Chapter from the book *Glioma - Exploring Its Biology and Practical Relevance*

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Role of the Centrosomal MARK4 Protein in Gliomagenesis

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1. Introduction

Human gliomas are the most frequent tumours of the central nervous system (Kleihues & Cavenee, 2000). They are of neuroectodermal origin and present as different histological types and malignancy grades (Louis et al., 2007). According to the WHO (world health organization) system, astrocytoma, oligodendroglioma and mixed oligoastrocytoma are classified as differentiated gliomas, while anaplastic glioma and glioblastoma show increasing grades of malignancy (Box 1).

Box 1.

Gliomas are composed of different cell types displaying, even within low-grade tumours, a wide spectrum of heterogeneity regarding morphology, genotype, invasive potentiality, and treatment sensitivity (Noble & Dietrich, 2004). The development and progression of glioma malignancies is driven by accumulation of genomic alterations, including both mutations and chromosomal instability (CIN).

2. Chromosomal instability (CIN) in glioma

CIN refers to the rate of lost or gained chromosomes and/or structural chromosome anomalies and ploidy changes during cell divisions (Geigl et al., 2008; Lengauer et al., 1998). Structural chromosome anomalies (translocations, deletions, insertions, inversion and additions) may be balanced or unbalanced and involve one or more chromosomes (Bayani et al., 2007). Chromosomal instability in glioma is mainly characterized by aneuploidy (Bigner et al., 1988; Hecht et al., 1995; Jenkins et al., 1989; Lindstrom et al., 1991; Magnani et al., 1994; Park et al., 1995; Thiel et al., 1992) affecting in particular glioblastoma, the most
malignant glioma. Gliomas frequently display near-diploid (2n+/-) and/or near-tritetraploid (3n+/-)/(4n+/-) karyotypes, implicating aberrant mitotic divisions, in addition to chromosomal rearrangements. Highly polyploid subpopulations and the presence of apoptotic nuclei are also reported (Figures 1a-d).

Fig. 1. (a) The G-banded, near diploid karyotype of MI-4 GBM (glioblastoma multiforme) cell line (Magnani et al., 1994), showing trisomy of chromosome 7, monosomy of chromosome 10 and a complex rearrangement involving chromosomes 1, 9 and 19. (b) The G-banded, near tetraploid karyotype of MI-4 cell line, displaying several chromosome losses and structural rearrangements including marker chromosomes. (c) Representative polyploid metaphase from MI-60 GBM cell line, characterized by a high frequency of hyperdiploid cells. (d) Apoptotic and large nuclei of MI-60 cell line.

Low-grade astrocytomas and oligodendrogliomas (WHO grades I-II) show a number of chromosome aberrations quite low. When present, they involve the gain of chromosome 7, the loss of chromosomes 10, 22 and one sex chromosome (see Figures 1a, b), while structural changes affect in particular 1p (Figure 2a) and 9p (Figure 2b) chromosome arms. These chromosome abnormalities are qualitatively similar to those found in anaplastic astrocytoma (WHO grade III) and glioblastoma (WHO grade IV), but their frequency is
increased in the latter and multiple chromosomal rearrangements are also present. The finding of common abnormalities associated to both low- and high-grade glioma has suggested a progressive chromosomal evolution during tumour growth (Bigner et al., 1988; Jenkins et al., 1989; Magnani et al., 1994; Thiel et al., 1992) even though it has been demonstrated that a subset of glioblastomas arises clonally de novo, further emphasizing the genetic heterogeneity of glioma (Kleihues & Ohgaki, 2000; von Deimling et al., 1993). Given that numerical CIN features many cancer cells, it has been hypothesized that it may have a primary role in tumorigenesis (Duesberg et al., 2006; Weaver et al., 2007). Recently it has been shown that the main pathway to aneuploidy in cancer cells is triggered by extra centrosomes that, increasing improper merotelic attachments of kinetochores to spindle microtubules, cause chromosome mis-segregation (Meraldi et al., 2002) (Figure 3) (see Box 3 for centrosomes and Box 4 for mitotic spindle).

Fig. 2. (a) Chromosome 1 rearrangements of both p and q arms observed in different glioma cell lines by G-banding. (b) Rearrangements of chromosome 9p, sharing the loss of p21 band, observed in different glioblastoma cell lines by G-banding.

At early mitosis, the merotelic orientation escapes the spindle mitotic checkpoint thus representing the major mechanism of chromosome mis-segregation in non-cancer cells. Usually these errors are corrected before cells enter anaphase, to preserve genome stability (Cimini et al., 2004).
Fig. 3. Proposed events of lagging chromosomes in cancer cells with extra centrosomes through merotelic kinetochore orientation. (top) In the presence of extra centrosomes (three instead of two, as example), merotelic kinetochore orientation may occur: one kinetochore is bound by spindle microtubules from two centrosomes (right) instead of just one (left). (bottom) As cells move to mitosis and cluster extra centrosomes in a bipolar spindle, many attachment errors persist into anaphase, leading to lagging chromosomes.

3. Tetraploidy, centrosome amplification and spontaneous chromosomal instability in glioma

A relationship between extra centrosomes and the formation of multipolar spindles in cancer cells has been proposed by different authors (Basto et al., 2008; Cimini et al., 2004; Saunders, 2005; Sluder & Nordberg, 2004). Multipolarity in cancer cells is considered an essential transient stage prior to clustering extra centrosomes in a bipolar fashion (Brinkley, 2001). Multiple centrosomes have been detected in many types of cancer cells including glioma (Figure 4) and strongly linked to aneuploidy in a variety of studies (D’Assoro et al., 2002; Ganem et al., 2009; Ghadimi et al., 2000; Katsetos et al., 2006; Lingle et al., 2002; Magnani et al., 2009; Pihan et al., 1998).

A positive linear correlation between the percentage of cells with supernumerary centrosomes and the extent of aneuploidy within a panel of glioblastoma cell lines is shown in Figure 5.

In tumour development, aneuploidy is frequently preceded by tetraploidy, often with prolonged tetraploid precancerous status, a feature that makes it of central importance to cancer research (Margolis et al., 2003). It has been proposed that failure of cytokinesis is a key step in the formation of tetraploid karyotypes and in tumour initiation (Fujiwara et al., 2005). A tetraploid cell inherits twice the normal complement of centrosomes, a condition assessed to generate chromosomes mis-segregation in subsequent cell divisions (Ganem et al., 2007). However, tetraploid cells are observed in some normal tissues including liver and heart, indicating that cytokinesis is physiologically regulated. The possible fate of a tetraploid progeny is shown in Figure 6.
Fig. 4. Immunofluorescence with anti-γtubulin antibody (red) of representative glioblastoma cell lines, showing (a) multiple centrosomes; (b) multipolar spindles; (c) a mitotic bipolar spindle in which centrosomes are larger than the normal one (likely extra centrosomes clustered into two spindle poles), a condition that favours mitotic stability and neoplastic growth; (d) normal centrosomes and a mitotic bipolar spindle configuration. The nuclei are counterstained with DAPI (4’,6-diamidino-2-phenyl indole) (blue).

Fig. 5. Regression analysis between aneuploidies and centrosome aberrations in glioma cell lines, showing a statistically significant positive correlation. OA: oligoastrocytomas; A: astrocytomas; GBM: glioblastoma multiforme; GC-GBM: giant cell-GBM.
Fig. 6. Fate of a tetraploid cell: if extra centrosomes coalesce, a bipolar spindle assures the progeny maintains a tetraploid set, while lack of this *escamotage* gives rise to aneuploid progeny through a multipolar mitosis.

Binucleated tetraploid cells with multiple centrosomes are frequently observed in glioma cell lines, as illustrated by a representative image in Figure 7.

![Image](image_url)

Fig. 7. Immunofluorescence with anti-\(\gamma\text{-tubulin}\) antibody (red) of a binucleated, tetraploid-derived glioblastoma cell line, showing coalesced centrosomes in one (left) of the two nuclei. Nuclei are counterstained with DAPI (blue).

To measure the occurrence of DNA damage in once-divided binucleated (BN) cells, the cytokinesis-block micronucleus cytome (CBMN Cyt) assay, an established biomarker to detect spontaneous genomic instability (Fenech, 2007), can be used. Application of CBMN Cyt to a series of glioma cell lines evidenced a high rate of micronuclei (MNi), a biomarker of chromosome breakage and/or whole chromosome loss, and chromosome aberrations.
such as nucleoplasmic bridges (NPBs), a biomarker of DNA misrepair and/or telomere end-fusions determining the furrow regression, and nuclear buds (NBUDSs), a biomarker of elimination of amplified DNA and/or DNA repair complexes (Figure 8a, b).

Fig. 8. Photomicrographs of glioma cell lines showing (a) typical binucleated cells with nucleoplasmic bridges and (b) binucleated cells with micronuclei and nuclear buds.

Thus, binucleated tetraploid cells may be transmitted to the progeny and enhance subsequent rounds of aberrant mitosis.

4. Cytogenomics of gliomas

Chromosomal instability can be detected by different techniques, including conventional karyotyping, fluorescence in situ hybridization (FISH), spectral karyotyping (SKY) and array-based comparative genomic hybridization (aCGH) analyses.

The classic assay to monitor and quantify chromosome aberrations is karyotyping (see Figure 2).

The in situ hybridization technique with fluorescently labelled probes targeting specific chromosomes is commonly applied on fixed glioma cells, allowing the analysis of chromosomes of interest cell by cell. Examples of FISH analysis in glioma cell lines are shown in Figure 9.

Aneuploidies are rapidly detectable by interphase FISH as well as by quantification of micronuclei formed by chromosomes that lagged behind during a previous mitosis (Figure 10).

The technique of array-CGH is considered the most powerful tool for identifying copy number changes of genetic material, since it combines high resolution and large scale genomic analysis, characteristics that are not combined by conventional approaches. Since it allows a quantification of amplifications and deletions, pointing through human genome databases directly to the affected genes, aCGH technology is more and more used in the study of tumours for the identification of potentially causative cancer genes.
Fig. 9. Partial karyotype of MI-4 GBM cell line displaying chromosome 1 alterations by (a) whole chromosome 1 painting probe and (b) dual colour FISH of YACs 745h6, spanning the 1p36.3 breakpoint (green), and 957f12, mapping to 1p36.1 (red), showing a transposition of 1p36.1 material to der (1)(p22). DNA is counterstained with DAPI (blue). Interphase dual colour FISH of RP11-111p21, mapping to 3p21 control clone (red), and RP11-172g5, mapping to 3q26.3 (green), (c) in a normal diploid cell and (d) in MI-60 GBM cell line showing amplification of the region targeted by RP22-172g5 (green).

Fig. 10. (a) Interphase FISH with centromeric probes of chromosomes 7 and 10 showing trisomy of chromosome 7 and monosomy of chromosome 10 in MI-4 GBM cell line. (b) Interphase FISH with whole chromosome 19 painting probe showing a micronucleus labelled by chromosome 19 material. DNA is counterstained with DAPI (blue).
aCGH studies have been applied to gliomas and have successfully complemented previously published metaphase-CGH, SKY and LOH (loss of heterozigosity) analyses (Bredel et al, 2005; Cowell et al., 2004a, 2004b; Kitange et al., 2005; Nigro et al., 2005). Integration of the results has demonstrated an excellent correlation between the findings obtained through this genomic approach and those obtained by alternative techniques, stressing the usefulness and overall accuracy of aCGH as compared to classic previously widely employed analyses (Cowell et al., 2004a, 2004b). Comparative analysis of elaborated aCGH data led to identify copy number changes shared by various glioma grades as well as aberrations apparently related to progression to glioblastoma (GBM) (Roversi et al., 2006).

5. Non-random chromosomal aberrations in gliomas: The 19q13 abnormalities

Over the last decade, molecular approaches including mutation screening, LOH and aCGH analyses have led to identify the most frequently recurring genomic imbalances associated with each WHO glioma subtype (Kitange et al., 2005; Koschny et al., 2002; Shapiro, 2002) and hence the driver genes acting in pathways involved in glioma development, either in the initiation stages (Tp53 and Ras by PDGF-NF1) or in malignant progression (Rb-CDKN2-CDK4) (Collins, 2004; Zhu & Parada, 2002). Comprehensive genomic characterization by integrative analysis of DNA copy number, gene expression and DNA methylation aberrations in >200 glioblastomas has then refined the definition of human glioblastoma genes and core pathways (The Cancer Genome Atlas [TGCA] Research Network, 2008). Deletion of chromosome 19q is nevertheless of particular interest, as it is shared by all three glioma subtypes, occurring in approximately 75% of oligodendrogliomas, 45% of mixed oligoastrocytomas and 40% of astrocytomas (von Deimling et al., 1992, 1994), where it is associated with the transition from low-grade to anaplastic tumours (Ohgaki et al., 1995; Ritland et al., 1995; Smith et al., 1999) (Box 2).

Box 2. TSGs: tumour suppressor genes.

At the cytogenetic level, chromosome 19q abnormalities are more frequently detectable in GBM than in low grade glioma, with 19q13 as the most affected region, as shown in Table1.
<table>
<thead>
<tr>
<th>Case no.</th>
<th>Diagnosis</th>
<th>Partial Karyotypes</th>
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</thead>
<tbody>
<tr>
<td>D-245</td>
<td>GBM</td>
<td>49,XY,... +19</td>
</tr>
<tr>
<td>D-250</td>
<td>GBM</td>
<td>47,XY,... +19</td>
</tr>
<tr>
<td>D-256</td>
<td>GBM</td>
<td>44,XY,... t(9;19)(p13;q13)</td>
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<td>D-290</td>
<td>GBM</td>
<td>45,X,-X,... t(1;19)(q21;q13)</td>
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<tr>
<td>D-299</td>
<td>GBM</td>
<td>46,XY,... der(19)t(17,19)(q11;q13)</td>
</tr>
<tr>
<td>D-304</td>
<td>AMG</td>
<td>43,XY,... -19</td>
</tr>
<tr>
<td>D-316</td>
<td>GBM</td>
<td>46,XX,... der(19)t(10;19)(q11;q11)</td>
</tr>
<tr>
<td>D-340</td>
<td>GBM</td>
<td>66~77,XXYY,... -19,-19, der(11)t(11q19p)x2, der(11)t(11p19q), der(11)t(11;19)(cen;q13)</td>
</tr>
<tr>
<td>D-320</td>
<td>GBM</td>
<td>47,XX,... der(19)t(5;10;19)(q15<del>21;q11</del>26;q13)</td>
</tr>
<tr>
<td>nr</td>
<td>GBM</td>
<td>47,XX,... der(19)t(19;?)(?)(q13;?)</td>
</tr>
<tr>
<td>nr</td>
<td>GBM</td>
<td>79~83,Y,... -19,-19, der(19)t(19;?)(q13.3;?)x2</td>
</tr>
<tr>
<td>37</td>
<td>PA</td>
<td>46,... der(19)t(19;?)(q13.1;?), der(19)t(19;?)(p13.3;?)</td>
</tr>
<tr>
<td>43</td>
<td>O</td>
<td>83~88,XXYY,... -19, der(19)t(19;?)(q13.3;?)</td>
</tr>
<tr>
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<td>GBM</td>
<td>39~45,der(X)t(X;?),... der(19)t(19;?)(p13.3;?)</td>
</tr>
<tr>
<td>39</td>
<td>GBM</td>
<td>43,X,-X,... tdic(19;22)(p13.3;q13)</td>
</tr>
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<td>33</td>
<td>GBM</td>
<td>43,XX,... +19</td>
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<tr>
<td>36</td>
<td>GBM</td>
<td>44,XY,... der(19)t(9;19)(q13;q13)</td>
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<tr>
<td>37</td>
<td>GBM</td>
<td>47~48,XY,... +19</td>
</tr>
<tr>
<td>40</td>
<td>AA</td>
<td>65~75,XY,... +19,+19</td>
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<td>GBM</td>
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<td>AA</td>
<td>48,XY,... +19</td>
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<tr>
<td>56 AW</td>
<td>GBM</td>
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</tr>
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<td>MI-4</td>
<td>GBM</td>
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</tr>
<tr>
<td>MI-32</td>
<td>GBM</td>
<td>47,XX,... del(19)(q13.2)</td>
</tr>
<tr>
<td>MI-14</td>
<td>GBM</td>
<td>86~89,X,-X,... -19,-19</td>
</tr>
<tr>
<td>nr</td>
<td>O</td>
<td>45,XY,... der(1)t(1;19)(p11;q11)</td>
</tr>
<tr>
<td>T-60</td>
<td>GBM</td>
<td>45~46,XX,... -19,der(19)t(19;?)</td>
</tr>
<tr>
<td>3/T110</td>
<td>AO</td>
<td>44~46,XY,... -19</td>
</tr>
<tr>
<td>26/G227</td>
<td>GBM</td>
<td>44~45,X,-Y,... der(19)t(14;19)(q11.2;p13.1)</td>
</tr>
<tr>
<td>31/T35</td>
<td>GBM</td>
<td>41~45,XY,... der(19)t(14;19)(q13;q13.1)</td>
</tr>
<tr>
<td>36/T66</td>
<td>GBM</td>
<td>48~52,XY,... der(19)t(1;19)(q21;q13)</td>
</tr>
<tr>
<td>nr</td>
<td>PXA</td>
<td>46,XY,... t(1;11;19)(q24;q23;q13)</td>
</tr>
<tr>
<td>2</td>
<td>GBM</td>
<td>44,XX,... t(1;19)(q23;q13)</td>
</tr>
<tr>
<td>nr</td>
<td>GBM</td>
<td>... t(10;19)(q24;q13)</td>
</tr>
</tbody>
</table>

Table 1. Cytogenetic alterations of chromosome 19 in gliomas; 19q13 alterations are marked by red stars. GBM: glioblastoma multiforme; AMG: anaplastic mixed glioma; PA: pilocytic astrocytoma; O: oligodendroglioma; AA: anaplastic astrocytoma; AO: anaplastic oligodendroglioma; PXA: pleomorphic xanthoastrocytoma; nr: not reported.
Furthermore, similarly to oligodendroglioma, combined LOH of 1p and 19q was found to define a small subset of GBM patients with a significantly better survival, even if their tumours were not morphologically distinguishable from the bulk of GBMs (Schmidt et al., 2002). This finding has been translated into significant advance in the prognosis and treatment of oligodendrogliomas (van den Bent, 2004). A candidate tumour suppressor region has been assigned by LOH to 19q13.3 (Hartmann et al., 2002), but no positional or functional candidate gene in this band has yet been appointed.

Only recently an integrated analysis of human glioblastoma multiforme with the application of next generation sequencing technology disclosed a new marker associated with an increase in overall survival, represented by recurrent mutations in the active site of isocitrate dehydrogenase 1 (\(\text{IDH1}\)) in a large fraction of young patients with secondary GBM (Parsons et al., 2008).

6. Identification of \(\text{MARK4}\) gene through refined FISH mapping of 19q13 breakpoints

FISH studies of structural 19q chromosomal rearrangements in glioma (Magnani et al., 1999) and a detailed analysis of the breakpoints underlying the 19q13 alterations in the MI-4 glioblastoma cell line, led to identify a 19q13.2 intrachromosomal duplication of the MAP/microtubule affinity-regulating kinase 4 (\(\text{MARK4}\)) gene (Beghini et al., 2003) (Figure 11). Genomic profiling by means of array-CGH interrogation of 25 primary glioma cell lines including the MI-4 GBM cell line (Roversi et al., 2006) revealed that the BAC clone encompassing \(\text{MARK4}\) at 19q13.2 (Figure 12) is included in a “gain” region in a few of the tested cell lines and confirmed \(\text{MARK4}\) duplication in the MI-4 glioblastoma cell line (Figure 13).

![Fig. 11. 19q13.2 intrachromosomal duplication of \(\text{MARK4}\) in the MI-4 GBM cell line detected by G-banding and FISH analysis using a whole chromosome painting 19 probe and a \(\text{MARK4}\)-specific cosmid clone.](image-url)
Fig. 12. MARK4 genomic region (http://genome.ucsc.edu/). Clones of chromosome 19q full coverage (blue) overlapping MARK4 gene are circled in red: the gene is entirely encompassed by BAC clones RP11-746H08, RP11-568L16, RP11-202G02, RP11-752G09 RP11-584B04, RP13-647G21 and partially encompassed by RP11-577I16.

Fig. 13. (left) Chromosome 19q array-CGH of MI-4 GBM cell line, showing duplicated MARK4 gene (red star) and the common LOH region in glioma. (right) Schematic representation of MARK4 position on chromosome 19, at the boundary of 19q13.3 LOH region.
The combined FISH and array-CGH results provided the rationale for investigating a possible role of the serine-threonine kinase MARK4 in glioma. It’s worth of note that this gene, belonging to the so called “kinome”, maps at the centromeric boundary of the 19q13.3 LOH region in glioma.

7. The family of MARK kinases

MARK4 (MAP/microtubule affinity-regulating kinase 4) is a member of the MARKs family, constituted in mammals by four serine-threonine kinases (MARK1-4) which are able to phosphorylate the microtubule-associated proteins (MAPs, including Tau, MAP2, MAP4 and doublecortin) (Drewes et al., 1997). Microtubules (MTs) are cytoskeleton cylindrical structures formed by α and β tubulin dimers; dimers can quickly assemble or disassemble, causing the microtubules to grow or shorten and making them very dynamic. MAPs association stabilizes the MTs; when MARK kinases link a phosphate group to MAPs (phosphorylation), MAPs cannot associate to MTs any longer, thus microtubules become more instable and disassemble (Figure 14).

Fig. 14. Schematic representation of microtubules. Assembled α and β tubulin dimers form the microtubules, stabilized by MAP association. When MAPs are phosphorylated, they are no more able to bind microtubules, which disassemble.

7.1 MARKs protein structure

All MARK proteins have a very conserved structure, consisting of six sequence segments (Marx et al., 2010) (Figure 15):

- the N-terminal header, whose role is unknown;
- the catalytic or kinase domain, containing both the activation/inactivation loop (MARK kinases are in turn activated/inactivated by phosphorylation/dephosphorylation) and the catalytic loop, by which MARKs transfer a phosphate group to substrate proteins;
- a linker, that is a highly and negatively charged motif resembling the common docking (CD) site in MAP kinases; it may bind interactors;
- the UBA domain, a small globular domain with sequence homology to ubiquitin-associated proteins; it may exert an autoregulatory function through interaction with the catalytic domain;
- a spacer, the most variable region among MARK members; it is probably important for regulating MARK activity since it holds phosphorylation sites;
- the C-terminal tail, consisting of the kinase-associated (KA1) domain, whose function is still uncertain. It is characterized by a hydrophobic portion surrounded by positively
charged residues, which may interact with negatively charged regions of cytoskeletal proteins, MARK catalytic domain or MARK CD domain (Tochio et al., 2006) with an inhibitory effect. It has been proposed it could be involved in protein localization to the membrane, being identified as a domain that binds membrane anionic phospholipids, in particular phosphatidylserine (Moravcevic et al., 2010).

Fig. 15. Schematic representation of MARK protein structure. Boxes are not drawn to scale.

7.2 MARKs regulation
Being composed of several domains, MARK proteins are regulated by multiple mechanisms. All MARKs are activated by liver kinase B (LKB1) and MARK kinase (MARKK) by phosphorylation on the threonine residue in the activation loop (Timm et al., 2008); in addition, phosphorylation by CaMKI (calcium/calmodulin-dependent protein kinase I) activates MARK2 (Matenia & Mandelkow, 2009). On the contrary, phosphorylation by the glycogen synthase kinase 3β (GSK3β) on the serine residue in the activation loop, by aPKC (atypical protein kinase C) in the spacer region or by Pim1 kinase, down-regulates MARK activity (Matenia & Mandelkow, 2009; Timm et al., 2008). Finally, interaction between MARK catalytic domain and other proteins/MARK domains (such as 14-3-3 proteins, PAK5, MARK UBA and KA1 domains) inhibits MARK activity (Marx et al., 2010).

7.3 MARKs functions
Since MARK kinases regulate the affinity between MAPs and MTs, they are implicated in several cellular processes involving the microtubules, such as cytoskeleton dynamics, neuron motility (Schaar et al., 2004), and microtubule-dependent transport of proteins, vesicles and organelles (Mandelkow et al., 2004). Microtubules also play an important role in centrosome formation (Box 3) and in the correct distribution of the chromosomes in the two daughter cells during cell division (mitosis and cytokinesis; Box 4).

\( \text{Tau} \) is a microtubule-associated protein particularly expressed in the central nervous system. The aggregation of hyperphosphorylated \( \text{Tau} \) has been demonstrated to form insoluble neurofibrillary tangles (Chin et al., 2000; Gamblin et al., 2003) which are characteristic of Alzheimer’s disease. MARKs role in this pathology has been evaluated in many studies, demonstrating, as an example, MARK co-localization with neurofibrillary tangles (Chin et al., 2000).

MARK2 is involved in establishing cell polarity, cooperating in the organization of the epithelial structure of liver, kidney and stomach (Cohen et al., 2004; Matenia & Mandelkow, 2009), and regulating axon formation in neuronal cells (Chen et al., 2006). Experiments in mice demonstrated that MARK2 is also implicated in many physiological functions, such as fertility, homeostasis of the immune system, memory, growth and metabolism (Bessone et al., 1999; Hurov et al., 2001; Hurov & Piwnica-Worms, 2007; Segu et al., 2008). MARK3 plays an important role in cell signaling and cell cycle control: phosphorylation of some proteins by MARK3 induces their binding to 14-3-3 proteins thus regulating many cellular pathways (Bachmann et al., 2004; Müller et al., 2001).
8. MARK4

MARK4 is the less characterized member among MARK proteins. It has been discovered by Kato and colleagues in 2001 among a few genes whose expression resulted significantly increased in hepatocarcinoma cells with elevated β-catenin levels in their nucleus (Kato et al., 2001).

MARK4 gene is located on chromosome 19q13.2, consists of 18 exons and encodes at least two isoforms, namely MARK4S and MARK4L, originated by alternative splicing (Kato et al., 2001) (Figure 16). mRNA splicing is a complex process consisting in the removal of introns, which are non-coding sequences, and in the joining of exons, the coding sequences, to generate the “edited” mRNA ready to be translated into a protein.

- MARK4S (“short”) protein is the native isoform, consisting of all the 18 exons, and is 688 aminoacid-long with predicted molecular weight of 75.3 kilo Daltons (kDa);
- MARK4L protein derives from skipping of exon 16, which causes a shift of the reading frame\(^1\) with a downstream stop codon, originating a longer protein (752 aminoacids; predicted molecular weight: 82.5 kDa).

![Fig. 16. Alternative splicing of exon 16 gives origin to MARK4 isoforms. When exon 16 is included in the mRNA, the stop codon is inside exon 18 and the encoded protein, MARK4S, lacks the KA1 domain at the C-terminal tail (left); when exon 16 is skipped, a shift of the reading frame occurs, changing the stop codon and generating a longer MARK4L protein, which has the classical KA1 domain (right).](image)

Both MARK4L and S share the same protein structure of MARKs, with 90% sequence homology in the kinase domain. The two isoforms differ in the C-terminal tail, since MARK4L includes the kinase-associated 1 domain as the other MARK proteins, whereas MARK4S contains a domain with no homology to any known structure (Kato et al., 2001; Moroni et al., 2006) (Figure 16). Actually, MARK4 has less sequence homology in the C-terminus compared to the other MARKs; nevertheless MARK4L tail seems to fold in a similar shape, suggesting that the role of the C-terminal region may apply also to MARK4L (Marx et al., 2010).

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\(^1\) The mRNA sequence is “read” by an enzyme which matches a determinate “codon”, made by three nucleotides, with its respective aminoacid. There are two particular codons, namely the start and the stop codon, which mark the beginning and the end of the protein.
8.1 MARK4 regulation
Phosphorylation by LKB1, in the activation loop, activates MARK4, while polyubiquitination of MARK4 inhibits the kinase activation (Al-Hakim et al., 2008). Furthermore, as MARK4 interacts with aPKC (Brajenovic et al., 2008), it could be phosphorylated and inactivated by this kinase as reported for MARK2 and MARK3.

8.2 MARK4 interactors and hypothetical functions
By tandem affinity purification and immunoprecipitation experiments, near twenty proteins have been identified as putative MARK4 interactors (Brajenovic et al., 2008). Among them, PKCλ and Cdc42 are implicated in cell polarity control and TGFβIAF (transforming growth factor β-inducing anti-apoptotic factor) is thought to be a homologue of Miranda, a protein involved in the asymmetric division of neuroblasts in Drosophila. MARK4 interacts with the 14-3-3η isoform (Angrand et al., 2006; Brajenovic et al., 2008) of 14-3-3 proteins, which control multiple cellular processes by binding phosphorylated proteins and could directly regulate MARK4 or act as bridges among different pathways. Other MARK4 interactors are ARHGEF2, a cytoskeleton binding protein, and Phosphatase 2A, which is associated to microtubules and regulates Tau (Brajenovic et al., 2008). MARK4 protein has been also found to co-localize and co-precipitate in complex with α, β, and γ tubulin, myosin and actin (Brajenovic et al., 2008; Trinczek et al., 2004).

As the other MARK members, MARK4 phosphorylates MAPs, increasing microtubule dynamics; therefore, as also suggested by the interactions above reported, MARK4 may be involved in many processes involving microtubules, such as cytoskeleton dynamics.

9. Up-regulation of MARK4L in glioma
MARK4 gene is expressed ubiquitously in human tissues, with particularly elevated levels in brain and testis (Kato et al., 2001).

Few MARK4 expression studies are reported in literature; they were performed with non-quantitative methods, such as northern blot (Kato et al., 2001; Schneider et al., 2004; Trinczek et al., 2004) and semi-quantitative competitive PCR (polymerase chain reaction) (Moroni et al., 2006), on different organisms (human, rat and mouse tissues) not always allowing to discern between the two MARK4 isoforms. MARK4 transcriptional variants are differentially regulated in human tissues, especially in the central nervous system: MARK4S is the predominant isoform in mouse and human brain, while MARK4L has been found highly expressed in neural progenitors and in gliomas (Beghini et al., 2003; Moroni et al., 2006).

By a semi-quantitative approach MARK4L has been found up-regulated in glioma tissue samples (fragments of glial tumours excised from patients) and glioma cell lines, of different malignancy grades, including the MI-4 GBM cell line carrying the MARK4 duplicated gene as detected by FISH and aCGH analysis. MARK4L has been also found highly expressed in neural progenitors and down-regulated during their glial differentiation into astrocytes, suggesting that it might be necessary for proliferation, being thus highly enriched in proliferating or undifferentiated cells (Beghini et al., 2003) (Figure 17).

Protein kinase activation, often caused by gene amplification and/or mutation, is frequently associated to cancer initiation and progression, as most kinases are involved in cell proliferation. Although array-CGH analyses on glioma cell lines showed that the BAC clone encompassing MARK4 at 19q13.2 is included in a “gain” region in a few of the tested cell
lines, it did not evidence MARK4 copy number variations, except for the MI-4 GBM cell line (Roversi et al., 2006). Only a few MARK4 alterations are reported in the literature, namely two missense mutations (aminoacidic substitution) in exon 12 (R377Q and R418C in the spacer region), two silent mutations (no aminoacidic substitution) in exons 5 (Y137Y) and 9 (I286I) (kinase domain), while one intronic mutation (exon 8 +5 C>T; kinase domain) has been found in a few tumour samples (Greenman et al., 2007). In addition, only a splice-site mutation (exon 13 +1 G>A; spacer region) has been identified in one among 91 glioblastoma samples (TGCA Research Network, 2008). However, CpG methylation and/or promoter amplification have not yet been investigated. Based on this evidence, neither amplification nor mutations of MARK4 gene seem to be the cause of its reported sustained expression in glioma samples.

Fig. 17. (a) Semi-quantitative Reverse Transcription-PCR of MARK4S and MARK4L isoforms in whole normal brain (WNB) and in 32 glioma cell lines, subdivided according to WHO grade (A: astrocytoma; AA: anaplastic astrocytoma; OA: oligoastrocytoma; AOA: anaplastic oligoastrocytoma; O: oligodendroglioma; GBM: glioblastoma multiforme). (b) Downregulation of MARK4L expression during glial differentiation of human neural progenitors: semi-quantitative RT-PCR (top) and graph representation (bottom) of MARK4L expression in neural progenitors at times 0, 10 and 28 days of induced differentiation.

10. MARK4 sub-cellular localization in glioma cell lines

Recently, immunofluorescence analyses with a specific anti-MARK4L antibody highlighted multiple sub-cellular localizations for the endogenous MARK4L protein in glioma cell lines (Magnani et al., 2009).
10.1 Centrosome localization
It has been assessed that, under microtubule-stabilizing conditions, MARK4L localizes in the perinuclear region of glioma cell lines. By co-localization experiments with both anti-MARK4L and anti-γ-tubulin (the main centrosomal protein) antibodies, this perinuclear localization has been demonstrated to correspond to the centrosome (Magnani et al., 2009), as shown in Figure 18 (Box 3). This result confirms previous data referring to exogenous MARK4 protein conjugated to GFP (green fluorescent protein), which has been shown to co-localize with microtubules and centrosomes of CHO (Chinese hamster ovary) and neuroblastoma cell lines (Trinczek et al., 2004), in contrast to MARK1, MARK2 and MARK3 that exhibit uniform cytoplasmic localization. Furthermore, it has been demonstrated that the association with the centrosome is independent from microtubules, since it is not abolished when microtubules are depolimerized by nocodazole treatment (Magnani et al., 2009).

Fig. 18. Anti-MARK4L (green; left) and anti-γ-tubulin (red; right) antibodies showing co-localization signals in interphase (top) and mitotic (bottom) centrosomes of glioma cell lines.

The centrosome
The centrosome is a little organelle, not bound by membrane, positioned centrally in the cell near the nucleus. It is the primary Microtubule Organizing Center (MTOC), as it can nucleate and organize microtubules. It consists of two distinct domains:
• the centriolar domain, including the centrioles, which are cylindrical organelles important for centrosome organization and replication. Each centriole consists of 9 triple microtubules;
• the pericentriolar domain, consisting of many fibers and proteins that surround the centriole. In this domain microtubules are nucleated, by associating α and β-tubulin dimers from a γ-tubulin ring (Doxsey, 2001).

The centrosome plays a key role in organizing the interphase cytoskeleton (regulating cell polarity, adhesion and motility) and the mitotic spindle (Kramar et al., 2002). It also contributes to cell cycle progression and cytokinesis (Martinez-Ganey et al., 2006) and is involved in cell cycle transitions, in the cellular response to stress and signal transduction (Doxsey et al., 2005). The centrosomes duplicate only once in the cell cycle, during G1/S transition and in S phase, and form a strictly bipolar spindle during mitosis.
The endogenous MARK4L localizes both at normal interphase centrosomes (Figure 18) as well as at the aberrant centrosomes frequently observed in glioma cell lines (see Figure 4), suggesting a possible link between the alternatively spliced kinase and the mitotic instability frequently observed in human glioma. Two abnormal centrosome configurations are reported: a random one (multiple centrosomes randomly distributed) and a clustered one (multiple centrosomes collected in a single large aggregate) (Magnani et al., 2009), as depicted in Figure 19.

Fig. 19. Anti-MARK4L (green; left) and anti-γtubulin (red; middle) antibodies showing co-localization signals in abnormal centrosomes of glioma cell lines. Both the abnormal centrosome configurations are reported: the random one (top) and the clustered one (bottom). The nuclei are counterstained with DAPI (blue, right).

10.2 Midbody localization
The centrosome association is maintained during the entire course of mitosis, as MARK4L co-localizes with γtubulin in all the cell cycle phases. The anti-MARK4L antibody is also detected in the midbody, a microtubule structure forming at the contact point between the two daughter cells at the end of the cell division. These data demonstrate that the kinase is endogenously associated with the centrosomes during the whole cell cycle and concentrates thereafter into the midbody during cytokinesis (Magnani et al., 2009) (Figure 20) (Box 4).

Fig. 20. Co-localization of MARK4L (green) and γtubulin (red) proteins at the midbody (arrow) during the cytokinesis of a glioma cell. The nuclei are counterstained with DAPI (blue).
10.3 Nucleolar localization
Under standard immunofluorescence conditions, anti-MARK4L antibody is also detected in the nucleoli (Box 5). Silver-colloid method, which allows visualizing the nucleolar organizing regions (NORs), and co-localization experiments with anti-nucleolin (a nucleolar protein) antibody allowed to assess that the nuclear structures bound by MARK4L antibody are indeed the nucleoli (Magnani et al., 2009) (Figure 21) (Box 5).

Fig. 21. Co-localization of MARK4L (green) and nucleolin (red) proteins in the nucleoli of glioma cells. The nuclei are counterstained with DAPI (blue).
Box 5.

The overall immunofluorescence data on endogenous MARK4L protein confirm the previous evidence on its centrosome association and highlight two novel localization sites of MARK4L: the nucleolus and the midbody (Magnani et al., 2009).

Immunoblotting with anti-MARK4L antibody on centrosomes, midbody and nucleoli isolated by biochemical fractionation from glioblastoma cell lines confirmed the presence of MARK4L protein in each fraction, validated by antibodies specific for each cell structure: anti-αtubulin antibody for centrosomes, anti-βtubulin for the midbody (βtubulin, together with αtubulin, accounts for 30% of midbody proteins) and anti-nucleolin for the nucleolus (Magnani et al., 2009).

The localization pattern of MARK4L delineated by the above studies suggests that the kinase may take part in cell cycle progression and influence the microtubules, particularly those affecting the centrosome and midbody.

MARK4L association with the nucleolus in glial tumours is very interesting, since MARK4L could have a functional impact on this organelle, being requested for its building and maintenance like other protein kinases, as well as it could be spatially regulated by alternate translocation in and out the nucleolus. Many proteins are indeed sequestered in the nucleolus and then released according to a temporally regulated activity, since they must exert their function in certain phases of the cell cycle (Visintin & Amon, 2000). Last, the nucleolar localization of a protein may also influence its stability, protecting the protein from proteasomal degradation, since proteasomes are present in the nucleoplasm but not in nucleoli (Wojcik & DeMartino, 2003).
11. Conclusion

A few remarks can be drawn from the above synthesis on cytogenomics of human gliomas and the MARK4 cell cycle gene as a likely “player” in gliomagenesis. Gliomas are one of the most intractable tumours due to their “complex identity”: as it has been beautifully underlined, the generation - since the earliest glioma stages - of multiple cell populations with different genotypic and phenotypic features makes unlikely to succeed therapeutic strategies targeting only clones with “dominant” or “average” characteristics of the cell population (Noble & Dietrich, 2004). The intrinsic genomic heterogeneity of human glioma has first been disclosed cytogenetically, as documented by a huge number of studies which across two decades have used the cytogenetic tools suitable to monitor the intratumour cell heterogeneity and to discern “recurrent” and potentially causative chromosomal rearrangements. A few of these rearrangements entered the diagnostic and prognostic flow chart of gliomas, others allowed to identify crucial genes which mutations or imbalance are the signature of a specific glioma type or glioma malignancy stage. In line with a research pathway that has been reiterated for several genes of relevance in cancer, focus on MARK4 has been pinpointed by cytogenetics and deepened by multiple tools ranging from gene-targeted molecular to genomic and cytogenomic analyses. Despite its nature of serine-threonine kinase gene, MARK4 has not be found mutated or affected by copy number alterations in glioma, while its encoded proteins represented by two different isoforms, MARK4S and MARK4L, could be featured as a potential target of dysregulation in tumours due to its dual nature. The latter isoform, produced by alternative splicing, has been found up-regulated in glioma and shown to display sub-cellular localizations, namely the centrosome, the midbody and the nucleolus, which strictly associate it with the process of cell division. Interestingly, alternative mRNA splicing has been considered a mechanism not only increasing proteomic complexity but also involved in cancer, through mechanisms of oncogenes/tumour suppressors activation/inactivation or through the generation of CIN (López-Saavedra & Herrera, 2010). CIN is a general property of aneuploid cancer cells and is generated by defects in different processes, among which the regulation of the number of centrosomes, the dynamics of microtubules attachment to the kinetochores and the overall control of cell cycle. Defects in centrosomal number and structure have been well documented in gliomas (D’Assoro et al., 2002; Katsetos et al., 2006; Magnani et al., 2009) raising the issue whether the increased MARK4L isoform, a gene involved in microtubule dynamics, may concur to errors in chromosomal segregation driving gliomagenesis. Recent application of multidimensional technological approaches has comprehensively highlighted the scenario of glioma genes and core pathways. However, despite the impressive advances, the links between genes alteration and cellular behavior are yet hampered by the multiplicity of the genetic lesions and the interconnections among the different affected pathways. Hopefully ongoing and next years research will compose the puzzle promising to translate into the clinical set the unraveled glioma pathomechanisms.

12. Acknowledgement

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13. Methods

13.1 Cell cultures and preparation of human metaphase chromosomes
Glioma cell lines were derived from primary tumour post-surgery specimens and subsequently maintained by serial passages in RPMI 1640 medium containing 5% Fetal Calf Serum at 37°C in a 5% CO₂ atmosphere. Most of the cell lines were used within the first 30 passages.
Metaphase spreads were obtained on both fresh tumours and cultured cell lines, harvested when “peak” mitotic activity was observed; usually, a 16-hour treatment with Colcemid at a final concentration of 0.01-0.02 mg/ml is employed (Magnani et al., 1994).

13.2 Fluorescence in situ hybridization (FISH) analysis
Fluorescence hybridization with genomic DNA has proven to be a powerful tool for identification of chromosome rearrangements in cancer cells. Potential applications include detection of chromosome-specific aneuploidy in metaphase and interphase cells, quantification of the frequency of chromosome translocations and/or aneuploidy as a measure of genetic damage, and detection of diagnostically and prognostically relevant chromosomal lesions. Detection of translocations between human metaphase chromosomes is possible by using cocktails of chromosome-specific sequences that hybridize more or less uniformly along the chromosome. Depending on the aberration, its detection may be by visual fluorescence microscopy (see Figures 9, 10). In brief: slides carrying interphase or metaphase spreads are washed in 2x SSC (1 x SSC is 0.15 M NaCl/0.015 M sodium citrate), dehydrated in an ethanol series and denatured [70% (vol/vol) formamide/2x SSC (final concentration), pH 7, at 70°C for 2 min]. The hybridization mix consists of (final concentrations) 50% formamide, 2x SSC, 20% dextran sulfate, carrier DNA (sonicated herring sperm DNA), and biotin-labeled human genomic DNA. The mixture is applied to the slides under a glass coverslip. After overnight incubation at 37°C, the slides are washed at 45°C (50% formamide/2x SSC, pH 7), and immersed in BN buffer (0.1 M sodium bicarbonate, 0.05% Nonidet P-40, pH 8). The slides are never allowed to dry after this step. The coverslips are then removed and fluorescein-avidin DCS is applied. The coverslips are put back in their original places and the slides incubated 20 min at 37°C. They are then washed in BN buffer at 45°C. The intensity of biotin fluorescence is amplified by adding a layer of biotinylated goat anti-avidin antibody followed, after washing as above, by another layer of fluorescein-avidin DCS. After washing in BN buffer a fluorescence anti-fade solution is added. The DNA counterstain [4,6-diamidino-2-phenylindole (DAPI) or propidium iodide] is included in the anti-fade solution (Magnani et al., 1999; Pinkel et al., 1986).

13.3 Immunofluorescence
Immunofluorescence analyses enable to visualize, by fluorescence microscopy, the subcellular localization of a specific protein in cultured cells. The target protein is recognized by an antibody, which in turn is conjugated to a fluorochrome emitting fluorescent light. Briefly, cells are grown on glass chamber slides, then permeabilized (with solvents that extract lipids from the membranes allowing antibodies to reach a sub-cellular structure) and fixed (in order to protect the cell structure from eventual damages and to “freeze” cells in their current state). Afterwards, cells are incubated with bovine serum albumin (BSA) to block non-specific binding of antibodies. Glass slides are then incubated with a primary
antibody specific to the target protein, then with a secondary antibody conjugated to the fluorochrome and finally observed under the microscope (Magnani et al., 2009).

13.4 Biochemical fractionation and immunoblotting

By biochemical fractionation we mean the whole techniques that allow to separate and isolate intact cellular components. It usually consists in carefully breaking the cell membrane with homogenizers and isotonic/hypotonic solutions, so that intact organelles can come out, and in separating cellular components by centrifugation, on the basis of differences in their mass and specific weight. Centrosome, midbody and nucleoli isolation protocols are described in Magnani et al., 2009 and based on methods respectively by Moudjou & Bornens, 1994; Chu & Sisken, 1977; Muramatsu et al., 1963. In particular, for midbody isolation cells are synchronized in mitosis by nocodazole treatment and then released from mitotic arrest in nocodazole-free medium, so that after 30 minutes near 90% of cells had formed the midbody.

After membrane breaking, all the passages are done at 4°C and with protease inhibitors, in order to prevent protein degradation, possibly exerted by released proteases. Proteins extracted from centrosome, midbody and nucleolus fractions are then analyzed by immunoblotting. Proteins are first separated, according to their molecular weight, by SDS-PAGE (Sodium Dodecyl Sulphate - PolyAcrilamide Gel Electrophoresis): this technique allows proteins to migrate, driven by electric current, in a porous gel, with speed depending exclusively on their size. Afterwards, separated proteins are transferred onto a membrane, incubated with a blocking solution (BSA or milk) to prevent non-specific binding of antibodies and then incubated with appropriate antibodies (immunoblotting). The primary antibody is specific to the target protein and is recognized by the secondary antibody conjugated to HRP (horse radish peroxidase). Antibodies are detected by covering the membrane with a peroxide/enhancer solution, which is oxidized by HRP and emits light signals.

14. References


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The title ‘Glioma - Exploring Its Biology and Practical Relevance’ is indicative of its content. This volume contains 21 chapters basically intended to explore glioma biology and discussing the experimental model systems for the purpose. It is hoped that the present volume will provide supportive and relevant awareness and understanding on the fundamental advances of the subject to the professionals from any sphere interested about glioma.

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