method for the identification of genes important in tumorigenesis (Clark *et al.*, 1994; Ohta *et al.*, 1996). Using this approach we have recently identified a glioblastoma cell line, T98G, which carries a t(10;19)(q24;q13) translocation and no normal copy of chromosome 10 (Chernova and Cowell, 1998). Retention of only translocated copies of chromosome 10 in these tumor cells suggests that the primary event was whole chromosome loss and that the second event is specific gene inactivation as a result of the structural chromosome rearrangement. To investigate this possibility, we previously identified YAC clones from chromosomes 10 and 19 which crossed the translocation breakpoints in T98G glioblastoma cells (Chernova and Cowell, 1998). Here we describe the isolation and characterization of a novel gene from chromosome 10, encoding a leucine-rich repeat-containing protein, which is associated with this translocation. Normal expression of this gene is highly specific for neural tissue and is absent, or significantly down-regulated, in malignant gliomas but not in low grade gliomas. We have called this gene *LGII* (Leucline-rich gene–Glioma Inactivated).

Results

Isolation of the *LGII* gene

In a FISH analysis of the t(10;19)(q24;q13) rearrangement from GBM cell line T98G (Chernova and Cowell,

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1998), we demonstrated that the non-chimeric, chromosome 10-specific YAC, 912c7, crossed the translocation breakpoint. From an analysis of overlapping YACs spanning this region, and YACs prepared by fragmenting 912c7, it was demonstrated that D10S185 and NIB1686 were the closest proximal and distal markers to the breakpoint, respectively (Chernova and Cowell, 1998). To define the position of the chromosome 10 breakpoint more accurately, a series of somatic-cell hybrids have now been generated between T98G cells and mouse 3T3 cells. PCR analysis of individual hybrid clones identified two hybrids, TGF3 and TGF7, which apparently contained the der(19) and der(10) chromosomes, respectively, but not the homologous normal chromosomes. The presence of these derivative chromosomes was confirmed using FISH with paints for human chromosomes 10 and 19 (data not shown). These hybrid clones were used in all subsequent PCR analyses of the translocation breakpoint and allowed us to determine the presence/absence of individual STS markers on the derivative chromosomes. This analysis allowed us to determine the precise position of the breakpoints relative to the physical map of the region (Figure 1a). We found that D10S185 lies above the breakpoint and NIB1686 lies below the breakpoint. One other marker, IB1099, which has been mapped to YAC 912c7, produced an amplification product which was the same size from both human and mouse DNA and so could not be positioned relative the breakpoint. Both IB1099 and D10S185, however, did map to a 225 kb fragmented YAC, F36 (Figure 1), which was shown by FISH to cross the 10q24 breakpoint (data not shown), indicating that D10S185 and IB1099 lay in the vicinity of the breakpoint.

Figure 1 Summary of the physical map surrounding the 10q24 breakpoint region in T98G cells. In (a) the orientation of individual YACs relative to the STS map of the region is shown. YAC F36 is a subclone from 912c7 both of which cross the translocation breakpoint. BACs 306o10 and 341b18 were isolated with IB1099 and D10S185 respectively, and by FISH 306o10 crosses the breakpoint. In (b) the genomic position of *LGII* gene (shown as a hatched bar), within 306o10, is shown relative to the restriction enzyme map of the region. This analysis shows that cosmid 17/306, which also crosses the breakpoint, contains most of the genomic structure of *LGII*. The selected restriction endonuclease sites are: Bs, *BssHII*; Eg, *EagI*; M, *MluI*; N, *NarI*; Xh, *XhoI*. cen., centromere; tel., telomere

To obtain smaller clones which crossed the translocation breakpoint we used PCR primers for D10S185 and IB1099 to screen a human BAC library and isolated one BAC for each marker (Figure 1). FISH analysis demonstrated that BAC 306o10, which contained IB1099, crosses the breakpoint but that BAC 341b18, which contains D10S185, hybridized exclusively to the (der)10 and so, did not cross the breakpoint (data not shown). FISH analysis of normal chromosomes confirmed that BAC 306o10 localizes to 10q24 and is not chimeric. The IB1099 STS is one of the gene-based STSs which was derived from short sequences of 3' untranslated regions of random, uncharacterized human brain cDNAs (Berry *et al.*, 1995). Homology searches of the EST database using the IB1099 sequence identified three clones from the IMAGE consortium (Lennon *et al.*, 1996). The longest clone, 35825, which was approximately 2.0 kb, was sequenced on both strands. This sequence shows no homologies in the databases. Using the 35825 cDNA as a probe, and a combination of rare cutting restriction endonucleases which recognized CpG-rich sequences, we generated a restriction map of BAC 306o10 and demonstrated that most of the CpG-rich sites cluster within a 30 kb region which also contained the 35825 cDNA (Figure 1b). Pulse field gel analysis of BAC 306c10 demonstrated that it was 170 kb long. To obtain smaller genomic clones, and to confirm the translocation of the *LGII* gene, BAC306 was subcloned into the pWE15 cosmid vector. FISH analysis of cosmid 17/306, which contained the *LGII* gene, showed the presence of specific hybridization signal on both derivative chromosomes (data not shown) demonstrating that this cosmid clone crossed the translocation breakpoint.

Although our primary aim was to isolate the chromosome 10 gene associated with the t(10;19) translocation in T98G cells, we also analysed the translocation breakpoint region on chromosome 19. Previously we demonstrated that the CEPH YAC, 751e12, crossed the t(10;19)(q24;q13) translocation breakpoint in T98G (Chernova and Cowell, 1998). This YAC, however, was clearly chimeric and, in addition, it contained an internal deletion making it unsuitable for use in further analysis. Using the somatic cell hybrid analysis with the PCR primers specific for STSs and genes which had been mapped to the YAC 751e12 region allowed us to position the chromosome 19 breakpoint to a relatively small region flanked by the *ATP4A* and *APLP1* genes (Chernova and Cowell, 1998) according to the chromosome 19 map compiled by Ashworth *et al.* (1995). Using primers from the *APLP1* gene, we isolated BAC clone, 316i22. FISH analysis demonstrated that this BAC crossed the translocation breakpoint and confirmed its location at 19q13.1. Knowing the approximate position of the breakpoint allowed us to use sequence data recently released by the LLNL Human Genome Center for the chromosome 19q13.1 region (unpublished, see www-bio.llnl.gov/genome/genome.html) to create a set of PCR primers for more detailed analysis of the somatic cell hybrids. We found that the breakpoint occurred within a 50 kb region, which is spanned by cosmid F25965 and is flanked by cosmids F24109 and F19541 (Figure 2). Individual primer pairs, which were designed from throughout the region defined by these three cosmids,

were used in a PCR analysis of the somatic cell hybrids, TGF3 and TGF7. This analysis demonstrated that the proximal part of cosmid F24109, including the *U2AF* gene, was present only on the der(19) chromosome and so lay above the breakpoint (Figure 2). The distal part of cosmid F19541, extending from nucleotide 14400, was detected only on the der(10) chromosome and so is translocated from chromosome 19. However, all of the PCR products from: (1) the distal end of cosmid F24109, (2) the entire cosmid F25965 and (3) the proximal portion of cosmid F19541, were detected on both derivative chromosomes, suggesting that duplication of this region had occurred during the evolution of this rearrangement. Although this secondary rearrangement complicated further analysis of the chromosome 19 breakpoint region, we found that one of the consequences of the translocation/rearrangement is the interruption of a hypothetical proline oxidase-like gene (accession number U95090) which was identified in cosmid F19541 as a result of the LLNL sequence analysis. Further analysis of this predicted gene in tumor samples, however, will be required to determine whether it has a role in tumorigenesis.

Expression and structure of *LGII* gene

To analyse the expression profile of *LGII*, clone 35825 was used to probe a Northern blot containing RNA

Figure 2 Cosmid map of the region spanning the 19q13.1 breakpoint in T98G. Cosmids F24109, F25965 and F19541 (accession numbers AD000671, AC002398 and U95090, respectively) are not drawn to scale. The position of the PCR-based DNA markers (filled circles) used to characterize the position of the 19q13.1 breakpoint using somatic cell hybrid analysis is indicated. The genes identified by their DNA sequence are; *U2AF*, alpha-B crystallin and nephrin; X, is a predicted gene similar to proline oxidase

from multiple human tissues (Figure 3a). Transcripts were only detected in brain and skeletal muscle but not in heart, kidney, lung, placenta, liver, or pancreas. In brain and skeletal muscle, two mRNA species were detected; an abundant transcript which was approximately 2.4 kb and a 1.6 kb transcript which was much less abundant. This smaller transcript may represent a splicing variant of the gene. However, using the more sensitive RT-PCR procedure we detected low levels of *LGII* mRNA in heart, liver and pancreas. Analysis of RNA isolated from peripheral nerve showed levels of *LGII* mRNA which were intermediate between that seen in brain and other tissues. From this analysis it seems probable that the *LGII* mRNA detected in skeletal muscle originates from the peripheral nerves present in this tissue. Northern blot analysis of RNA derived from several different regions of human brain revealed a widespread expression of both transcripts, although with different intensity (Figure 3b). The highest abundance was found in total brain, cerebral cortex, hippocampus, and putamen. The lowest expression was detected in corpus callosum. The levels of expression were intermediate in the other regions.

The complete 1.8 kb sequence from the 35825 cDNA and overlapping IMAGE clones, 44117, 43427 and 47189, was established. The compiled consensus gene sequence contained a 1.5 kb open reading frame and a relatively short, 262 bp, 3' untranslated region, although the initiation methionine was still missing (Figure 4). Sequencing data revealed that at least three EST clones, including the 35825 cDNA, contained unspliced introns at their 5'-ends. To obtain the most 5' sequence, an oligonucleotide designed from close to the 5' end of clone 35825 (see primers GTmR in Materials and methods) were used in a 5'-RACE experiment using human brain Marathon-Ready cDNA (Clontech) as a template. The resultant PCR products were cloned into the pGEM-T vector and two clones were analysed. DNA sequencing of the longest clone, 5'RACE, confirmed its overlap with cDNA 35825 (Figure 4) and identified the initiating methionine codon of *LGII*. To obtain a full length cDNA clone, a fetal brain cDNA library was screened, resulting in the isolation of five positive clones. The longest clone hFB6d has a 2.3 kb insert and overlaps with the 5'RACE clones and

Figure 3 *LGII* expression in human tissues. Northern blots, containing poly(A)⁺ RNA (2 µg) from (a) multiple tissues or (b) different brain regions, were probed with the 35825 cDNA insert. Transcripts of approximately 2.4 and 1.6 kb were detected in most brain regions and skeletal muscle. In (b, lower panels) the same filter membranes were reprobed with the beta-actin probe as a loading control

identifies an additional EST sequence, D45285 (see Figure 4), in GDB. Analysis of these clones produced the final 2254 bp consensus cDNA sequence (Figure 5), which was consistent with the size of the *LGII* polyadenylated transcript detected by Northern blot analysis (see Figure 3), suggesting that this represents the full-length cDNA. The complete cDNA sequence, therefore, contains a 1674 bp open reading frame which starts at position 225 with an ATG codon and terminates with TGA stop codon at position 1895 (Figure 5). A polyadenylation site (AATAAA) is located in the 3'-untranslated region, 20 nucleotides upstream of the poly-A tail. Three ATTTA motifs, which are potentially involved in mRNA destabilization (Chen and Shyu, 1995), were also found in the 3'-untranslated region.

The predicted protein sequence produced a 557 amino acid precursor polypeptide with a calculated molecular mass of 64 kD (Figure 5). The protein amino terminus contains a stretch of hydrophobic amino acids which represents a signal peptide with a predicted cleavage site between Gly34 and Lys35. The molecular mass of the mature *LGII* protein, after the cleavage of the signal peptide, is 60 kD. Another hydrophobic segment (amino acids 288–309) represents a putative transmembrane domain. These structural features indicate that *LGII* is a potential transmembrane protein with the amino terminus located outside the cell. A search for motifs inside the gene revealed the presence of three and a half tandemly arrayed leucine-rich repeats (LRRs) in the amino-terminus of *LGII*. These LRRs are flanked on both sides by conserved cysteine clusters. The LRRs in *LGII* consist of 24 amino acids and have a strong homology (Figure 6) to the LRRs of the slit, tall, osteomodulin, biglycan (38–41% identity and 58–67% similarity over LRR region) and other small proteoglycans, which belong to the 'F-20' family of the LRR-containing proteins (for review see Buchanan and Gay, 1996; Kobe and Deisenhofer, 1994). These proteins are characterized by the conservation of a phenylalanine at position 20 in the LRR. The presence of cysteine clusters flanking the LRRs is highly conserved in these transmembrane and extracellular proteins (Figure 6c,d) which function as adhesion proteins and receptors. Two potential N-glycosylation sites are located in the C-terminal flanking region (Asn192) and upstream of the transmembrane region (Asn277). The cytoplasmic portion of *LGII* revealed no

homology to known proteins. However, there are potential phosphorylation sites for cyclic AMP-dependent protein kinase (Ser313), tyrosine kinase

Figure 4 Relationship between the individual *LGII* cDNA clones (identified by their IMAGE #) used to compile the full length sequence. The position of the open reading frame (ORF) and IB1099 STS used to isolate the gene are shown. hFB6d is the longest cDNA clone isolated from a human fetal brain cDNA library; 5'RACE is the longest cDNA we obtained from RACE and D45285 is an EST derived from a cerebral cortex cDNA. The broken lines at the 3' ends of three cDNAs indicates the presence of intronic sequences

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GATCCCTCCCATCTCAGTAACCTCAGGTCCTCTCCCGAGCAGTTCCTGCTGAG 60
CGAGGAGAGCTCAGCAATCAGCTCAGGTCCTCTCTTTGAAAAAGCAGATACAGG 120
CAGAGGAAGGGGGTGGACTCTCTATGTCAGCTGCTCTAGAGCAGCAATCAGCATCTGA 180
ATTCCAGAGGCGCTGTCATGGTGGGGATTTTCTCGACTGCATGAATCAGAAAGAA 240
M E S E R
GCAAAAGGATGGGAATGCTGCAATCCCTGAAAGAAATGCTTATTTCTATGCTCT 300
S K R M G N A C L P L K B T A Y F L C L
TATCTGCGCTTTTGTCTGACTGAGGGGAGAAACCGAGAACCAAAATGCGCTGCTGT 360
L S A L L L T E G K K P A K P K C P A V
GTACTGTACCAAGATAATGCTTTATGTCAGAAATGCGCAATCCATCCAGCAGCGTTC 420
C T C T K D N A L C E N A R S I P R T V
CTCTGATGTTATCTCATTTATCTTTGTGAGATCTGGTTTACTGAAATCTCAGAGGGA 480
P P D V I S L S F V R S G F T E I S E G
GTTTATTTCAGCCATCGCTGAGCTCTTGTTTATTCATCATGCACTCTTTGATGIGA 540
S F L F T P S L Q L L T F T S N S F D V
TCAGTGATGTTGCTTTTATGCTCTTCCATCTACAGTATTTATTCATAGAAACACA 600
I S D D A F I G L P H L E Y L F I E N N
ACATCAAGTCATTTCAAGACATCTCTCTCGGAGCAATTAATTCATTTCTGAGCC 660
N I K S I S R H T F R G L K S L I H L S
TTGCAAAACACATCTCCAGACATCCCAAAAGATTTTTCAGAGGCGTGGATTCCTTAA 720
L A N N N L Q T L P K D I F K G L D S L
CAATGTGTCAGCTGAGGGTAAATTCATTTATGTCAGTAAATGCTGCTAGTGG 780
T N V D L R G N S F N C D C K L K W L V
AATGGCTTGGCCACACCAATGCACTGTTGAAGACATCTACTGCGAGGCGCGCCAGAT 840
E W L G H T N A T V E D I Y C E G P P E
ACAAGAAGCGCAAAATCAATAGTCTCTCTCTGAGGATTTGATTCATTTATTCAGAT 900
Y K K R K I N S L S S K D F D C I I T E
TTGCAAACTCTCAAGACCTGCTTATCAATCATTTGTCATAGACATCTTTTCTTATTTGA 960
F A K S Q D L P Y Q S L S I D T F S Y L
ATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT 1020
N D E Y V V I A Q P F T G K C I F L E W
ACCATGTGGAAGAGCTTCCGAAATTTATGACACATTTACAGGCATCATCTGATGAT 1080
D H V E K T F R N Y D N I T G T S T V V
GCAAGCTTATGATCAATGAACTCAGCTCTATGTCAGTGGCCAGCTGTTTGGTGGCT 1140
C K P I V I E T Q L Y V I V A Q L F G G
CTCAGCTTATTAAGCGAGCAGTTTTCGAATTAATTCATTAATAAATCAGGATTTGAAA 1200
S H I Y K R D S F A N F I K I Q D I E
TTCTCAAAATCCGAAACCAATGACATTTAAACATTTCAAGTTGAAACCACTGGTACT 1260
I L K I R K P N D I E T F K I E N N W Y
TTGTGTTGCTGACAGTTCAAAAGCTGGTTTACTACCATTTACAAATGGAACGGAAAG 1320
F V V A D S S K A G F T T I Y K W N G
GATTTCTACTCCCTCATCTTACAGCGGCTGTTACAGGACACTGATGTCGATATCTAG 1380
G F Y S H Q S L H A W Y R D T D V E Y L
AAATGATCAGAACCTCAGACATCTCAGAACCGCTCTTAATTTCTGCTAGTGTATTOC 1440
E I V R T P Q T L R T P H L I L S S S
AGCGTCTGTATTTATCTAGTGAACCAAGCAGCAATTTATCTACTAAGCAACTGACA 1500
Q R P V I Y Q W N K A T Q L F T N Q T D
TTCTTAACCTGAGCAGTGTGACCGAGTGAAGCTCTCTCAGTGAAGGGGAGGTGAT 1560
I P N M E D V Y A V K C F I T S V G K D V Y
TTTGCTTGAAGATTTATGTTGTTTCAAGTCTGATGATGATGATGATGATGATGATGAT 1620
I C L T R F I G D S K V M K W G G S S F
AGATTTTCAAGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT 1680
Q D I Q R M P S R G S M V F Q P L Q I N
ATTACCAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT 1740
N Y Q Y A I L G S D Y S F T Q V Y N W D
CAGAGAAGCAATTTGTGAAATTTTCAAGATTAATGTTTCAAGCAACCAAGATCAATCA 1800
A E K A K F V K F Q E L N V Q A P R S F
CACATGTGTCATTAATTAAGGATTAATTTCTTTTGTCTCAGTTTATAGGGAATACAC 1860
T H V S I N K R N F L F A S S F K G N T
AGATTTACAAACATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT 1920
Q I Y K H V I V D L S A *
ATCAGAAATTTTCTCAGTACATGACCGGATGACCAATGCAATGATGATGATGATGAT 1980
CACACTTGCAATTAATGCTTTTCAACATTTGAGATGCTAGAACCAAGCACTAACAGTA 2040
TCTCCATCTTACTGTCAGTCCAGTGAATGAGTGAATGATGATGATGATGATGATGATGAT 2100
TAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT 2160
ACTCCAAAAGAAATTTAATGATGATGATGATGATGATGATGATGATGATGATGATGAT 2220
ACTGCCAATAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT 2254

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Figure 5 The human *LGII* cDNA nucleotide and deduced amino acid sequence. The predicted signal peptide is underlined with a broken line; the position of the 3.5 leucine-rich repeats are underlined with a solid line, and the conserved Cys residues in the regions flanking the LRR are indicated in bold. (*), TGA stop codon. A polyadenylation site (AATAAA) and ATTTA-destabilization motifs located in the 3'-UTR are also in bold

Figure 6 A schematic representation of the various domains of the *LGII* protein is shown in (a). N-flank and C-flank indicate the cysteine-rich regions flanking the leucine-rich repeats (LRR); TM, transmembrane region. In (b), the comparative alignments of the three LRRs in *LGII* are compared with various other homologous proteins. Comparative alignments for the amino-flanking (c) and the carboxy-flanking (d) regions, relative to other LRR-containing proteins, are also shown. The most conservative, consensus residues are shown in gray boxes. 'a' represents aliphatic residues such as L, I, A, V, F, Y and M. The consensus sequences have been reported previously (Buchanan and Gay, 1996; Kobe and Deisenhofer, 1994)

(Tyr384), and several sites for PKC and casein kinase II. From the results of this structural analysis, it became apparent that *LGII* is a novel integral membrane protein belonging to the LRR-protein superfamily.

Analysis of *LGII* in brain tumors

To investigate the frequency of *LGII* rearrangements in glioblastoma cells, we used Southern blot analysis of DNA from seven glioblastoma cell lines (Figure 7a,b) digested with *Xba*I or *Bam*HI. Rearrangements of *LGII* were seen in two (29%) cell lines, T98G and A172. Cell lines U87-MG, U118-MG, U251-MG, U373-MG, and GB-1, showed no abnormal bands. The rearrangement in the T98G cells confirmed that *LGII* is a target for the translocation in these cells. The rearranged fragment in A172 cells was different from that seen in T98G cells. FISH analysis of the chromosomes from the A172 cells, using the cosmid 17/306 which contains *LGII*, revealed an intra-chromosomal rearrangement in 10q, possibly an inversion, which resulted in a more proximal location for *LGII* on the marker chromosome (in preparation). We also examined genomic DNAs from 19

primary GBM and four anaplastic gliomas for rearrangements of the *LGII* gene by blot hybridization. Genomic DNA from 9/23 (39%) tumors, digested with the *Xba*I restriction endonuclease, showed abnormal bands. Representative examples of the rearranged bands are shown in Figure 7c. To determine whether any of the abnormal restriction fragments represent polymorphic variants, we analysed 17 control DNA samples, isolated from blood lymphocytes of normal individuals, using the *Xba*I enzyme. In the majority of these normal DNAs (15/17, 88%), *LGII* cDNA hFB6d recognizes two high molecular weight bands, approximately 10 kb and 13 kb long. Two (12%) of the DNA samples, however, showed an additional 19 kb band suggesting the possibility that these represent polymorphic variants (Figure 7c). The same 19 kb band was also seen in 13% of malignant glioma DNA. Even when the 19 kb band was excluded, however, the various rearranged restriction fragments were still detected in 26% (five GBMs and one anaplastic oligodendroglioma) of tumors, suggesting that they represent tumor-specific rearrangements.

Despite the abundant expression of *LGII* in brain tissue, Northern blot analysis of RNA from glioblastoma cell lines failed to detect any *LGII*-specific transcripts (data not shown) at this level of sensitivity. To investigate whether lower levels of expression could be detected, we used primers specific to different regions of the *LGII* cDNA to perform RT-PCR using total RNA from glioblastoma cell lines and normal brain tissue. To increase the specificity of the RT-PCR analysis, and therefore to avoid amplification from potential contaminating genomic DNA, only PCR primers amplifying across one or two splicing sites were used. PCR products of the expected sizes were generated from normal brain RNA, but not from RNA isolated from T98G and A172 cells (Figure 8). This lack of *LGII* expression suggests that the rearrangements in these cells were responsible for gene inactivation. GBM-specific PCR products were almost undetectable in any of the other glioblastoma cell lines.

We also examined a series of nine low grade gliomas, ten anaplastic gliomas and 15 glioblastoma multiforme by RT-PCR for *LGII* expression. For this, primary brain tumors were cultured *in vitro* for a few passages to enrich for tumor cells. All low grade gliomas (four juvenile pilocytic astrocytomas, two adult astrocytomas and three oligodendrogliomas) and one benign acoustic neuroma (schwannoma) had decreased but clearly detectable expression of *LGII* mRNA when compared with normal brain RNA (Figure 8). In contrast, four/five anaplastic oligodendrogliomas had very low levels of *LGII* expression and four/five anaplastic astrocytomas had no detectable *LGII* transcripts. Similarly, PCR analysis showed that three of 15 (20%) GBM tumors were negative and the other 12 (80%) tumors had very low levels of *LGII* transcripts. Both the absent, or reduced, expression of the *LGII* gene in GBMs and anaplastic gliomas, together with the relatively frequent rearrangements of the genomic sequences and its localization in the region of frequent LOH on chromosome 10 in gliomas, suggests that *LGII* is a target gene for inactivation in malignant brain tumors.

Figure 7 Southern blot analysis of the *LGII* gene in glioblastomas. Genomic DNAs from GBM cell lines and peripheral blood (normal control) were digested with *Bam*HI or *Xba*I and probed with (a) cos17/306 or (b) hFB6d cDNA. In (c), genomic DNAs from primary GBMs (patients 35, 3, 36, 8 and 48) and normal samples were digested with *Xba*I and probed with the hFB6d cDNA. Arrows indicate the restriction fragments observed in normal DNA samples; lane 1 shows the restriction pattern (10 and 13 kb) found in the majority of normal DNA samples, lane 2 shows the rare polymorphic variant indicated by the presence of an additional 19 kb band. Abnormal bands were detected in DNAs from the T98G and A172 cells, and GBM tumors 35, 36 and 48 (indicated by the arrowheads)

Figure 8 Analysis of *LGII* expression in various brain tumors, human tissues and glioblastoma cell lines using RT-PCR followed by agarose gel electrophoresis. The 450-bp GT3-4 product spans the interval between nucleotides 50...509 of the *LGII* cDNA (see Figure 5). A 600-bp RT-PCR product from beta-actin was used for normalization of cDNA levels. Lane 2 contains the sample from tumor CCF4 and is negative. GBM, glioblastoma; AA, anaplastic astrocytoma; AO, anaplastic oligodendroglioma; A, astrocytoma; O, oligodendroglioma; PA, pilocytic astrocytoma; AN, acoustic neuroma; Gen. DNA, human genomic DNA (negative control); M, 100-bp size marker

Discussion

We have used a positional cloning approach to isolate a novel gene, *LGII*, which is interrupted by the t(10;19)(q24;q13) translocation in the T98G glioblastoma cell line. *LGII* was localized to 10q24, a region which is commonly deleted in malignant gliomas (Albarosa et al., 1996; Karlbom et al., 1993; Rasheed et al., 1995) and other malignant tumors (Gray et al., 1995; Herbst et al., 1994; Peiffer et al., 1995). The highest levels of *LGII* expression is seen in different brain regions and at lower levels in peripheral nerve and skeletal muscle. Using RT-PCR, low levels of expression were detected in some other tissues (liver, pancreas, thymus).

We confirmed the rearrangement of *LGII* in T98G cells using Southern blot analysis and also detected rearrangements of the genomic copy of *LGII* in another GBM cell line, A172, and in six primary malignant gliomas. Analysis of chromosomes from A172 revealed a complex intrachromosomal rearrangement of one of the copies of chromosome 10. Because the ability to detect genomic rearrangements using blot hybridization with a cDNA probe depends greatly on a choice of restriction endonucleases used to digest the DNA, the exact frequency of rearrangements in tumors may still be higher.

Analysis of expression in brain tumors demonstrated reduced levels of *LGII* mRNA in low-grade pediatric and adult gliomas and in one schwannoma. In anaplastic gliomas (both, astrocytic and oligodendrocytic lineage) and glioblastoma multiforme tumors, however, *LGII* expression was absent or barely detectable, even using RT-PCR. A similar expression pattern was observed in GBM cell lines. Remarkably, in two glioblastoma cell lines, T98G and A172, in which *LGII* expression was completely lost, structural rearrangements in the *LGII* gene were observed. A limited cytogenetic analysis of primary tumors also identified one glioblastoma, CCF4, in which one copy of chromosome 10 was lost and the other was involved in a t(10;11)(q24-25; q11) translocation (Chernova and Cowell, 1998). These tumor cells were also negative for *LGII* expression (see Figure 8). Although preliminary FISH analysis indicated that *LGII* does not cross the translocation breakpoint in CCF4 (unpublished observations), it seems likely that the relocation of *LGII* close to the pericentric heterochromatin in the centromere of chromosome 11, may affect expression of *LGII* in this tumor. Blot hybridization analysis of DNA from the other glioblastoma cell lines showing

very weak *LGII* expression, however, did not show changes in the restriction pattern. Similar observations of reduced expression in the absence of deletions or mutations have been reported for the *BRCA1* gene in non-hereditary forms of breast cancer (Thompson *et al.*, 1995), the p16INK4/MTS1 gene in a number of tumors (Asamoto *et al.*, 1997; Marchini *et al.*, 1997), and the *DCC* gene in neuroblastoma (Kong *et al.*, 1997). One of the mechanisms of down-regulation of the p16INK4 gene was shown to be due to hypermethylation (El *et al.*, 1997; Myohanen *et al.*, 1998). However, because down-regulation of p16INK4 was also observed in the absence of hypermethylation in some cases (Ryan *et al.*, 1998; Schmidt *et al.*, 1997), it seems that other, as yet identified, mechanisms may also be involved. Further analysis of the promoter region of the *LGII* gene and its methylation pattern will be required to understand the mechanisms of its down-regulation in malignant tumors.

The *LGII* cDNA encodes a predicted 557 amino acid polypeptide. The deduced protein contains a signal peptide and a transmembrane domain, suggesting a cytoplasmic membrane localization of the mature 60 kDa protein. *LGII* contains 3.5 LRRs flanked on both sides by conserved cysteine-rich regions. The LRR structure allows *LGII* to be classified as a new member of the group of extracellular LRR proteins. The presence of the LRRs flanked by the conserved cysteine-rich regions is a structural feature of the extra-cytoplasmic domains of these secreted or transmembrane proteins (Buchanan and Gay, 1996; Kobe and Deisenhofer, 1994). Many of these proteins are involved in control of cell proliferation, differentiation, cell-cell and cell-matrix recognition and adhesion. The known function of the LRR domains is either protein-protein interaction or specific receptor-ligand binding. LRRs have been identified as the high affinity binding sites in the receptors for neurotrophin (Windisch *et al.*, 1995a,b) and gonadotrophin (Braun *et al.*, 1991) as well as in the proteoglycans (Svensson *et al.*, 1995). A number of LRR-containing proteins such as slit, toll, tartan, connectin and the Trk family of proteins are involved in the development and differentiation of the neural system (Chang *et al.*, 1993; Halfon *et al.*, 1995; Meakin and Shooter, 1992; Nose *et al.*, 1997; Rothberg *et al.*, 1990).

The evidence for a glioma-related tumor suppressor gene in 10q24 is now overwhelming. However, the long arm of chromosome 19 also shows frequent LOH in brain tumors, especially oligodendrogliomas and oligoastrocytomas where LOH for 19q markers occurs in 70–80% of tumors (Kraus *et al.*, 1995; von Deimling *et al.*, 1994). The frequency of LOH on 19q in GBM, however, is smaller. Because of this observation, however, the question arises whether the t(10;19) translocation in T98G cells also interrupts a gene on chromosome 19 which may be important for tumorigenesis. The minimal, commonly deleted region has been assigned to 19q13.3 and spans about 1 Mb (Rosenberg *et al.*, 1996). Our cytogenetic interpretation of the translocation breakpoint in T98G cells is that it occurs in 19q13.1 (Chernova and Cowell, 1998) and this has now been confirmed by the molecular studies here, which places it proximal to the commonly deleted region. Unfortunately, even though the position of the

19q breakpoint in T98G is still a provocative observation, the presence of a secondary rearrangements at the site of the breakpoint makes it difficult to establish the exact consequences of the rearrangement at this time. The fact that *LGII* is interrupted by chromosome translocations and is almost invariably inactivated in malignant gliomas, indicates that this gene is probably the most important gene partner in this translocation. This suggestion is further supported by the observation that rearrangements of *LGII* occur in tumor cells which do not show structural rearrangements involving chromosome 19.

Recently, two candidate tumor suppressor genes, *PTEN/MMAC* (Li *et al.*, 1997; Steck *et al.*, 1997) and *DMBT1* (Mollenhauer *et al.*, 1997), have been isolated from the 10q23 and 10q25-26 regions, respectively. *PTEN/MMAC* has been shown to be mutated in 17–24% of primary GBMs, 14% breast cancer tumors, and 25% of kidney carcinomas (Steck *et al.*, 1997). This gene, however, clearly maps outside of the region of frequent LOH in gliomas. The localization of another candidate gene, *DMBT1*, does coincide with the region of frequent LOH in 10q25-26. Intragenic, homozygous deletions in *DMBT1* gene were detected in 23% of GBMs (Mollenhauer *et al.*, 1997). Nevertheless, unlike *LGII*, it is clear that neither of these genes are inactivated in the majority of GBM. In summary we believe that, because *LGII* is localized to the 10q24 region, shows a brain specific pattern of expression, and is inactivated and rearranged in malignant gliomas, it is a strong candidate tumor suppressor gene involved in the malignant progression of glial tumors.

Materials and methods

Tumor samples and cell lines

Primary tumor samples were obtained from an unselected series of patients treated at Cleveland Clinic Foundation. Tumors were graded according to the revised WHO classification (Kleihues *et al.*, 1993). Primary explant cultures were established in MEM containing 10% fetal calf serum, from tumor samples as described elsewhere (Cowell and Franks, 1984). Following outgrowth of the tumor cells, they were passaged four to eight times following a 1:3 split ratio to increase cell numbers and to enrich for tumor cells. Established glioblastoma cell lines were obtained from ATCC, except for GB1 cells, which were a gift from Dr B Barna.

BACs and cDNA isolation

The human BAC library and I.M.A.G.E Consortium (LLNL) cDNA clones (ESTs) were purchased from Research Genetics, Inc. (Huntsville, AL, USA). The BAC library was screened using PCR with primers for IB1099 and D10S185. A human fetal brain cDNA library (Clontech, Palo Alto, CA, USA) was screened by hybridization using cDNA clone 35825 (EST). Inserts from positive clones were subcloned into pBluescript SKII(–) and sequenced. 5'RACE-products were generated using Marathon-Ready cDNA from human brain (Clontech) according to the manufacturer's instructions. The *LGII*-specific primer used in the 5'RACE procedure was GTmR (5'-AGGTCTTGTCACATGGTCC-3'). PCR products were cloned into the pGEM-T vector (Promega, Madison, WI, USA).

DNA sequencing

DNA sequencing of all cDNA clones was performed on both strands using an ABI377 automated DNA sequencer (Applied Biosystems, Foster, CA, USA) by the CCF sequencing core facility. Information for EST clones and expressed sequence tags (dbEST) was obtained from public databases using the network service at the National Center for Biotechnology Information (NCBI). cDNA and predicted protein sequences were analysed using a variety of generally available prediction programs, in particular, BLAST, BLOCK, and TMPred. GenBank accession number for the human *LGII* gene is AF055636.

Expression analyses

Northern blots containing the RNA from the tissues of interest were obtained from Clontech. Hybridizations were carried out at 68°C according to the ExpressHyb-protocol provided by the manufacturer. Expression analyses in brain tumors and cell lines was done by RT-PCR. Total RNA was isolated from primary tumor cultures using the Tryzol reagent (Life Technologies). The first-strand cDNA was generated from total RNA using random primers and Superscript revertase (Life Technologies) according to manufacturer's protocol. A 1/10 fraction of the first-strand cDNA was amplified with *LGII*-specific primers designed from different exons such that they did not generate a PCR-product from genomic DNA. *LGII*-specific primer pair sequences were as follows: GT1 5'-CAT-TGCTGGAGCGAGGAGAA-3' and GT2, 5'-GAGCTG-

CAGCGATGGCGTGA-3'; GT3 5'-CGAACTCCTTT-GATGTGATC-3' and GT4 5'-CTTCGCAGTAGATGT-CTTCA; GT5 5'-GGACCATGTGGACAAGACCT-3' and GT6 5'-CACATCAGTGTCCCTGTACCA-3'. After an initial denaturation (94°C for 3 min), 35 cycles of amplification (94°C for 1 min, 57°C for 30 s, 72°C for 1 min) were carried out, followed by a final extension for 5 min at 72°C. For a normalization control, 1/20 fraction of the same samples was amplified using primers specific for the β -actin cDNA (Raff et al., 1997).

Southern blot analysis

Samples containing 7 μ g of genomic DNA were digested with different restriction enzymes and electrophoresed on 0.8% agarose gels, transferred to nylon membranes, and hybridized with the ³²P-labeled cDNA probes following standard protocols. The hybridization mixture for cosmid 17/306 contained 50 μ g/ml sheared human DNA to compete out repetitive sequences. Hybridization was carried out in 5 $\times$ SSC at 65°C, with a final wash at 0.2 $\times$ SSC at 65°C.

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