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The neural progenitor-restricted isoform of the *MARK4* gene in 19q13.2 is upregulated in human gliomas and overexpressed in a subset of glioblastoma cell lines

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Alterations of 19q13 are frequently observed in glial neoplasms, suggesting that this region harbors at least one gene involved in gliomagenesis. Following our previous studies on structural 19q chromosome rearrangements in gliomas, we have undertaken a detailed FISH analysis of the breakpoints and identified a 19q13.2 intrachromosomal amplification of the MAP/microtubule affinity-regulating kinase 4 *(MARK4)* gene in three primary glioblastoma cell lines. Recent data suggest that this gene is involved in the Wnt-signaling pathway. We observed that the expression of the alternatively spliced MARK4L isoform is upregulated in both fresh and cultured gliomas and overexpressed in all of the above three glioblastoma cell lines. Interestingly, we also found that MARK4L expression is restricted to undifferentiated neural progenitor cells or proliferating glial precursor cells, whereas its expression is downregulated during glial differentiation. Perturbation of expression using antisense oligonucleotides against *MARK4* in glioblastoma cell lines, consistently induced a decreased proliferation of tumor cells. Taken together, these data show that *MARK4*, which is normally expressed in neural progenitors, is re-expressed in gliomas and may become a key target of intrachromosomal amplification upon 19q rearrangements.

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Introduction

Gliomas are the most common primary neuroepithelial tumors that develop in the central nervous system (CNS). Premalignant stages have not been recognized, but the multistep process underlying development and progression of glial neoplasms has been dissected through the analysis of large series of tumors of different malignancy grade (Louis *et al*., 2002). Whether gliomas arise from a glial-restricted progenitor or from a dedifferentiated oligodendrocyte or astrocyte, molecular analysis has shown that specific pathways that control the differentiation of glial precursor cells become disregulated (Nobel and Mayer-Proschel, 1997). Molecular genetic studies have demonstrated that both inactivation of tumor-suppressor genes as well as oncogene amplification are important in the malignant progression of human gliomas (Holland, 2001). In particular, critical genes coding for growth factors, and their receptors (Ekstrand *et al*., 1991; Takahashi *et al*., 1992; Wong *et al*., 1992; Guha *et al*., 1995), as well as genes involved in cell cycle regulation (El-Azouzi *et al*., 1989; Jen *et al*., 1994; Henson *et al*., 1994; Reifenberger *et al*., 1994) are differentially overexpressed or altered in glial tumors. The progression to glioblastoma multiforme (GBM) involves losses of expression of tumor-suppressor proteins on chromosome 10 in a subset of tumors (Mollenhauer *et al*., 1997; Chernova *et al*., 1998; Sano *et al*., 1999). Despite these observations, however, there are still other critical genes that map to other amplified or deleted chromosomal regions, which have not yet been identified (Schrock *et al*., 1994). Of all of the recurrent structural chromosome aberrations that have been described in gliomas, abnormalities involving 19q13 occur in virtually all stages and variants of gliomas, suggesting that genes on this chromosome have a fundamental importance in gliomagenesis and normal glial differentiation (von Deimling *et al*., 1992; Ritland *et al*., 1995). Where deletions have been observed, the minimal deletion interval has been progressively narrowed down to the cytogenetic band 19q13.3, between the D19S412 and STD markers (Rosenberg *et al*., 1996; Smith *et al*., 2000).

We previously described clonal chromosomal rearrangements in three glioblastoma cell lines (Magnani *et al*., 1999) that affected both homologs of chromosome 19. We have now analysed these rearrangements further using FISH mapping of the 19q breakpoints. Using three overlapping cosmids we have identified an

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amplified region in 19q13.2 that lies centromerically to the frequent LOH region in gliomas. The amplified region turned out to contain only MAP/microtubule affinity-regulating kinase 4 (*MARK4*) gene. This gene participates in the Wnt-signaling pathway and is considered a β-catenin/Tcf/LEF-regulated gene (Kato *et al*., 2001). *MARK4* is expressed in two alternatively spliced isoforms: MARK4S, exclusively expressed in the brain, and MARK4L, which results from alternative splicing that leads to exon 16 skipping (Kato *et al*., 2001).

In the present work we show that MARK4L isoform, which is downregulated in normal brain, is upregulated in all gliomas and overexpressed by intrachromosomal duplication in three independent glioblastoma cell lines.

Results

FISH mapping of 19q breakpoints in the MI-4 glioblastoma cell line

Using conventional cytogenetic and FISH analysis we previously reported that the glioma cell line, MI-4, carries several complex chromosomal rearrangements affecting both homologs of chromosome 19 with breakpoints in the pericentromeric 19q region underlying the formation of three derivative chromosomes. One copy of chromosome 19 undergoes a split and translocation to chromosomes 1 and 9, while the other copy carries an interstitial deletion where part of the deleted region has become inserted into a derivative unidentified chromosome (Magnani *et al*., 1999). To characterize the chromosome 19q breakpoints more accurately, we used YACs from the WC19 contig (generated by the Lawrence Livermore National Laboratory) in order to define clones that crossed the translocation breakpoints. YAC 784g9, which maps to 19q13.1 (Figure 1), demonstrated hybridization signal on both the der(19) and der(9), indicating that it spans the 19q breakpoint (Figure 2a). YAC 877f9, which maps more centromerically, gave a fluorescent signal only on der(19), whereas a more telomeric YAC, 896g5, hybridized only to the der(9), indicating that they lie proximally and distally to the breakpoint, respectively (data not shown).

The breakpoints associated with the interstitial q13.2–13.31 deletion on the other copy of chromosome 19 were also analysed, as shown in red in the ideogram in Figure 2. In this case, YAC 857c10 demonstrated a split signal between 19q and a chromosome defined as der(1) by using a whole chromosome paint (WCP) (data not shown), thus defining the centromeric breakpoint position on 19 (Figure 2b). The position of the proximal breakpoint for this deletion was shown by FISH to lie within YAC 202h12. Indeed, this clone gave a split signal on der(1) and der(19(q13.1)) confirming the

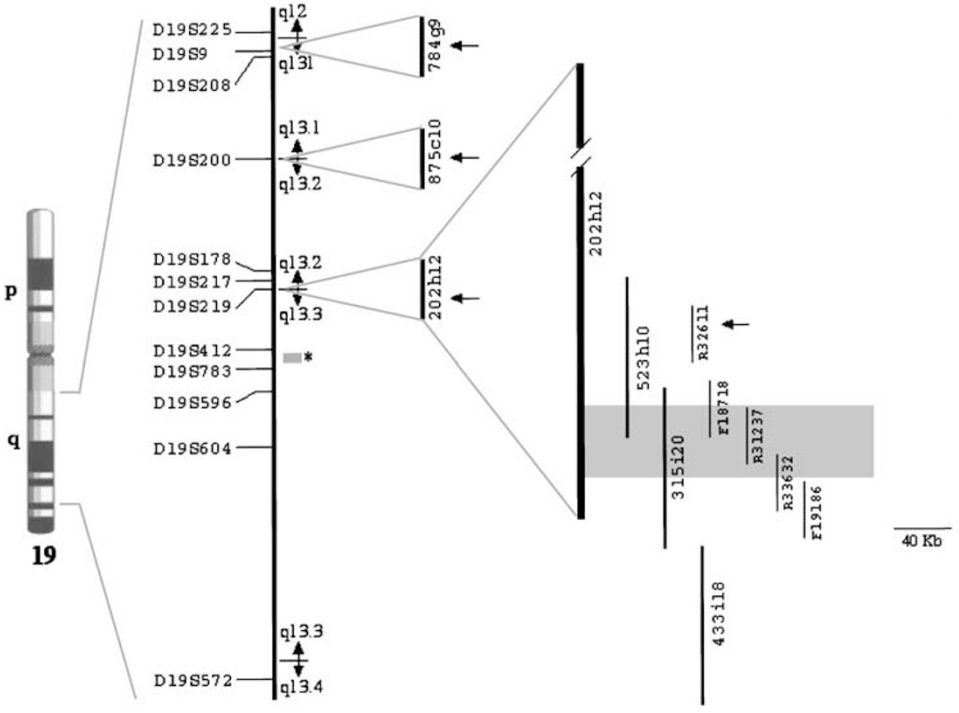

Figure 1 Ideogram of chromosome region 19q12–q13.3. The location of the STS loci is shown on the left. The relative positions of the YACs 784g9, 875c10 and 202h12 are shown on the right of the STS. The positions of the 19q chromosomal breakpoints in the MI-4 glioblastoma cell line are indicated by the arrows throughout and the corresponding genomic clones are highlighted. The region spanned by YAC 202h12 is enlarged on the far right to show the relative spatial orientation of the BACs and cosmids used in the FISH analysis. The shaded box indicates the amplified region mapping to 19q13.2. The dark gray box area (asterisked) indicates the region of common LOH in gliomas. Only those YACs, BACs and cosmids relevant to the characterization of the breakpoint are positioned on the map

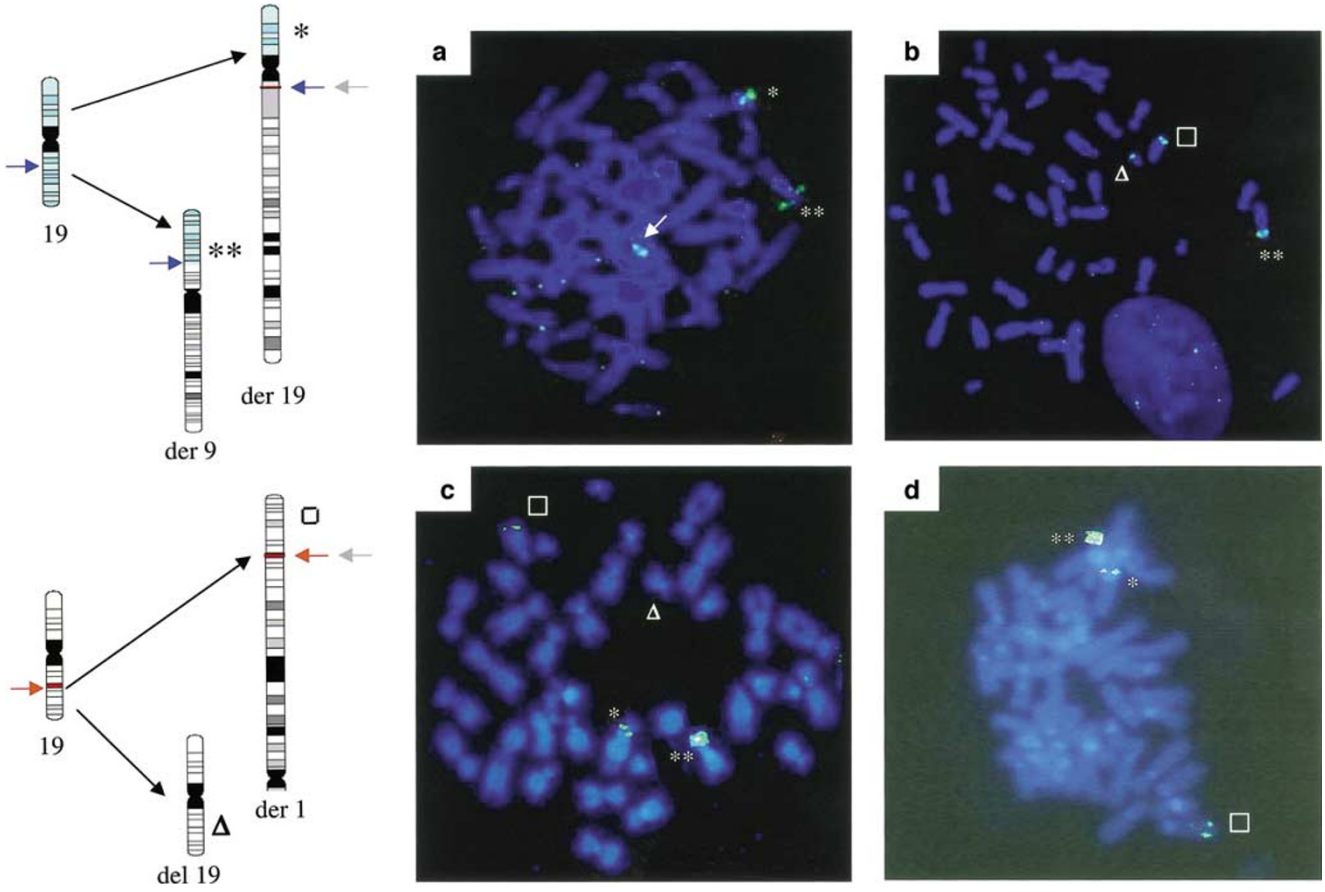

Figure 2 Complex chromosomal rearrangements affecting both 19 homologs in the MI-4 glioblastoma cell line. Ideograms of chromosome 19 indicating the breakpoint positions (arrows). The derivative chromosomes are shown on the right. Different symbols label the rearranged 19q derived chromosomes present in the MI-4 cell line in order to facilitate their identification by FISH in the representative metaphases. (**a**) Yac 784g9 shows two fluorescent signals on der(19) (*) and der(9) (**) indicating that it encompasses the 19q13.1 breakpoint (blue arrow). An arrow indicates the native position of the chromosome 19 not involved in this chromosomal rearrangement. (**b**) Yac 875c10 gives a split signal on 19q- and a recipient chromosome identified as der(1) (□), identifying it as the clone spanning the centromeric breakpoint (19q13.2) of the interstitial 19q deletion (red arrow). A standard fluorescent signal can be seen on der(9). (**c**) Yac 202h12 covering the proximal breakpoint for the deletion (19q13.2) shows a split on der(1) and der(19) that a further rearrangement affected the 19q13.2 region excised from chromosome 19q (light gray arrow). A much more intense signal than expected is visible on der(9). (**d**) Bac 523h10 spanning a telomeric portion of Yac 202h12 reproduces the same FISH pattern giving a split signal on der(1) and der(19) and a highly amplified signal on der(9)

insertion of chromosome material from the homologous 19q13.2–13.31 region in der(19). The signal intensity on der(9), however, was much stronger than expected (Figure 2c). The position of the distal breakpoint of the interstitial q13.2–13.31 deletion remains to be identified. To define the region of 19q that is apparently amplified on the der(9) chromosome more accurately, we identified a series of overlapping BACs spanning the genomic interval defined by YAC 202h12. BAC 523h10 showed an identical hybridization pattern on der(1) and der(19) to YAC 202h12, where again a highly amplified signal was seen on der(9) as compared to that observed on normal metaphase chromosomes (Figure 2d).

Defining the over-represented 19q13.2 interval in the MI-4 cell line

FISH analysis of MI-4 chromosomes using BAC 179k24, which overlaps with BAC 523h10 at the centromeric side, demonstrated a signal of regular intensity on der (9) (data not shown), suggesting that the amplified region involves only the telomeric portion of BAC 523h10. Using BAC 315i20 (Figure 1), an amplified signal was also seen on the der(9) chromosome but no split signal was observed (data not shown). Finally, FISH analysis of BAC 433i18, which overlaps at the very proximal (telomeric) end of b315i20 (Figure 1) showed only normal signal intensities demonstrating that it was not amplified (data not shown). To further refine the extent of the amplified region, cosmid R32611 was hybridized to MI-4 chromosomes and shown to cross the 19q13.2–13.31 breakpoint, although it did not give an enhanced signal (data not shown). Thus, the combined FISH results suggest that the amplified signal is located in a 150 kb region, covered by the contig of cosmids F18718, R31237, R33632 and F19186 (Figure 1). FISH analysis of the R13237 cosmid gave a signal on der(19(q13.1)) and a

duplicated signal on der(9) in all of the metaphases analysed (Figure 3a). A quadruplet of close spots plus an additional spot could be seen on the nuclei as well (Figure 3b), confirming that 19q13.2 amplification is homogeneously present in the MI-4 cell line.

Further refinement of the amplified region was obtained by using the F18718 and R33632 cosmids that overlap both ends of the amplified R31237 cosmid (Figure 1). Both of these clones gave a single signal on both der(19) and der(9) (data not shown). However, in a few metaphases, the signals on der(9) appeared duplicated but with low intensity, suggesting that only a small portion of these probes is included in the 19q13.2 amplicon (data not shown).

Duplication/amplification of 19q13.2. in the glioblastoma cell lines

Since chromosome 19 alterations occur frequently in gliomas, we extended our FISH analysis to other glioblastoma cell lines. Using a whole chromosome 19 paint we identified two other cell lines, MI-7 and GBM, which carried chromosomal rearrangements involving chromosome 19 that had previously escaped cytogenetic detection (Magnani *et al*., 1994; Perego *et al*., 1994). The MI-7 cell line has a modal chromosome number of 59. Within the parental population there are two clones, one with two der(19) chromosomes and another with three or four copies of an apparently normal chromosome 19. The GBM cell line also displays a der(19) in some metaphases together with three or four copies of chromosome 19. Examples of these chromosome 19 abnormalities are shown in the insets of Figure 3c, d and f.

To investigate whether the genomic amplification that was seen in 19q13.2 in the MI-4 cell line was also present in these cell lines we applied the FISH analysis. Using the R31237 cosmid on MI-7 metaphases, a duplicated signal was seen on both the der(19) (Figure 3c) as well as on an iso19q-like chromosome (Figure 3). This latter hybridization pattern was also observed in some metaphases from the GBM cell line (Figure 3e). A subclone of this cell line showed a cluster of fluorescent spots on the derivative chromosome 19 in some metaphase spreads (inset of Figure 3e).

We also hybridized the MARK4 cDNA probe to a Southern blot from MI-4 and GBM cell lines and confirmed the increased dosage of the gene as compared to normal control and the G32 glioblastoma cell line, which is devoid of 19q chromosomal rearrangements (Figure 4c).

Gene content of the contig cosmids R31237 and R33632, and cloning of the alternatively spliced isoforms of human MARK4

Computer-assisted analysis of the DNA sequence from cosmids R31237 and R33632, which centered on the amplified region in glioma cell lines, predicted several potential exons. BLAST analysis showed that these potential exons matched three independent cDNA clones; two partial coding sequences (CDS) isolated from anaplastic oligodendroglioma (AI869600, AI939391), and a complete CDS isolated from normal human brain (Nagase *et al*., 2001). Comparison of the complete 3.6-kb cDNA with the genomic clone-derived sequence revealed the presence of a gene with 18 exons, containing a 688 amino-acid ORF (Figure 4a). The independent cloning of the same gene has been recently described, which was identified as the MAP/microtubule affinity-regulating kinase 1 (MARK4) gene (Kato *et al*., 2001). By using the cDNA isolated from the MI-4 glioblastoma cell line as a FISH probe to normal lymphocyte metaphases, we confirmed that the native location of the MARK4 gene is on chromosome 19q13.2 (data not shown).

A distinctive structural feature of human *MARK4* is the alternative splicing-skipping of exon 16, which leads to a change in the reading frame and the generation of two different transcripts (Figure 4a). One alternatively spliced isoform (MARK4L), which does not include exon 16, is predicted to encode for a 752 amino-acid peptide containing an ELKL-box/KA1 kinase-associated domain in the C-terminal region (Figure 4b). The other isoform, named MARK4S, includes exon 16.

Upregulation and overexpression of MARK4L isoform in glioma

Using RT–PCR we investigated the expression profile of the *MARK4* gene on tumor samples from eight glioma patients, including two OA (Figure 4d, lanes 1 and 2), three AA (Figure 4d, lanes 3, 4 and 8) and three GBM (Figure 4d, lanes 5–7), as compared to whole normal brain (WNB). The MARK4S isoform is prevalent in WNB, as previously described (Kato *et al*., 2001), where the MARK4L isoform, lacking exon 16, can be only detected at low levels. Conversely, all tumors expressed the MARK4L isoform; detection of MARK4S isoform in four cases may be imputed to the admixture of other cell types, which is common in fresh tumors (Figure 4d). Semiquantitative RT–PCR analysis was then performed on 26 glioma cell lines. Reaction conditions and the number of cycles were adjusted so that the reaction fell within the linear range of product amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a gene with constitutive expression, served as an internal control (Fan *et al*., 1998). Results confirmed the exclusive expression of the transcript lacking exon 16 (MARK4L) in all cases with variable transcript levels. Pooled RT–PCR data from the above glioma cell lines, subdivided into WHO grade I and II ($n=10$), grade III ($n=11$) and grade IV ($n=5$), showed expression levels of MARK4L isoform increasing with malignancy (Figure 4e).

We next investigated whether the intrachromosomal amplification of the *MARK4* gene, observed in three human glioblastoma cell lines, might be associated with gene overexpression. *MARK4* expression was evaluated in seven primary glioma cell lines, including five glioblastoma (WHO grade IV), one anaplastic oligoastrocytoma (WHO grade III), and one pilocytic

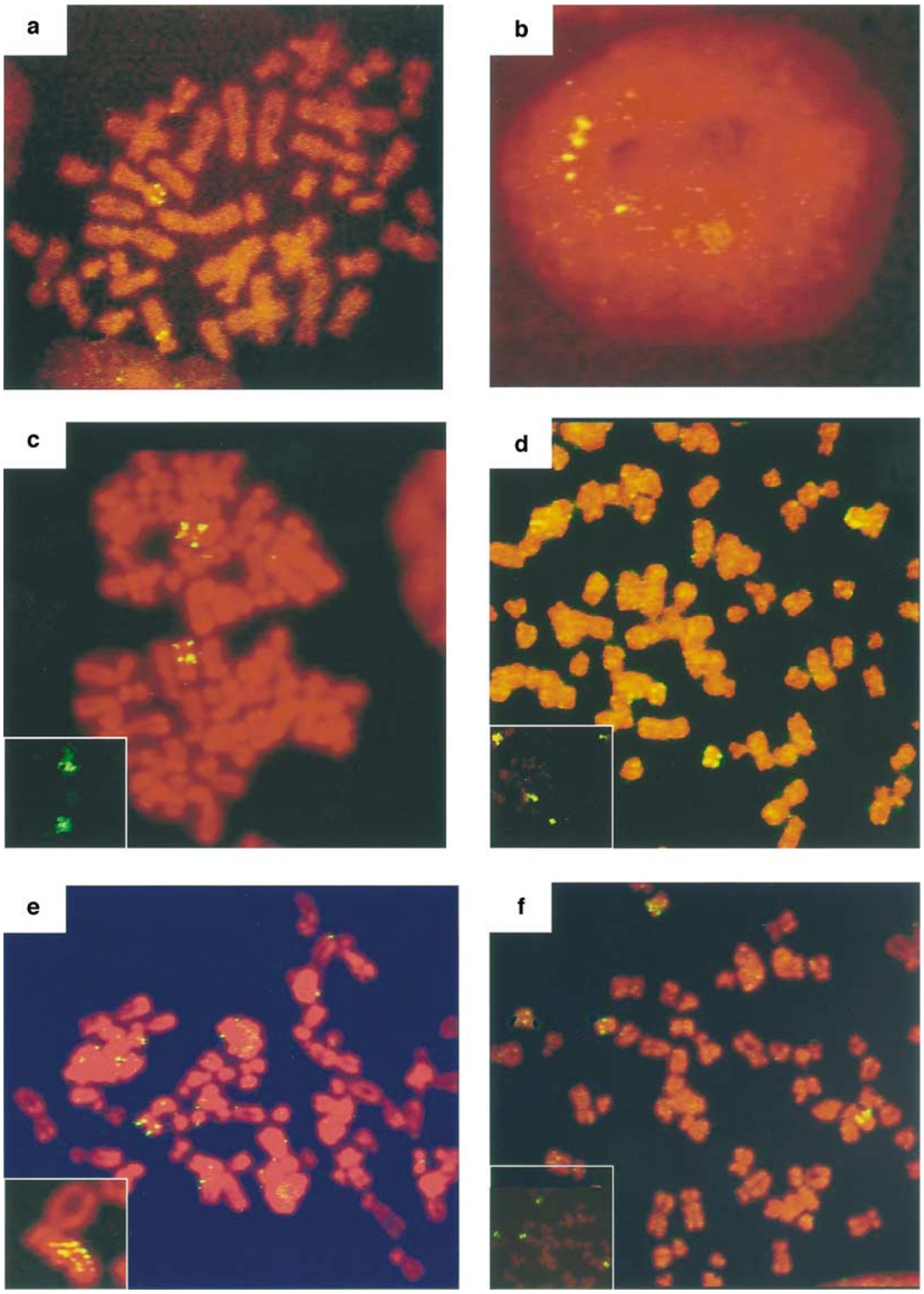

Figure 3 Amplification of the MARK4 gene in MI-4, MI-7 and GBM glioblastoma cell lines. FISH analysis of cosmid R31237 in: (**a**) a MI-4 metaphase counterstained by propidium iodide shows a single signal on der(19) and a duplicated signal on der(9), demonstrating the presence of MARK4 within the translocated and the duplicated 19q13.2 region, (**b**) an interphase nucleus from the MI-4 cell line shows a quadruplet of close signals plus an additional spot, (**c**) in MI-7 metaphases duplicated signals are seen on both of the two der(19) chromosomes, or (**d**) a 19q isochromosome-like marker present together with other three chromosomes 19. The WCP-19 identification of der19/iso19q is shown in the insets (**c,d**). GBM metaphases show duplicated signals on either a 19q isochromosome-like marker (**e**), or a der(19) chromosome (**f**). The inset in **e** shows a large marker chromosome with ladder-like signals, while the inset in **f** shows WCP-19 which identifies the 19 derivation of the marker

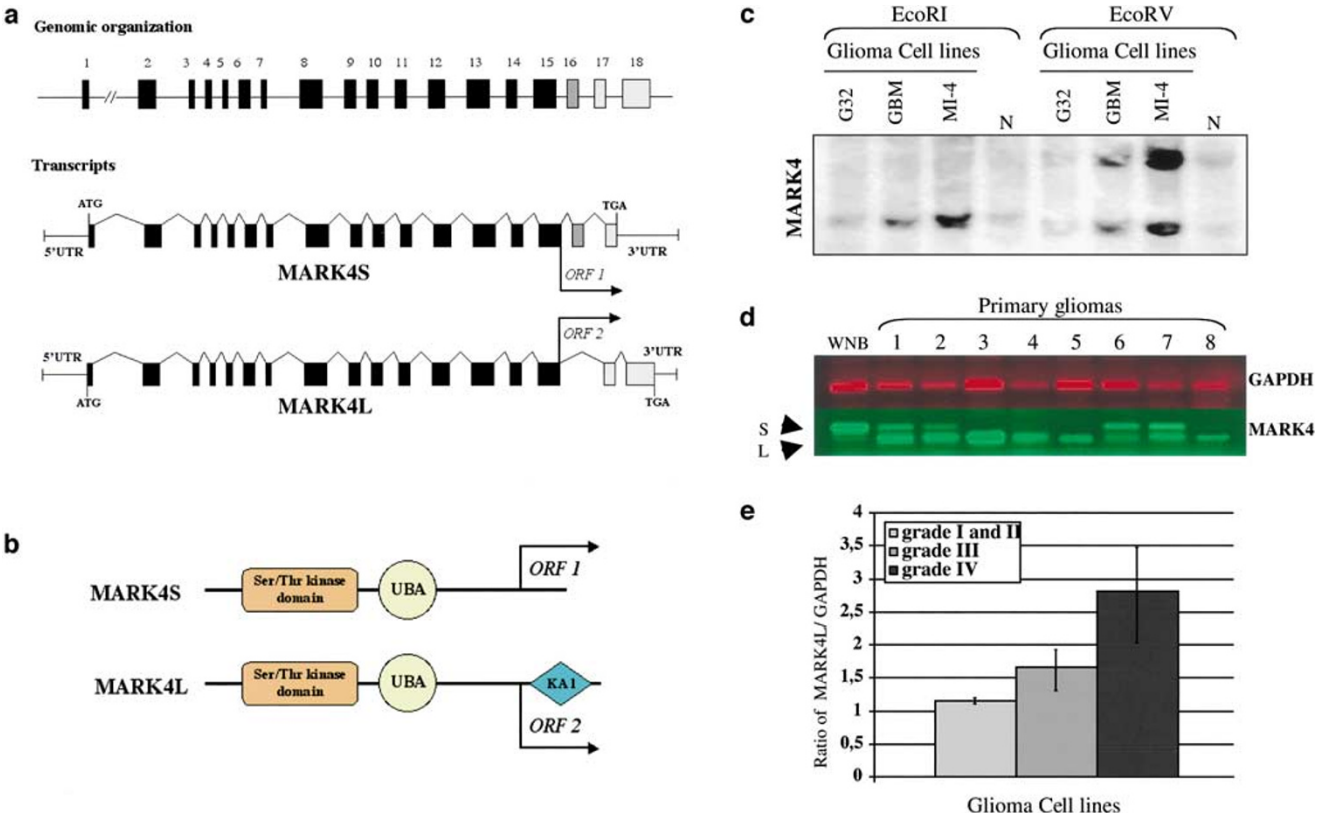

Figure 4 Upregulation of MARK4L in gliomas and amplification in glioblastoma cell lines. (**a**) Intron/exon organization of the MARK4 gene. Solid bars represent constant exonic elements (dark), alternative spliced exon 16 (dark gray) and variable exons with changes in the reading frame (light gray). (**b**) Schematic structure of the two isoforms of MARK4 protein showing conserved serine/threonine kinase, ubiquitin-associated (UBA) and kinase-associated (KA1) domains. (**c**) Southern blot analysis using a cDNA probe spanning exons 1–12 of MARK4. DNA from peripheral blood lymphocytes of a healthy donor served as a normal control (N). MARK4 is clearly amplified in the glioblastoma cell lines MI-4 and GBM. (**d**) Semiquantitative RT–PCR analysis of MARK4 isoforms expression in WNB and human gliomas (lanes 1–8). Total RNA was isolated from WHO IV glioblastoma (lanes 1 and 2), WHO III anaplastic astrocytoma (lane 3), WHO III anaplastic oligoastrocytoma (lane 4). L isoform expression is detectable at variable levels in all gliomas. In WNB only the S isoform is observed. (**e**) Semiquantitative pooled RT–PCR data from glioma cell lines subdivided according to WHO grade into three groups indicated by the light gray ($n=10$), gray ($n=11$) and black ($n=5$) bars, respectively. Results shown are the mean $\pm$s.d.

astrocytoma (WHO grade I), with proliferating astrocyte precursors derived from cultured normal human progenitors (dHNPCs), included as positive control for MARK4L isoform expression. The fluorescent end-labeled products from all these cell lines (Figure 5a) were measured by densitometric analysis and four of them showed increased expression compared to dHNPCs (Figure 5b). The highest levels of *MARK4* expression were detected in glioblastoma cell lines MI-7, GBM, and MI-4 and G32, including all cell lines carrying 19q amplification. Nevertheless, all the remaining cell lines showed definitely increased expression levels as compared to the proliferating control (Figure 5a and b).

Expression of MARK4 mRNA isoforms during glial differentiation from neural progenitor cells

In order to check whether the *MARK4L* isoform upregulated in glioma tumors is normally expressed in undifferentiated or proliferative cells, we established a neural progenitor cell culture.

Normal human neural progenitor cells (HNPCs) were cultured as free floating spheroids (neurospheres) for 40 days (Svendsen *et al*., 1998) (Figure 6a). RT–PCR analysis of HNPC cells showed high expression of the MARK4-L isoform only (Figure 6e). To determine whether MARK4 expression is subject to regulation during glial differentiation, and whether the L isoform is downregulated during terminal differentiation, we plated HNPCs under culture conditions that induce glial differentiation. Neurospheres exposed to medium supplemented with fetal calf serum (FCS) rapidly attached to the plate, where cells with a glial morphology could be observed (Figure 6b). Immunohistochemical analysis of glial acidic fibrillary protein (GFAP; an astrocyte marker; Marsden *et al*., 1983) 2 weeks after addition of FCS showed that these cells were weakly positive (Figure 6c). The same cells were strongly positive for vimentin (Figure 6d) and nestin (data not shown), a pattern restricted to the precursor that generates only astrocytes (Lee *et al*., 2000). At 4 weeks after addition of FCS, the partially differentiated HNPCs (dHNPCs) proliferated to a limited degree

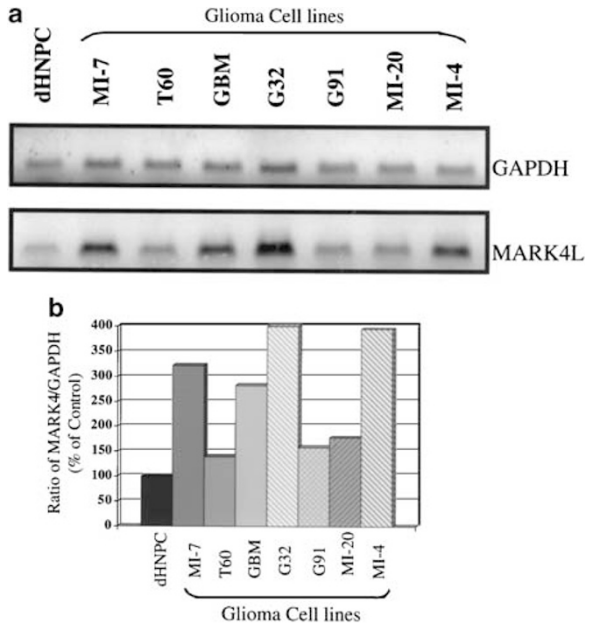

Figure 5 Semiquantitative RT–PCR analysis of the MARK4 gene in glioma cell lines. (**a**) RT–PCR assays were performed on RNA collected from confluent proliferating cells of the seven glioma cell lines indicted and from dHNPCs (HNPCs following 14 days in differentiation conditions), which represent the closest reference normal glial cell counterpart; GAPDH was used as in internal control. (**b**) End-labeled RT–PCR products were quantitated by densitometry and expressed relatively to GAPDH

and showed barely detectable levels of the MARK4L isoform (Figure 6e and f). Thus, the MARK4L transcript was detected at high levels in HNPCs and its expression significantly downregulated upon their differentiation into glia.

Perturbation of MARK4 expression alters the proliferation of glioblastoma cells

To establish whether MARK4 regulates the proliferation capacity of glioblastoma cells, we perturbed its expression in the GBM and MI-7 cell lines, which overexpress it, using antisense oligonucleotides. Following transfection of phosphorothioate sense and antisense oligodeoxynucleotides (ODNs) into the GBM and MI-7 cell lines, only those cells treated with the antisense ODN suppressed MARK4 expression in both cell lines, as determined by RT–PCR analysis (Figure 7a). To assess the effects of inhibition of MARK4 expression on tumor cell growth in both cell lines, we analysed ^{3}H-thymidine incorporation rate using a semiautomated cell harvesting system which was used to lyse the cells and precipitate the labeled DNA 1, 2 and 3 days after transfection. The radioactivity was then counted in a β-counter. Both cell lines treated with antisense ODN showed a significant reduction in ^{3}H-thymidine incorporation into cellular DNA, which indicates a reduction in cell proliferation (Figure 7b and c).

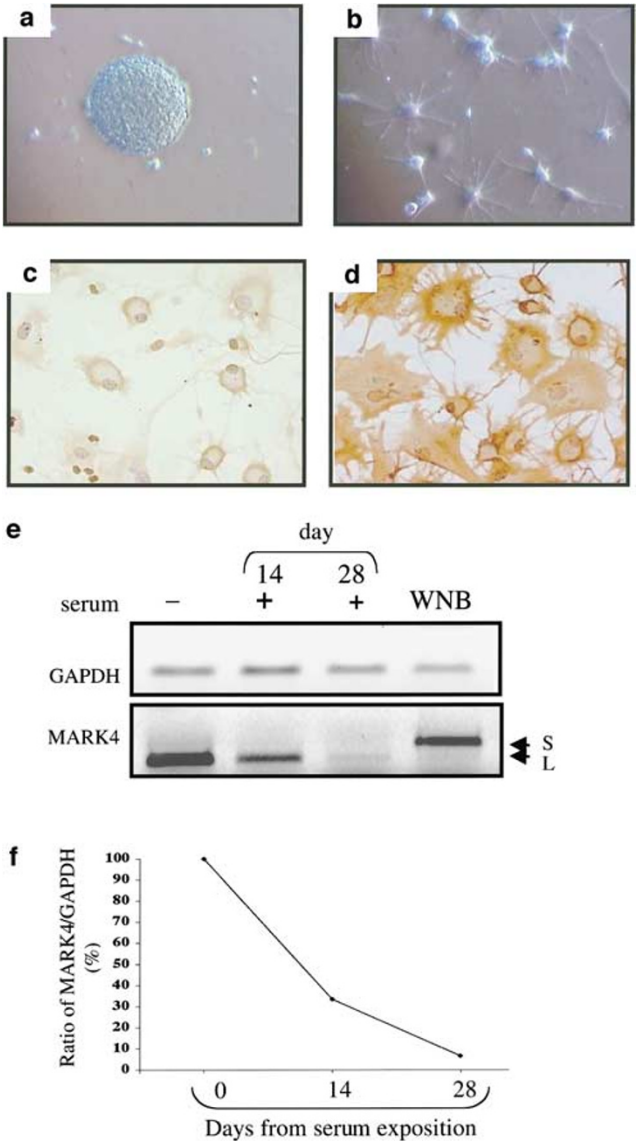

Figure 6 Downregulation of MARK4 expression during glial differentiation. (**a**) Phase-contrast photomicrograph of a primary neurosphere established from HNPCs. (**b**) Phase-contrast photomicrograph of glial-morphology cells originating from HNPCs plated in the presence of 5% FCS and cultured for 14 days (dHNPCs). (**c**) dHNPCs processed for immunohistochemistry demonstrated weak positivity for GFAP and (**d**) strong positivity for vimentin, indicating an early stage of astrocyte differentiation. (**e**) Semiquantitative RT–PCR analysis of MARK4L expression (below) in HNPCs (lane1), HNPCs following 14 (lane 2) and 28 (lane 3) days after plating with FCS and WNB expression (lane 4); GAPDH was used as an internal control (above). (**f**) Quantitative measurements by densitometry of fluorescent end-labelled RT–PCR products, expressed relative to GAPDH, as a percentage of the HNPC value

Discussion

Structural alterations of chromosome 19q are frequently observed in human gliomas (Chernova and Cowell, 1998; Magnani *et al*., 1999; Smith *et al*., 2000; Hartmann *et al*., 2002) and may represent an early oncogenic event

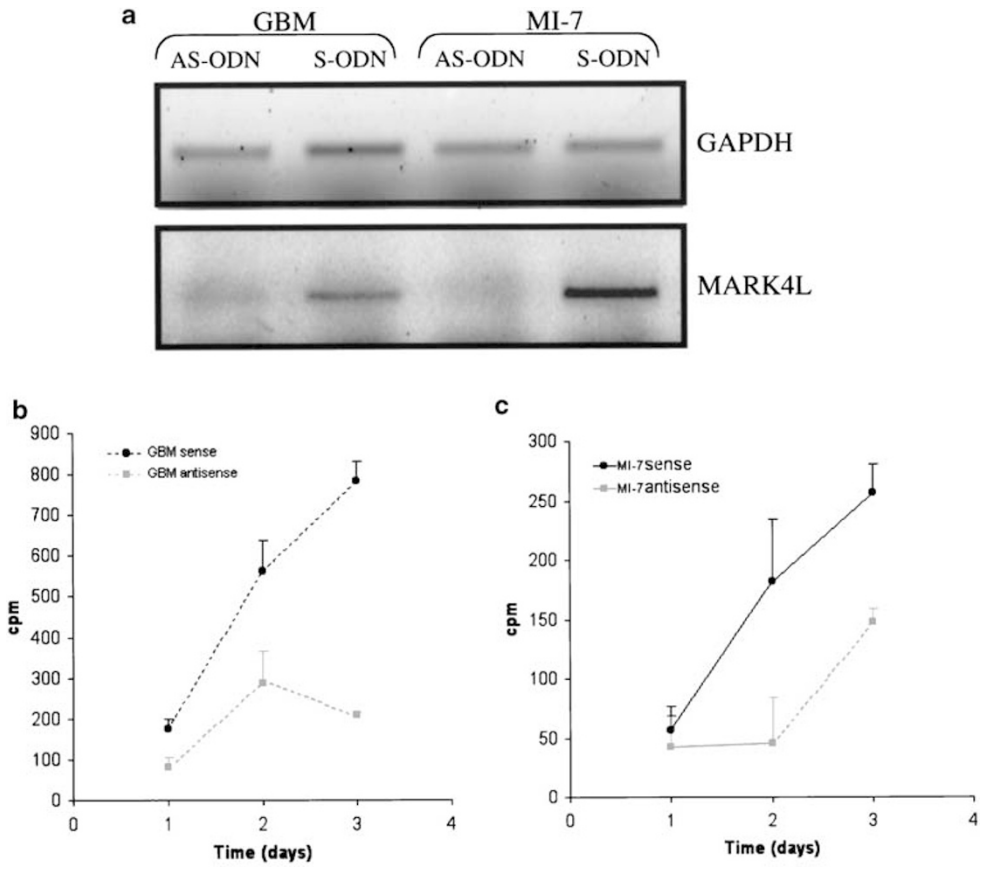

Figure 7 ^{3}H-thymidine incorporation in glioma cell lines MI-7 and GBM. (**a**) RT–PCR assays performed on RNA collected from MI-7 and GBM cell lines after transfections with sense (S-ODN) and antisense ODNs (AS-ODN) show an antisense-specific effect on MARK4 expression, GAPDH was used as an internal control. (**b**) ^{3}H-thymidine incorporation on glioma cell lines GBM and MI-7 after transfections with S-ODN and AS-ODN. A significant negative effect of AS-ODN on proliferation rate is shown as measured by a decrease in the ability of MI-7 and GBM to incorporate ^{3}H-thymidine

in oligodendrogliomas (Bello *et al*., 1995). We have previously identified a recurrent 19q11–12 breakpoint in glioblastoma cell lines that displayed several abnormalities involving chromosome 19q. In particular, the MI-4 glioblastoma cell line contained 19q rearrangements involving both homologs which resulted in the formation of three derivative chromosomes (Magnani *et al*., 1999). In the present study, we mapped by FISH analysis the breakpoints underlying the complex 19q rearrangements in the MI-4 cell line and identified the 19q13.2 band as target of amplification, a feature significantly shared by the MI-7 and GBM glioblastoma cell lines.

Attempts at defining the amplified chromosomal domain led to identify the R31237 cosmid as responsible for the multiple FISH signals. This result shown in all cell lines by a variety of typical chromosomal figures indicates the presence of DNA amplification in forms other than DMs and HSRs that represent the early evidence of gene amplification in gliomas (Bigner *et al*., 1988).

Interestingly the R31237 cosmid turned out to contain only one gene, *MARK4*, which was recently cloned and has been shown to be downregulated in response to decreased Tcf/LEF1 activity. This feature suggests that it may play a role as a messenger in the Wnt-signaling pathway (Kato *et al*., 2001). This pathway is involved in various developmental and oncogenic processes, including the positive regulation of cell cycle progression and the negative regulation of cell cycle exit by the β-catenin/TCF pathway in neural precursors (Roth *et al*., 2000; Satoh and Kuroda, 2000; Megason and McMahon, 2002). To further characterize the expression profile of *MARK4* in both committed neural progenitors as well as postmitotic differentiated cells, we exposed cultured neurospheres to differentiation conditions. A correlation between *MARK4* expression and the rates of proliferation/differentiation was also found in these cells, since constitutive expression in HNPCs was downregulated as a result of differentiation into glial cells. These expression data, together with the suggested role of *MARK4* in Wnt signaling, imply that *MARK4* expression might be associated with G1 to S cell cycle progression of HNPCs. Conversely, loss of expression might correspond to G1 to G0 cell cycle exit of differentiated glial cells. These suggestive findings are supported by two recent lines of evidence showing that: (i) activation of Wnt signaling primarily regulates cell

cycle progression by decreasing the passage through S phase and (ii) other TCF transcriptional targets, rather than cyclins D1 and D2, are involved in the mitogenic response of neural precursors to Wnts (Megason and McMahon, 2002).

Expression results that we obtained on glioma cell lines and primary gliomas showed as a common trend the upregulation of *MARK4* mitogenic-associated isoform, usually switched off in nonproliferating glial cells. The re-expression of MARK4L in tumor cells, normally restricted to undifferentiated or proliferative cells, may imply that MARK4L is necessary to allow glial proliferation.

MARK4 was also found to be target of intrachromosomal amplification leading to MARK4L overexpression in at least three glioblastoma cell lines. To evaluate the growth-promoting activity of *MARK4*, we have perturbed its expression by transfecting antisense ODNs into the glioblastoma cell lines showing *MARK4* amplification and monitored the acquired loss in the proliferation capacity. Given the *MARK4* is a proliferation-associated gene, its overexpression might be linked to selective advantage and clonal expansion. Other mechanisms such as aberrant Wnt pathway activation may also be involved in MARK4L overexpression as suggested by the observation of a fourth overexpressing glioblastoma cell line (G32) not displaying cytogenetic amplification (Zurawel *et al*., 1998; Koch *et al*., 2001). Although overexpression of the *MARK4* gene represents an entirely new pathogenetic mechanism in gliomagenesis, the fact that this is achieved mainly by intrachromosomal amplification, closely resembles for the situation of genes such as *MET* in human gastric carcinoma and more generally of genes residing within fragile sites in a variety of human and experimental systems (Coquelle *et al*., 1997; Hellman *et al*., 2002).

Allelic loss at 19q13.3, a well-documented finding in gliomas (Hartmann *et al*., 2002), may be accounted for by mitotic recombination that may be enhanced in this highly unstable region (Vrieling, 2001). We propose a role for the 19q13 fragile site (Sutherland and Richards, 1995) in driving recurrent breaks responsible to induce *MARK4* amplification accompanied by LOH at loci proximal to the amplification site (Reifenberger *et al*., 1995).

Materials and methods

Cell cultures

The following cell lines: MI-4, MI-7, GBM, G32, T60, MI-20, G91 as well as all the others glioma cell lines used for expression analysis were obtained from biopsy specimens as described elsewhere (Magnani *et al*., 1994; Perego *et al*., 1994). Subsequently, all cell lines were maintained by serial passages in RPMI 1640 medium containing 5% FCS at 37°C in a 5% CO_2 atmosphere. The MI-4 and GBM cell lines were used at 30th passage and all other cell lines within the first 20 passages. Cytogenetic analysis was performed on both fresh tumors and cell lines as described previously. Tumor tissues were mechanically ground, resuspended in complete medium and plated onto coverslips. Chromosomes spreads were set up from cultures harvested before they become confluent (Magnani *et al*., 1994; Perego *et al*., 1994).

Cell lines MI-4 and GBM presented the same stemline karyotype primarily described and MI-7 only presented a sideline of 59 chromosomes when examined by FISH.

HNPCs (Clonetics) were grown in the form of spheroids for 40 days in serum-free neural progenitor cell maintenance medium, BulletKit (Clonetics). Every 14 days the spheres were mechanically dissociated using a Pasteur pipette with a flame-reduced bore size, split 1 : 2 and reseeded into fresh medium. After 40 days, cells were processed for expression analysis of MARK4 gene. Moreover, a T75 flask and few chamber-slides were set up with a suspension of spheres and maintained in NPBMtm medium supplemented with 5% FCS to allow *in vitro* attachment. On day 14, HNPCs were processed for analysis of expression levels of the MARK4L isoform and chamber-slides were used for immunocytochemistry analysis.

Fluorescent in situ *hybridization*

CEPH YAC clones and CITB human BAC clones were obtained from Research Genetics. Chromosome 19 cosmids, LLNL-R32611, LLNL-F18718, LLNL-R31237, LLNL-R33632, LLNL-F19186, LLNL-F15123, LLNL-F10080, LLNL-F13544, LLNL-F20720 and LLNL-R32889 were obtained from the Lawrence Livermore National Laboratory. A WCP1 probe, to identify chromosome der(1), was purchased from Cambio (Cambridge, GB). A MARK4 cDNA probe lacking exon 16 was isolated from HNPCs to establish its endogenous location on normal lymphocyte metaphase chromosomes. YAC, BAC and cosmid extractions were carried out according to standard alkaline lysis procedures (Chernova and Cowell, 1998). Total DNA from the yeast host cell containing the YAC of interest was used as a template to synthesize a human-specific probe from the YAC DNA by using inter-ALU polymerase chain reaction (PCR) as described previously (Chernova and Cowell, 1998). Standard techniques were used to prepare metaphase chromosome spreads from glioma cell lines and lymphocyte cultures. All probes, except wcp1, were labeled with digoxigenin-11-dUTP by using the Dig-Nick translation Mix (Roche). From the labeled probes 200 ng of YAC or BAC DNA, or 350 ng of cosmid DNA were mixed with 25-fold excess of human Cot-1 DNA in a final volume of 15 μl of hybridization solution. For *in situ* experiments with the cDNA probe, we omitted the Cot-1 DNA in the hybridization mix. The FISH procedure, as well as the detection of digoxigenin-labeled probes, has been described elsewhere (Chernova and Cowell, 1998). Chromosome spreads were viewed using a CCD camera (Photometrics) and Smartcapture software (Vysis). The images were edited using HighFISH software (Casti Imaging).

Southern blot hybridization

We used Southern blotting to investigate the status of amplification of MARK4 gene. For Southern blot, 15 μg of genomic DNA from each cell line or from normal lymphocytes were digested with *Eco*RI and *Eco*RV and separated on 0.8% agarose, then transferred onto a nylon membrane (Amersham) with SSC20X o/n. The DNA was crosslinked to the membrane in an oven at 80°C for 8 h. The α^{32}P-labeled probe (including exons 1–12) used for hybridization was prepared by random priming using the Random Primed DNA Labelling Kit (Roche) and then purified on a Nick Column containing Sephadex G-50 (Pharmacia Biotech). Hybridization was performed at 68°C for 2 h with Express Hyb hybridization

solution (Clontech). The blot was then washed twice 20 min in a solution of $2 \times$ SSC/0.05% SDS at room temperature (RT) and twice 20 min in a solution of $0.1 \times$ SSC/0.1% SDS at RT, before exposure to film for 72 h at $-80°C$.

Semiquantitative RT–PCR analysis

Total RNA was extracted from all glioma cell lines using RNAqueous™-4 PCR (Ambion) according to the manufacturer's instructions. The extracted RNA was treated with DNase (Ambion) to exclude false results by DNA contamination. Aliquots of RNA (0.8 μg) were then reverse transcribed using ThermoScript™ reverse transcriptase (Invitrogen). Aliquots of cDNA corresponding to 20 ng of original RNA were used for PCR amplification of MARK4 and GAPDH. GAPDH was amplified as a control, resulting in a 312 bp product with fluorescent-labeled forward (5′-Hex-ACAACAGCCTCAAGATCATCAG-3′) and reverse (5′-GGTCCACCACTGACACGTTG-3′) primers with the following PCR conditions: 24 cycles at, 94°C, 30 min; 57°C, 30 min; 72°C, 30 min. MARK4 PCR, detecting both the MARK4S (363 bp) and MARK4L (263 bp) isoforms, was performed using fluorescent-labeled forward (5′-Fam-CTGACCTCCAAACTGACCCG) and reverse (5′-CGAAGTGGGACAGGGGCTC-3′) primers, flanking the alternative exon 16, with the following conditions: 27 cycles at 94°C, 35 min; 61°C, 25 min; 72°C, 15 min. Preliminary RT–PCR experiments were conducted using various cycle numbers and then one of these was chosen. Various amounts of the RT reaction were used to determine the amount of cDNA for analysis of PCR within the linear range. Fluorescent PCR products were analysed, after electrophoresis through 1.8% agarose gels, using Typhoon 9200 fluorescent densitometry (Amersham Pharmacia) and quantitated by Image Quant Software (Molecular dynamics, Amersham Pharmacia).

Immunocytochemistry

We performed an immunocytochemical study for GFAP and vimentin to determine the presence of glial differentiation. Monolayer cells cultured on coverslips were rinsed in Tris buffer and fixed in Carnoy for 10 min, then rehydrated, immersed for 10 min in 0.05% Triton solution, rinsed in distilled water, incubated for 10 min in 3% hydrogen peroxide and washed in Tris buffer three times for 5 min. The slides were covered with 5% normal goat serum in Tris buffer for 20 min, then incubated for 2 h at 37°C with a 1 : 100 dilution of primary anti-GFAP monoclonal antibody (DAKO) or a 1 : 20 dilution of primary antivimentin monoclonal antibody (DAKO). After three washes in Tris buffer, the slides were incubated 1 h in EnVision$^+$, Peroxidase, Mouse secondary (DAKO). Finally, the slides were washed and reacted with diaminobenzidine in 0.1 M Tris buffer with 0.03% hydrogen peroxide, rinsed in distilled water, counterstained and mounted. The cells weakly expressed GFAP, and showed marked immunostaining for vimentin, displaying a primitive glial differentiation.

ODNs transfection and proliferation assay

Glioblastoma cell lines MI-7 and GBM were cultured with Opti-MEM medium (Invitrogen) for 24 h during which the cells reached 80–90% confluence. Cells were transfected with synthetic phosphorothioate ODNs (0.1–0.2 μM final concentration) in LipofectAMINE™2000 (Invitrogen) containing Opti-MEM in accordance with the manufacturer's instructions and incubated for 6 h. For RT–PCR analysis, incubation was continued for 12 h before total RNA extraction.

After transfection 2×10^3 cells were plated in microtiter plates in four replicates. ^{3}H-thymidine (1 μCi/ml) was added 24 h before harvesting the cells. A semiautomated cell harvesting apparatus was used to lyse the cells with water and precipitate the labeled DNA on glass fiber filters. The filter pads were dried and counted in a β-counter after addition of the scintillation fluid.

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GenBank accession numbers
We obtained the genomic sequence spanning the human MARK4 gene from two cosmids: R31237 (AC005581) and R33632 (AC005781). Human MARK4 partial coding sequences isolated from anaplastic oligodendroglioma have been deposited into GenBank (AI869600, AI939391). MARK4S cDNA (KIAA1860, AB049127), MARK4L (AY120867). The cDNA referred in NCBI's GenBank as MARK4 corresponds to the MARK4L isoform (AY057448).

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