

Proliferation and Programmed Cell Death: Role of p53 Protein in High and Low Grade Astrocytoma

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Abstract. *p53 is a cell cycle regulator that has been well-recognized as the key molecule that triggers the induction and the control of cell proliferation and apoptosis in a wide variety of tumours, including astrocytoma. Apoptosis and proliferation are two processes intimately coupled, that occur simultaneously in tumour tissue. Previous studies of the correlations between proliferation and apoptotic index with p53 expression in astrocytic tumours have remained inconclusive. The aim of this study was to investigate the correlation of p53 expression with the apoptotic index (AI) and the cell proliferation index (PI) in pilocytic astrocytoma (PA) and glioblastoma multiforme (GBM). A correlation of p53 expression with AI and PI was found in pilocytic astrocytoma but not in glioblastoma, probably because of the mutated p53 phenotype in the latter.*

Proliferation and programmed cell death represent two cellular events that in physiological conditions are in dynamic balance, governing tissue homeostasis. The balance between positive and negative signals determines the decision between life and death (1), whereas an imbalance leads to diseases linked to unwanted apoptosis or unwanted cell growth. In the past it had been assumed that tumour aggressiveness was mainly related to cell proliferation (2, 3). More recently it has become apparent that tumour growth depends also on the rate of apoptosis (4). In particular, although it could be presumed that in tumours, in association with an increase of proliferation, there is also a decrease of apoptosis (5), several studies have demonstrated that this rarely happens and an increase of programmed death has been observed (6). The inability of cells to undergo apoptosis may advance cancer development, both

by allowing dividing cells to accumulate and by not eliminating genetic mutants that may harbour enhanced malignant potential (7).

It has been found that apoptosis and the cell cycle share several pathways, providing a rationale for linking these two cellular processes (1). In particular, the multifunctional tumour suppressor gene p53 has been well recognized as a key molecule directly involved in cell proliferation and apoptosis. Activation of p53 causes G1 arrest by inducing p21 expression and consequently inhibiting the cyclin/cyclin dependent kinase complex (8). In these conditions, the retinoblastoma protein (pRB) is not phosphorylated and cells do not progress through the cell cycle, thus allowing the activation of repair mechanisms. Once the cells have successfully repaired the DNA damage, their cell cycle is allowed to continue. In contrast, in the case of too extensive damage or the inactivation of repair mechanisms, the p53 protein induces cell death, through apoptosis protein cascade synthesis (*i.e.* caspases, Bax and Bak) (9).

p53 protein mutations have been observed in many neoplasms, including astrocytic tumours where they were the first genetic alterations identified (10). Furthermore, these alterations have been associated with the tumorigenesis, progression (11, 12), prognosis (13) and radiation response of tumours (14, 15).

Studies of the correlation between proliferation (PI) and apoptotic (AI) indices with p53 expression in astrocytic tumours have remained inconclusive so far (16-19). Pilocytic astrocytoma (PA) is a typically circumscribed, slow growing and non-aggressive brain tumour, mostly found in children and young adults (20). In contrast, glioblastoma multiforme (GBM) is one of the most aggressive primary brain tumours, with unfavourable prognosis despite maximal treatment (21).

In the light of these observations, the aim of our study was to investigate the correlation of p53 expression with the AI and PI in low and high grade astrocytomas in order to determine the role of p53 in the balance between proliferation and programmed cell death in these tumours.

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Materials and Methods

Tissue specimens. Forty-three archival formalin-fixed, paraffin-embedded human astrocytomas were obtained from the diagnostic biopsy or resection specimens of 16 patients at Azienda Ospedaliera Ospedale Maggiore di Parma (Parma, Italy) with the approval of the local research ethics committee. All the astrocytomas included in this study were histopathologically diagnosed by neuropathologists according to WHO classification as PA (7 patients, 27 samples) and GBM (9 patients, 16 samples).

Immunohistochemistry. To provide comparative data serial sections mounted on poly-L-lysine coated slides were immunostained using the following primary antibodies: for the proliferation indices anti-human Ki-67 antigen, mouse monoclonal (clone MIB-1, DakoCytomation, Carpinteria, CA, USA) and anti-human MCM-7, mouse monoclonal (USBiological, Swampscott, MA, USA) and for p53 expression anti-human p53 antigen (clone D0-7, DakoCytomation).

Five- μm paraffin-embedded sections were cut, dewaxed in xylene and rehydrated through a series of ethanols to water. The sections were microwaved (5 cycles of 2 minutes at 950 Watt) in 1% unmasking solution (Vector Laboratories, Burlingame, CA, USA) to facilitate antigen retrieval. The sections were then cooled down at room temperature for 20 minutes before immunostaining. For MCM-7 expression the immunostaining was performed with EnVision™ (DakoCytomation) according to the manufacturer's protocol followed by counterstaining with Mayer's haematoxylin. The Ki-67 and p53 immunostainings were performed with the standard streptavidin-biotin peroxidase technique, stained with 3,3'-diaminobenzidine (DAB), counterstained with haematoxylin and mounted. The negative controls were performed by omitting the primary antibodies.

TUNEL method. Apoptosis was determined through the TUNEL technique, using the *In Situ* Apoptosis Detection Kit (ApopTag Plus Peroxidase, United Chemi-con, Resemont, IL, USA). Five- μm paraffin-embedded sections were cut, dewaxed in xylene and rehydrated through a series of ethanols to water. The sections were incubated with K proteinase for 15 minutes at room temperature and endogenous peroxidase was blocked with a solution of PBS and 3% H₂O₂ for 5 minutes. The slides were then incubated with a solution of digoxigenin-conjugated nucleotides and terminal deoxynucleotidyl transferase (TdT) at 37°C for 60 minutes. Subsequently, the anti-digoxigenin antibody was applied and incubated for 30 minutes at room temperature. Detection of the antigen-antibody link was made through immunoperoxidase followed by 3,3'-DAB chromogen.

The sections were counterstained with 0.5% methyl green, rinsed in distilled water and mounted. The omission of the TdT enzyme was used as the negative control.

Quantification and statistics. The PI and AI scores were obtained by calculating the percentage of positively stained nuclei out of the total number of nuclei counted in representative microscopic fields (40x objective). For each tumour sample the labelling index (LI) quantification was performed in the same area, previously delineated by overlapping the serial slides. The sections were scored independently by two individuals and an inter-observer

variation of less than 5% was observed. A mean of 1,000 nuclei per sample was screened for each marker.

Statistical analysis. The correlations between proliferation index, apoptosis index and p53 labelling index were determined using Pearson's correlation method. A $p<0.05$ was considered significant.

Results

Immunohistochemistry. Representative images of PA and GBM are shown in Figure 1 a,b.

Immunohistochemistry for MCM-7, Ki-67 and p53 proteins showed a well-defined, strong, nuclear pattern of staining in the tumour cells with very low background staining. The distribution of stained cells was heterogenous, varying in the different fields (Figure 1 c-h).

The Ki-67 LI in the cases of PA varied from 0% to $9\%\pm3.7\%$ (mean $1.29\%\pm0.47\%$) whereas the GBM samples showed a range between $3.3\%\pm1.4\%$ and $27.6\%\pm9.5\%$ (mean $13.38\%\pm4.78\%$). The MCM-7 expression in the PA was between 0% and $9.2\%\pm3.7\%$ (mean $3.05\%\pm1.31\%$), while in the GBM it was between $9.1\%\pm3.9\%$ and $51.5\%\pm10\%$ (mean $27.09\%\pm6.62\%$). The p53 protein expression in the PA samples varied between 0% and $1.2\%\pm0.2\%$ (mean $0.29\%\pm0.14\%$). In the samples of GBM it was between $5.1\%\pm2.2\%$ and $48\%\pm9.1\%$ (mean $17.51\%\pm4.81\%$).

As illustrated in Figure 2, a statistically significant ($p<0.05$) direct correlation between p53 expression and the proliferation indices (Ki-67 and MCM-7) was shown in the PA samples. In the GBM, a positive correlation between these parameters was still found, although it was not statistically significant ($p>0.05$).

Apoptosis. The AI of PA and GBM showed significant differences (Figure 1 i and l). In the GBM samples a large number of apoptotic cells was observed ($7.5\%\pm2.3\%$ to $50.1\%\pm15.6\%$, mean $19.64\%\pm6.86\%$) and this was significantly different ($p<0.05$) from the PA samples (0% to $40\%\pm7.6\%$, mean $10.28\%\pm2.32\%$). Positive nuclei were absent in all the negative controls.

Discussion

The difference in correlation level between p53 and PI in the PA and GBM samples might be explained by the fact that p53 protein in pilocytic tumours is functional and not mutated (22), therefore, in the presence of DNA damage, the cell induces its expression and is consequently able to activate the repair mechanisms. On the other hand, p53 mutations inhibiting its function have been described in GBM (23). Thus the mutated protein is accumulated into the cell nucleus binding the normal isoform, whose function is consequently inhibited. Therefore, we

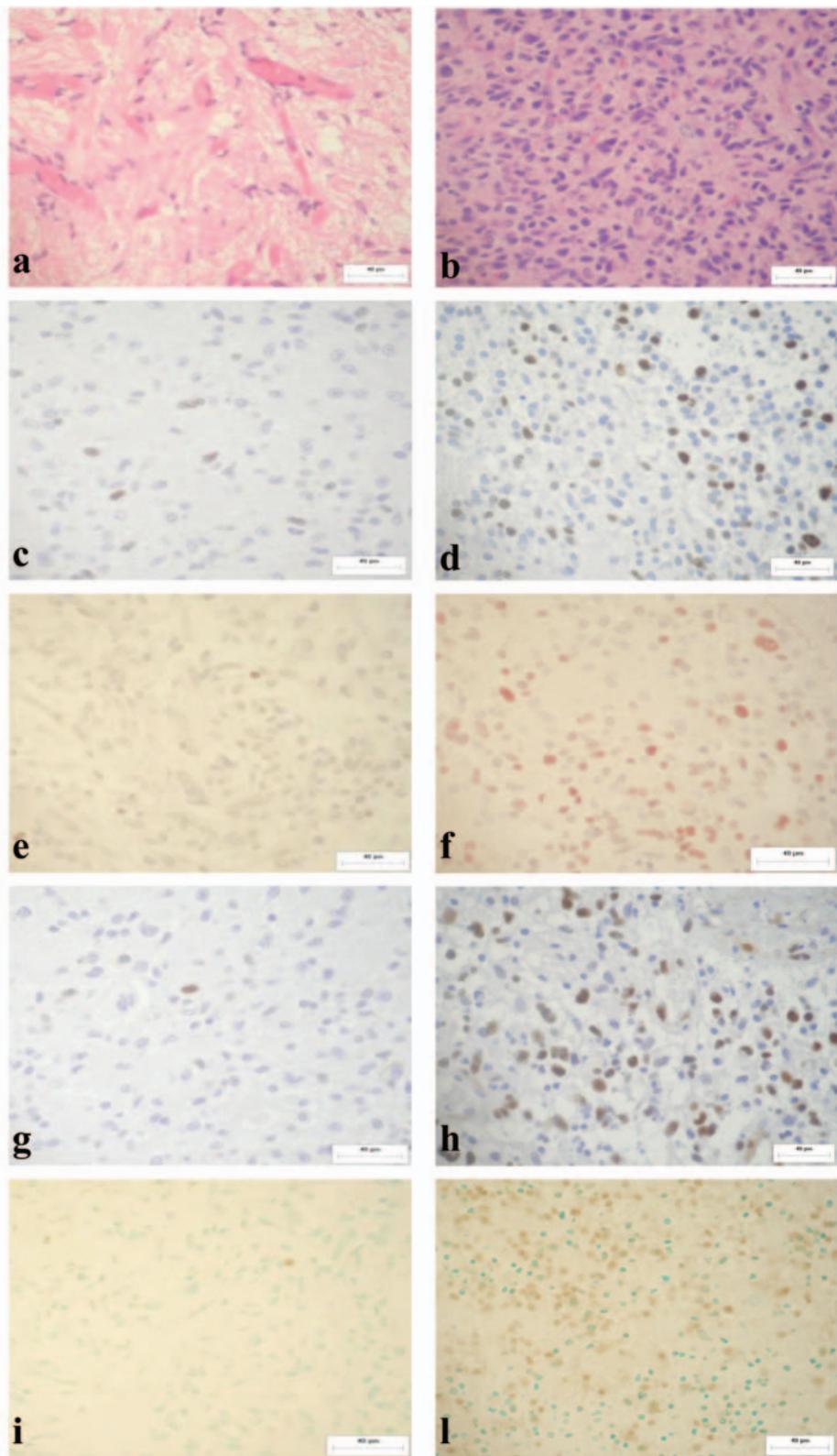


Figure 1. Representative images of pilocytic astrocytoma (left) and glioblastoma multiforme (right) formalin-fixed, paraffin embedded sections (original magnification x400): haematoxylin-eosin staining (a, b); anti-Ki-67 MIB1 immunostaining (c, d); anti-MCM-7 immunostaining (e, f); anti-p53 protein immunostaining (g, h); apoptosis staining by TUNEL (i, l).

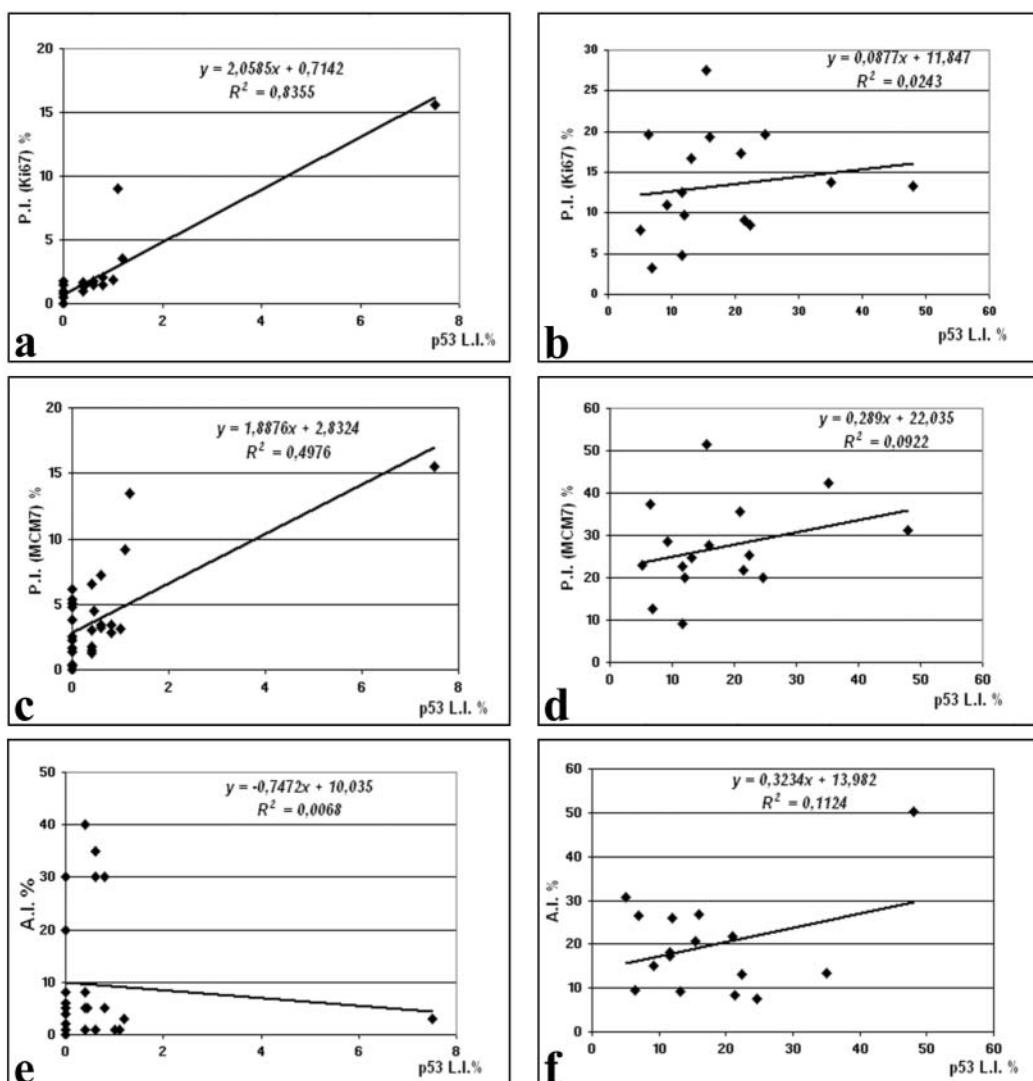


Figure 2. Scatterplots of pilocytic astrocytoma (left) and glioblastoma multiforme (right) data representing correlation found between proliferation index (PI) (Ki-67, a and b; MCM-7, c and d) and p53 labelling index (LI) and between apoptotic index (AI) and p53 labelling index (e and f).

hypothesize that the dramatic increase of p53 expression observed in GBM compared to PA (17.5% versus 0.55%) samples cannot be related to active control of proliferation.

There was a negative correlation between p53 expression and AI for PA and a positive one for GBM. Although the *p*-values were below the threshold of significance. The negative correlation observed might indicate that p53 protein, which is still functionally active in low grade astrocytoma (22) is able to drive the DNA repair mechanisms, allowing cell cycle progression and preventing apoptotic cell death. In contrast, in GBM no correlation was found between p53 expression and apoptosis probably because of functional alterations in the protein already

described for this malignancy (24). However, the observed increase in AI might be explained by the microenvironment adverse conditions, such as hypoxia and substrate adhesion loss, that are aggravated by malignancy (6, 25) rather than by activation of the p53 pathway.

In conclusion, a correlation of p53 protein expression with AI and PI was demonstrated in PA but not in GBM, probably because of mutated p53 in the latter.

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References

- 1 Vermeulen K, Berneman ZN and Van Bockstaele DR: Cell cycle and apoptosis. *Cell Prolif* 3: 165-175, 2003.
- 2 Majno G and Joris I: Apoptosis, oncosis, and necrosis. An overview of cell death. *Am J Pathol* 1: 3-15, 1995.
- 3 Kaufmann SH and Gores GJ: Apoptosis in cancer: cause and cure. *Bioessays* 22: 1007-1017, 2002.
- 4 Amirlak B and Couldwell WT: Apoptosis in glioma cells: review and analysis of techniques used for study with focus on the laser scanning cytometer. *J Neurooncol* 2: 129-145, 2003.
- 5 Harnden DG: The nature of ataxia-telangiectasia: problems and perspectives. *Int J Radiat Biol* 66(6 Suppl): 13-19, 1994.
- 6 McGill G and Fisher DE: Apoptosis in tumorigenesis and cancer therapy. *Front Biosci* 2: 353-379, 1997.
- 7 Forones NM, Carvalho AP, Giannotti-Filho O, Lourenco LG and Oshima CT: Cell proliferation and apoptosis in gastric cancer and intestinal metaplasia. *Arq Gastroenterol* 42: 30-34, 2005.
- 8 Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW and Vogelstein B: Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* 5393: 1497-1501, 1998.
- 9 Chipuk JE, Kuwana T, Bouchier-Hayes L, Droin NM, Newmeyer DD, Schuler M and Green DR: Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science* 5660: 1010-1014, 2004.
- 10 Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hostetter R, Cleary K, Bigner SH, Davidson N, Baylin S, Devilee P, Glover T, Collins FS, Weslon A, Modali R, Harris CC and Vogelstein B: Mutations in the p53 gene occur in diverse human tumour types. *Nature* 6250: 705-708, 1989.
- 11 Nozaki M, Tada M, Kobayashi H, Zhang CL, Sawamura Y, Abe H, Ishii N and VanMeir EG: Roles of the functional loss of p53 and other genes in astrocytoma tumorigenesis and progression. *Neuro-oncol* 2: 124-137, 1999.
- 12 Sarkar C, Ralte AM, Sharma MC and Mehta VS: Recurrent astrocytic tumours- a study of p53 immunoreactivity and malignant progression. *Br J Neurosurg* 4: 335-342, 2002.
- 13 Birner P, Piribauer M, Fischer I, Gatterbauer B, Marosi C, Ungersbock K, Rossler K, Budka H and Hainfellner JA: Prognostic relevance of p53 protein expression in glioblastoma. *Oncol Rep* 4: 703-707, 2002.
- 14 Heesters MA, Koudstaal J, Go KG and Molenaar WM: Proliferation and apoptosis in long-term surviving low grade gliomas in relation to radiotherapy. *J Neurooncol* 2: 157-165, 2002.
- 15 Fei P and El-Deiry WS: P53 and radiation responses. *Oncogene* 37: 5774-5783, 2003.
- 16 Carroll RS, Black PM, Zhang J, Kirsch M, Percec I, Lau N and Guha A: Expression and activation of epidermal growth factor receptors in meningiomas. *J Neurosurg* 2: 315-323, 1997.
- 17 Nakamura M, Konishi N, Tsunoda S, Hiasa Y, Suzuki T, Inui T and Sakaki T: Retinoblastoma protein expression and MIB-1 correlate with survival of patients with malignant astrocytoma. *Cancer* 2: 242-249, 1997.
- 18 Sarkar C, Karak AK, Nath N, Sharma MC, Mahapatra AK, Chattopadhyay P and Sinha S: Apoptosis and proliferation: correlation with p53 in astrocytic tumours. *J Neurooncol* 73: 93-100, 2005.
- 19 Ribeiro Mde C, Coutinho LM and Hilbig A: The role of apoptosis, cell proliferation index, bcl-2, and p53 in glioblastoma prognosis. *Arq Neuropsiquiatr* 62: 262-270, 2004.
- 20 Berroir S, Lafitte F, Heran F, Boissonnet H, Polivka M and Piekarski JD: Pilocytic astrocytoma: unusual feature. *J Neuroradiol* 28: 249-252, 2001.
- 21 Burger PC, Scheithauer BW and Vogel FS: *Surgical Pathology of the Nervous Systems and Its Coverings*. 3rd Ed. Churchill Livingstone, NY, 1991.
- 22 Patt S, Gries H, Giraldo M, Cervos Navarro J, Martin H, Janisch W and Brockmoller J: p53 gene mutations in human astrocytic brain tumours including pilocytic astrocytoma. *Hum Pathol* 27: 586-589, 1996.
- 23 Lang FF, Miller DC, Koslow M and Newcomb EW: Pathways leading to glioblastoma multiforme: a molecular analysis of genetic alteration in 65 astrocytic tumours. *J Neurosurg* 81: 427-436, 1994.
- 24 Ohgaki H, Schauble B, Zur H, von Ammon K and Kleishues P: Genetic alterations associated with the evolution and progression of astrocytic brain tumours. *Virchows Arch* 427: 113-118, 1995.
- 25 Kauffman-Zeh A, Rodriguez-Viciano P, Ulrich E, Gilbert C, Coffey P, Downward J and Evan G: Suppression of c-myc induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature* 385: 544-548, 1997.

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