Minichromosome Maintenance Protein 7: A Reliable Tool for Glioblastoma Proliferation Index

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Abstract. *At present there is increasing evidence concerning the value of minichromosome maintenance (MCM) protein expression as a novel indicator of proliferation. In the present study, 15 glioblastoma samples, classified according to WHO, were analysed to evaluate the expression of the principal proliferation markers. The samples examined were subdivided into 2 cytological subsets, small cell (SC) or multiforme cell (MC) glioblastoma, according to the predominant cell type defined in individual specimens. MCM7 detected more cells in the cycle than Ki67 and PCNA and all cases of SC glioblastoma, the most aggressive subset, displayed a significant increase of MCM7-stained nuclei versus those stained with Ki67. These results suggest that the cell cycle-associated proteins MCM are not only useful markers of proliferation, but also valid aids for diagnosis in cerebral glioblastoma.*

Glioblastoma is the most common neuroectodermal tumour and the most malignant astrocytic glioma. The diagnostic utility of various biological markers for glioblastoma cell proliferation has been broadly tested but the results obtained are still poor and controversial (1).

Minichromosome maintenance (MCM) proteins play an essential role in eukaryotic DNA replication since MCM2-7 proteins constitute the prereplicative complex that is formed at the replication origin. All 6 members of the MCM protein family, MCM2 to MCM7, function as heterohexameric complexes and their cell cycle-dependent regulation is essential for DNA replication, thus making antibodies against the human forms of the MCM proteins attractive as histological markers (2-4). In particular, there is increasing evidence supporting a strong correlation between pre-cancerous cells and cells that express high levels of MCM proteins (5, 6). For this reason, MCM proteins have recently been investigated as novel sensitive cellular markers to identify pre-cancerous cells before they become malignant (7).

In addition, proliferation markers, such as PCNA and Ki67, have, in many cases, provided a limited assessment of cell cycle status (8). Among these, staining for PCNA can be altered by a variety of factors, including fixation time (1), whereas the function of the Ki67 antigen remains largely unknown and there is evidence that the molecule is not essential for cell proliferation. Moreover, it has been reported that the expression of the Ki67 antigen can be affected by external factors, such as nutrient deprivation, which may operate regionally in solid tumours (9, 10).

Glioblastoma multiforme (GBM) is the most common form of malignant glioma and accounts for approximately 12-15% of all intracranial neoplasms and 50-60% of all astrocytic tumours (11). Glioblastomas are histologically strongly heterogeneous neoplasms, variably composed of several cell types. Little is known about the biological behaviour of the various cell types and cytological tumour subsets. It has been previously reported that glioblastomas composed predominantly of small cells exhibited more "aggressive" biological behaviour. On the other hand, the presence of giant cells revealed a positive correlation with survival for these patients (1).

The aim of our study was to investigate, by immunohistochemistry on paraffin-embedded tissues, whether MCM7 expression could be a more useful marker for proliferation in glioblastoma as compared to the routinely used Ki67 and PCNA expressions.

Materials and Methods

Tissue specimens. Fifteen archival formalin-fixed, paraffin-embedded human glioblastomas (WHO grade IV) were obtained from diagnostic biopsy or resection specimens from 10 patients at the Azienda Ospedaliera Ospedale Maggiore di Parma (Parma, Italy), after local research ethics committee approval. All glioblastomas

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Table I. *Characteristics of the enrolled patients and proliferation marker labelling indices (mean± SEM). The clinical outcome refers to 6 months after surgery.*

Patient	Sample	Gender and Age	Diagnosis	Recurrence	Clinical outcome	MCM7 LI (%)	Ki67 LI (%)	PCNA LI (%)
C.F.	1	F; 46	GBL-MC	Yes	poor	19.2±6	11.0±1.4	9.0±2.4
B.R.	2	M; 58	GBL-MC	No	fair	24.2±4.2	21.8±5.8	17.1±7.5
A.A	3	M; 42	GBL-MC	Yes	poor	4.8±3.2	2.5±1.8	7.2±1.8
S.B.	4	M; 41	GBL-MC	No	poor	58.4±8.4	30.5±9.3	8.4±3.1
R.M.	5 a	F; 72	GBL-MC	No	death	15.3±5	14.1±4.6	6.2±1.9
	5 b					13.8±5.3	16.4±3.8	9.1±1.4
D.F.	6 a	M; 38	GBL-SC	Yes	poor	14.6±4.7	14.5±3.3	10.5±3.9
	6 b					18.2±6.2	13.5±5.5	10.3±4.1
T.P.	7 a	F; 76	GBL-SC	No	fair	28.7±5.4	6.4±2.4	11.6±2.1
	7 b					9.6±2.1	6.1±3.4	7.1±1.4
C.P.	8 a	M; 60	GBL-SC	Yes	poor	33.1±9.4	17.4±5.9	18.2±5.4
	8 b		GBL-OLIGO			17.6±4.3	17.9±9.3	18.6±4
P.L.	9 a	F; 80	GBL-SC	No	poor	32.2±6.8	20.3±4.1	10.6±3.5
	9 b					22.5±5.8	18.2±4.2	12.6±3.8
C.M.	10	F; 38	GBL-SC	Yes	poor	28.5±7.1	15.1±3.1	13.0±2.4

SC, small cells; MC, multiforme cells; OLIGO, oligodendroglioma; LI, labelling index.

included in this study were histopathologically diagnosed by neuropathologists according to the WHO classification. The samples were morphologically subclassified as small cell (SC, n=9) or multiforme cell (MC, n=6).

Immunohistochemical staining. In order to provide comparative data, serial sections mounted on poly-L-lysine-coated slides were immunostained using the following primary antibodies: anti-human Ki67 antigen antibody (clone MIB-1, Dako), anti-PCNA, mouse monoclonal (Dako), anti-human MCM-7, mouse monoclonal (USBiological, MA, USA).

Five-μm paraffin-embedded sections were cut, dewaxed in xylene and rehydrated through a series of ethanol to water. The sections were microwaved (5 cycles of 2 min at 950 W) in 1% unmasking solution (Vector Laboratories) to facilitate antigen retrieval. The sections were then cooled down at room temperature for 20 min before immunostaining. For MCM7 expression, the immunostaining was performed with EnVision™ (Dako) according to the manufacturer's protocol and counterstaining with Mayer's haematoxylin. PCNA and Ki67 immunostainings were performed with the standard streptavidin-biotin-peroxidase technique. The slides were stained with 3,3'-diaminobenzidine, counterstained with haematoxylin and mounted.

Negative controls were performed by omitting the primary antibodies.

Quantification of immunohistochemical staining. A quantitative indication of the extent of staining for the nuclear markers under investigation was obtained by calculating the labelling index (LI) as the percentage of positively-stained nuclei out of the total number of nuclei counted in representative microscopic (40x objective) fields. For each tumour sample, the LI quantification was performed in the same area, previously delineated by overlapping the serial slides. The sections were counted independently by 2 individuals with an inter-observer variation of less than 3%. A mean of at least 1,000 nuclei was screened for each marker. For the PCNA LI evaluation, only those nuclei with a homogeneous diffuse dark brown staining were considered whereas nuclei with light and granular staining were excluded, according to Korshunov *et al.* and Pierce *et al.* (1, 12).

Statistical analysis. The paired *t*-test was performed to determine whether the differences were statistically significant. A p value less than 0.05 was considered statistically significant.

Results

The immunohistochemistry of the MCM7 and Ki67 proteins showed a well-defined, strong nuclear pattern of staining in tumour cells with very low background staining that was easy to quantify, whereas the PCNA patterns were less well-defined and some exclusion criteria were applied, as described in the Materials and Methods section (Figure 1).

As summarized in Table I, the Ki67 LI in the 15 samples studied ranged from 2.5% to 30.5%, that of PCNA from 6.2% to 18.2%, whereas the MCM7 LI in the same samples ranged from 9.6% to 58.4%, thus identifying a larger proportion of cells. A good correlation was observed between the MCM7 and Ki67 and PCNA labelling indices but, interestingly, MCM7 immunostained a higher proportion of cells in most of the samples, as summarized in Figure 2. The MCM7 LI was significantly higher ($p<0.05$) than the Ki67 LI in 9 samples out of 15 (7 patients out of 10).

In addition, in terms of the cytological subsets, the MCM7 expression was higher ($p<0.05$) compared to that of Ki67 in 7 out of 9 samples (5 patients out of 5) with SC glioblastoma, but in only 2 out of 6 samples (2 patients out of 5) with MC glioblastoma.

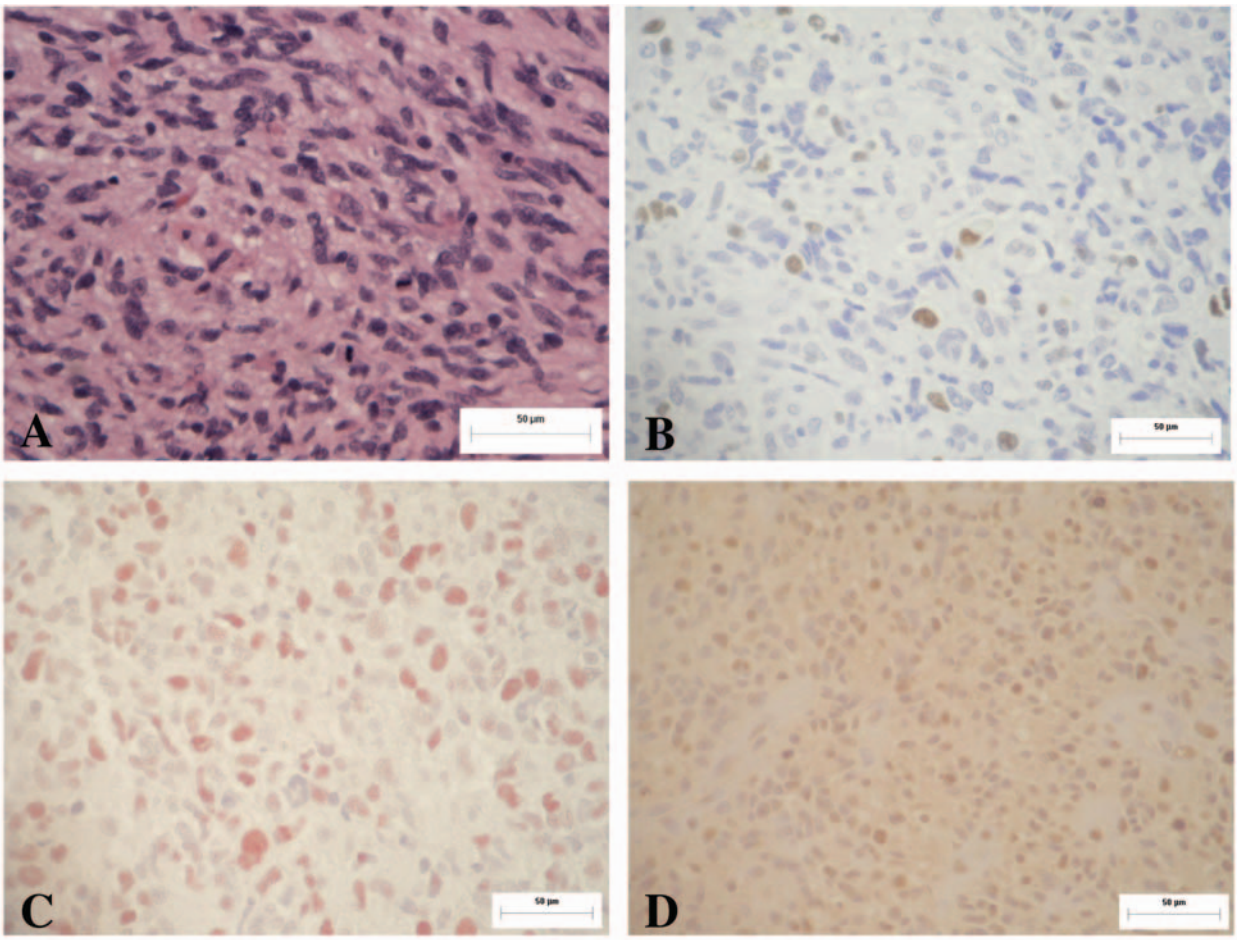

Figure 1. *Haematoxylin-eosin (A) and immunohistochemistry for Ki67 (B), MCM7 (C) and PCNA (D) in glioblastomas.*

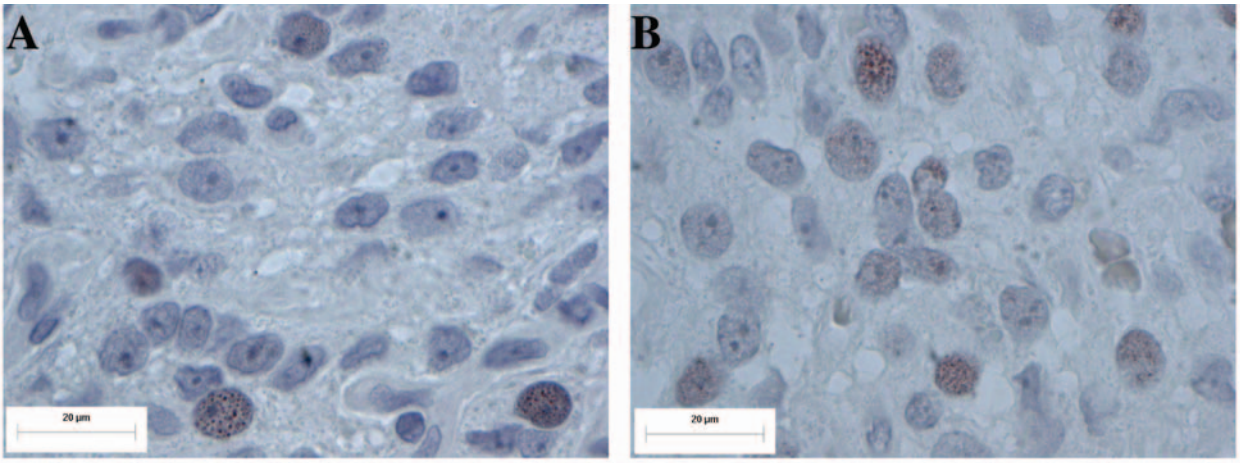

Figure 2. *Immunohistochemistry for MCM7 in glioblastomas: (A) Section with oligodendroglial component; (B) Section with small cell component.*

In 13 out of 15 samples (9 patients out of 10) MCM7 detected more cells than PCNA, without a clear distinction between the 2 cytological subsets (Figure 3).

An important observation was the difference in protein expressions for the patient C.P. : the MCM LI was capable of discriminating between 2 different histopathological

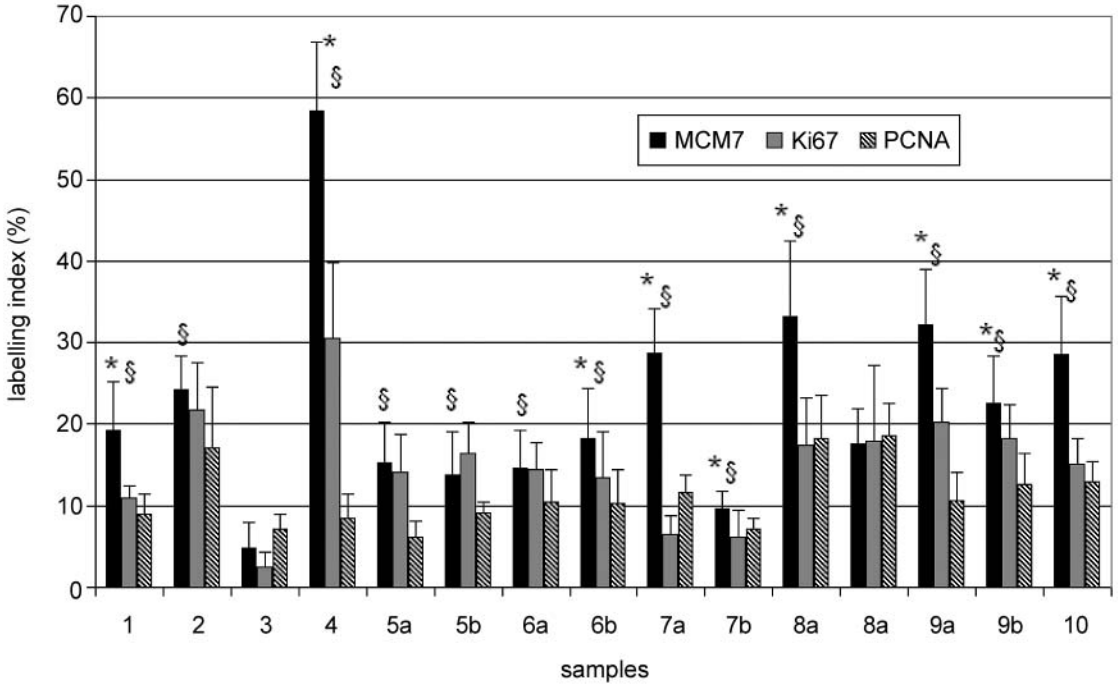

Figure 3. *Bar graph of MCM7 (black), Ki67 (grey) and PCNA (dashed) labelling indices for glioblastomas. *statistically significant ($p<0.05$) versus Ki67; §statistically significant ($p<0.05$) versus PCNA.*

samples (*i.e.,* significantly higher in the sample with the SC compared to the sample with the oligodendroglioma component) obtained from the same tumour mass, whereas Ki67 and PCNA failed (Figures 2 and 4).

No significant correlation between the MCM7 expression and the clinical outcome at 6 months after resection or recurrence was observed.

Discussion

In this investigation, attention was focused on the proliferative profile of 15 samples of glioblastoma by comparing different immunohistochemical proliferation markers. The evaluation of PCNA was not feasible because of heterogeneity in the staining pattern. In fact, the score was assessed, in agreement with other Authors considering the intermediate positivity as aspecific. However, the term "intermediate positivity" itself is subjective and uncertain.

Comparison with the Ki67 expression confirmed the superiority of MCM7 in the detection of proliferative cells in the majority of glioblastoma samples, although this protein is expressed in fewer cell cycle phases (G1 and S) compared to Ki67 (G1, S, G2 and M). A further indication for MCM proteins as good proliferative markers comes from the observation that the expression difference between MCM7 and Ki67 was significant in 7 out of 9 samples of the SC subset.

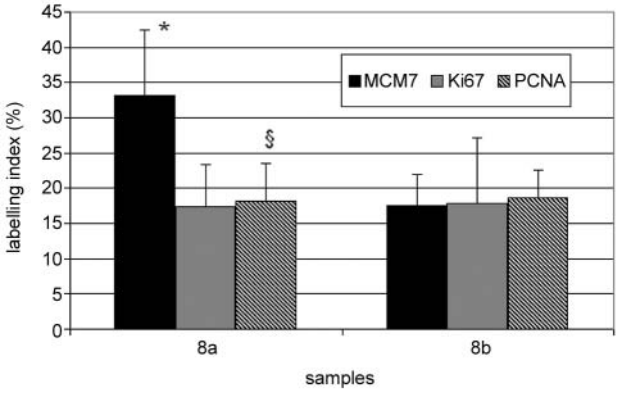

Figure 4. *Bar graph of MCM7 (black), Ki67 (grey) and PCNA (dashed) labelling indices in patient number 8 with a SC component (sample 8a) and an oligodendroglial component (sample 8b). *statistically significant ($p<0.05$) versus Ki67; §statistically significant ($p<0.05$) versus PCNA.*

These findings are of particular interest, since some evidence suggests that the pathological substrate of aggressiveness in this malignant glioma is largely related to the proliferation of a population of small anaplastic cells (13).

MCM proteins are known to be an important tool for estimating tumour proliferation and are a useful adjunct to the routinely used proliferation markers for glioblastoma diagnosis. However, these proteins do not provide information on glioblastoma patient survival.

In light of our findings, there are good indications that MCM7 LI can also be applied as a marker for glioma grading since it was able to discriminate between a sample with a prevalent oligodendroglial component and a sample with a SC component.

Knowledge of the cell cycle phases in which MCM proteins are expressed can have important implications for response to radiotherapy and long-term survival. In fact, MCM proteins, in contrast to Ki67 and PCNA which are expressed throughout the cell cycle, are mostly present in the radioresistant phases, *i.e.,* G1 and late S, suggesting a role as candidate predictive markers for the radiosensitivity of tumours (14, 15).

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