J Neurooncol (2007) 83:91–93
DOI 10.1007/s11060-006-9292-0

LETTER TO THE EDITOR

Frequent hypermethylation of the DNA repair gene *MGMT* in long-term survivors of glioblastoma multiforme

Ramon Martinez · Gabriele Schackert · Ricard Yaya-Tur · Iñigo Rojas-Marcos · James G. Herman · Manel Esteller

Received: 30 October 2006 / Accepted: 6 November 2006 / Published online: 13 December 2006

Abstract We have performed a methylation-specific PCR approach to comparatively analyze the *MGMT* promoter methylation status in 186 glioblastomas (GBM) from patients with classic survival and nine from patients with long-term survival (LTS GBM). The methylation rate in LTS GBM was significantly higher (77.8% vs. 39.2%, $P = 0.033$) which suggests that *MGMT* hypermethylation is a frequent hallmark of LTS GBM and contributes to characterize this intriguing GBM subtype.

R. Martinez · G. Schackert
Department of Neurosurgery, University of Dresden, Dresden, Germany

R. Yaya-Tur
Medical Oncology Department of Valencia, Valencia, Spain

I. Rojas-Marcos
Neurology Department, Hôpital Pitié-Salpetrière, INSERM U495, Paris, France

J. G. Herman
The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University, Baltimore, Maryland, USA

M. Esteller (✉)
Cancer Epigenetics Laboratory, Spanish National Cancer Centre (CNIO), Melchor Fernandez Almagro 3, E-28029 Madrid, Spain
e-mail: mesteller@cnio.es

R. Martinez (✉)
Department of Neurosurgery, Klinikum Fulda, Acad. Hospital Philipps University of Marburg, Pacelliallee 4, D-36043 Fulda, Germany
e-mail: voelter.martinez@arcor.de

To the editor:

Since our first report describing the strong association between *MGMT* (O^6-methylguanine DNA methyltransferase) methylation and positive clinical response to carmustine in gliomas [1], further studies have confirmed this observation and expanded the findings to other alkylating agents such as temozolomide, nimustine, procarbazine, and even cyclophosphamide [2]. Hegi and colleagues [3] recently described how *MGMT* methylation predicts positive outcome in those patients with glioblastoma multiforme (GBM) treated with temozolomide and radiotherapy. We would like to add an interesting twist to this emerging story by referring to a specific subgroup of GBM patients: the long-term survivors (LTS), defined as those patients with a median survival time of more than 3 years. Up to 5% of all GBM patients show such a long-term survival. Clinical parameters such as younger age, high Karnofsky performance status (KPS) score, and a radical extent of surgical resection are associated with a better prognosis. Molecular parameters such as the overexpression of the protein p53, unaltered copy number of the epidermal growth factor receptor (*EGFR*) gene, loss of heterozygosity at chromosome regions 1p/19q, and low rate of tumor proliferation appear to correlate with long-term survival. Nevertheless, they may also be detected in GBM patients succumbing at the statistically anticipated time. We thought that *MGMT* inactivation might also play a role in LTS-GBM.

To address this issue, we studied the CpG island promoter methylation status of *MGMT* in 195 GBM tumors from consecutive patients who had been referred to hospitals in Spain, France, Germany and

USA between 1993 and 2002. The age of the patients ranged from 28 to 79 years. Informed consent for samples and data analysis from each patient was obtained and tissue collection was approved by the corresponding Institutional Review Board. 186 were classic GBM with a survival time of ≤1 year, whereas, nine were GBM patients surviving >3 years (LTS GBM). Survival time was defined as the time lapse from initial surgery to patient's death or, in LTS patients, the last contact. The median age of the LTS GBM patients was 47 years (SD: 10.4), not significantly differing from that of classic GBM patients. The median survival time of LTS GBM was 53 months (*SD*: 14.1), with two patients alive at the time of the most recent control. All classic GBM patients succumbed ≤12 months after index surgery. All patients underwent a standard therapy consisting of a maximal surgical resection, followed by external fractionated radiotherapy (mean: 58 Gy) and chemotherapy with alkylating agents. Forty patients received temozolomide as first-line adjuvant chemotherapy. In the rest of the cases, patients have received ACNU/ nimustine, or BCNU/ carmustine or procarbazine as first-line chemotherapy. Seven of nine LTS-GBM patients had undergone ACNU-based adjuvant chemotherapy, the other two patients, temozolomide.

Tumor tissue samples were evaluated by experienced pathologists at the corresponding institutions according to the World Health Organization (WHO) criteria. Tumor tissue was frozen in liquid nitrogen after removal and stored at –80°C. DNA was extracted according to standard protocols. Furthermore, formalin-fixed, paraffin-embedded tumor tissue was also used as an additional source of DNA. Methylation patterns in the CpG islands of *MGMT* were determined by chemical modification of unmethylated, rather than methylated, cytosines to uracil, and subsequent PCR using primers specific for either methylated or modified unmethylated DNA. DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using a Wizard DNA purification resin (Promega, Madison, Wis., USA), treated again with NaOH, precipitated with ethanol, and resuspended in water. Primer sequences for the unmethylated reaction were 5′-TTGGTGTTTTG ATGTTTGTAGGTTTTTGT-3′ (forward primer) and 5′-AACTCCACACTCTTCCAAAAACAAAACA-3′ (reverse primer). For the methylated reaction, they were 5′-TTTCGACGTTCTAGGTTTTCGC-3′ (forward primer) and 5′-GCACTCTTCCGAAAACGAAACG-3′ (reverse primer). A second set of MSP primers adjacent

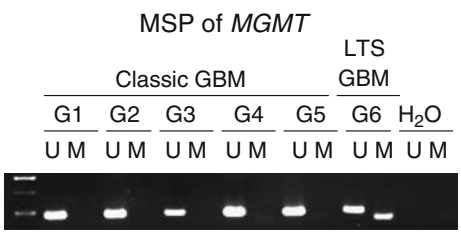

Fig. 1 Methylation analysis of the O^6-methylguanine DNA methyltransferase (*MGMT*) gene in classic glioblastoma (GBM) and long-term survivors (LTS GBM) by methylation-specific PCR. The presence of a PCR band under lanes M or U indicates methylated or unmethylated genes, respectively. Molecular weight marker is shown at the left and water control at the right. The LTS GBM sample G6 is methylated at the *MGMT* gene whereas the remaining classic GBM samples are unmethylated

to those aforementioned above were used and confirmed the results. These primer sequences are available upon request. The annealing temperature was 59°C. Placental DNA treated in vitro with *SssI* methyltransferase (New England Biolabs, Beverly, Mass., USA) was used as a positive control for methylated alleles, and DNA from normal lymphocytes was used as a negative control for methylated alleles. Controls without DNA were used for each MSP assay. Ten microliters of each 50 μl MSP product were loaded directly onto non-denaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination (Fig. 1).

In our set of GBM patients, we have found that *MGMT* methylation was present in 39.2% (73 of 186) of classic GBM survivor cases, as reported by Hegi et al. [3]. Strikingly, the long-term survival patients presented a significantly higher rate of *MGMT* hypermethylation, 77.8% (7 of 9 cases, $P = 0.033$, two-tailed Fisher's exact test). The presence of *MGMT* methylation was not associated with gender or age ($P > 0.05$, Chi square test).

Our data underscores the cumulative amount of reports that tight *MGMT* hypermethylation with sensitivity to alkylating agents and pinpoints that LTS GBM harbor *MGMT* hypermethylation as a frequent hallmark. The identification of further (epi)-genetic features related to this interesting subgroup of patients might be essential for the development of targeted therapies (for instance, *MGMT* inhibitors) for what is currently an incurable malignancy. Moreover, they may contribute to explain the broad spectrum of responses to alkylating drugs in *MGMT* unmethylated tumors, which suggests the existence of further chemoresistant factors.

References

1. Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, Baylin SB, Herman JG (2000) Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. N Engl J Med 343:1350-1354
2. Esteller M, Herman JG (2004) Generating mutations but providing chemosensitivity: the role of O^6-methylguanine DNA methyltransferase in human cancer. Oncogene 23:1–8
3. Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, Kros JM, Hainfellner JA, Mason W, Mariani L, Bromberg JE, Hau P, Mirimanoff RO, Cairncross JG, Janzer RC, Stupp R (2005) MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 352:997–1003