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CpG island promoter hypermethylation of the pro-apoptotic gene caspase-8 is a common hallmark of relapsed glioblastoma multiforme

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Glioblastoma multiforme (GBM) is an incurable malignancy with inherent tendency to recur. In this study, we have comparatively analyzed the epigenetic profile of 32 paired tumor samples of relapsed GBM and their corresponding primary neoplasms with special attention to genes involved in the mitochondria-independent apoptotic pathway. The CpG island promoter hypermethylation status was assessed by methylation-specific polymerase chain reaction and selected samples were double checked by bisulfite genomic sequencing. Thirteen genes were analyzed for DNA methylation: the pro-apoptotic *CASP8*, *CASP3*, *CASP9*, *DcR1*, *DR4*, *DR5* and *TMS1*; the cell adherence *CDH1* and *CDH13*; the candidate tumor suppressor *RASSF1A* and *BLU*; the cell cycle regulator *CHFR* and the DNA repair *MGMT*. The CpG island promoter hypermethylation profile of relapsed GBM in comparison with their corresponding primary tumors was identical in 37.5% of the cases, whereas in 62.5% of patients, differences in the DNA methylation patterns of the 13 genes were observed. The most prominent distinction was the presence of previously undetected *CASP8* hypermethylation in the GBM relapses ($P = 0.031$). This finding was also linked to the observation that an unmethylated *CASP8* CpG island together with methylated *BLU* promoter in the primary GBM was associated with prolonged time to tumor progression ($P = 0.0035$). Our data strongly suggest that hypermethylation of the pro-apoptotic *CASP8* is a differential feature of GBM relapses. These remarkable findings may foster the development of therapeutic approaches using DNA demethylating drugs and activators of the extrinsic apoptotic pathway to improve the dismal prognosis of GBM.

Introduction

Glioblastoma multiforme [GBM, World Health Organization (WHO) grade IV astrocytoma] is the most frequent primary brain tumor in adults. One of its characteristic features is the intrinsic tendency to recur despite aggressive therapy (1). Moreover, a considerable proportion of grade II and III astrocytomas undergoes progression to GBM (2). GBM, one of the most aggressive human malignancies, has a median survival time of 12 months. This has not significantly changed in the last 20 years despite advances in surgery, radiotherapy and chemotherapy (3,4). In a recent meta-analysis, a moderate increase in median survival time from 12.1 to 14.6 months was observed in GBM patients after multimodal therapy with gross total resection, radio- and chemotherapy with the alkylating drug temozolomide (5). Current anticancer therapy is directed to activate the mitochondria-dependent (intrinsic) apoptotic pathway (6), which will be stimulated by DNA damage induced by radiotherapy or chemotherapy drugs. This pathway activates *CASP9* through the release of mitochondrial cytochrome-c into the cytosol. The success rate of this treatment modality in GBM, with very few exceptions, is actually low. This therapy failure may be in part explained by the apoptosis-resistant phenotype of GBM, an extended hallmark in cancer (7). A previous study in relapsed GBM suggested that such a resistance to apoptosis is a phenomenon associated with the natural course of this malignancy rather than a consequence of external factors, like radiotherapy (8). These authors observed an up-regulation of the anti-apoptotic proteins BCL-2, BCLX and MCL-1 and down-regulation of the pro-apoptotic BAX in recurrent GBM compared with their primary tumors. This protein expression profile was encountered in GBM from both patients undergoing radiochemotherapy and those who did not.

Epigenetic alterations in DNA without mutations in the coding regions of associated cancer genes have been shown to be common events in the genesis and progression of tumors, especially the methylation-mediated silencing of tumor suppressor genes (TSGs) (9,10). In cancer cells, aberrant methylation of CpG islands located in the promoter regions of genes implicated in cell cycle, invasion, apoptosis or DNA repair is frequently associated with transcriptional silencing and gene repression (9,10). The former alterations of the 'epigenome' also contribute to define the biological behavior of the tumor and might modulate the response of tumor cells to anticancer therapies (11).

An evaluation of the epigenetic pattern associated with recurrent GBM with special attention to the extrinsic apoptotic pathway has not been performed before. The identification of these molecular features may be of capital importance, since better understanding of this process might provide clues for the development of efficient treatments. This scenario led us to examine the methylation pattern of a total set of 32 tumors consisting in relapsed GBM and their corresponding primary neoplasms that had undergone surgery and adjuvant therapy. In our study, we examined genes with critical cancer-related function. This included pro-apoptotic genes (*CASP8*, *CASP3*, *CASP9*, *DcR1*, *DR4*, *DR5* and *TMS1*), candidate TSGs (*RASSF1A* and *BLU*), cell adhesion-regulating genes (*CDH1* and *CDH13*), the cell cycle checkpoint regulator gene *CHFR* and the DNA repair gene *MGMT*.

Materials and methods

Patient population

All patients included in this series had undergone surgery with the goal of maximal possible tumor resection followed by external fractionated radiotherapy (mean dose: 58 gray). Thirty-two paired tumor samples were available from 16 patients, including 13 relapsed GBM and their corresponding primary glioblastoma, as well as three relapsed GBM locally progressing from WHO grade II astrocytomas. All patients were treated at the Department of Neurosurgery of the University of Dresden, Germany. Fresh tumor samples were frozen immediately in liquid nitrogen after removal and stored at −80°C. Representative tumor tissue specimens were evaluated by pathologists of the Institute of Pathology, University of Dresden, Germany, according to the WHO criteria. Informed consent for samples and data analysis from each patient or the patient's caretaker was obtained. DNA isolation was performed following standard procedures.

Analysis of CpG island promoter hypermethylation by methylation-specific polymerase chain reaction

DNA methylation patterns in the CpG islands of *CASP8*, *CASP3*, *CASP9*, *DcR1*, *DR4*, *DR5*, *TMS1*, *RASSF1A*, *BLU*, *CDH1*, *CDH13*, *CHFR* and *MGMT* were determined by chemical modification of unmethylated, but not the methylated, cytosines to uracil and subsequent polymerase chain reaction using primers specific for either methylated or the modified unmethylated DNA (12,13). One microgram of DNA was denatured by NaOH and modified

Abbreviations: GBM, glioblastoma multiforme; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TSG, tumor suppressor gene; TTP, time to tumor progression; WHO, World Health Organization.

by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega, Madison, WI), again treated with NaOH, precipitated with ethanol and re-suspended in water. Primer sequences for methylation analysis are shown in supplementary Table 1 available at *Carcinogenesis* online. Placental DNA treated *in vitro* with *Sss I* methyltransferase (New England BioLabs, Beverly, MA) was used as positive control for methylated alleles, and DNA from normal lymphocytes was used as negative control for methylated alleles. Controls without DNA were performed for each set of polymerase chain reaction. Ten microliters of each polymerase chain reaction was directly loaded onto non-denaturing 6% polyacrylamide gels, stained with ethidium bromide and visualized under UV illumination.

Statistical analysis

Mann–Whitney *U*-test, Student's *t*-test and chi-square test (with Mehta–Patel and Fisher–Yates corrections when appropriate) were performed to compare differences between groups depending on the analyzed variables. Spearman correlation test was used to assess the correlation between two parameters. Confidence interval was obtained through logistic regression. Kendall rank correlation test and sign test were performed to evaluate differences of methylation status of a given gene between primary and relapsed GBM. Kaplan–Meier analysis and log-rank tests were done to compare survival between groups defined by gene methylation status. The impact of gene methylation on time to tumor progression (TTP) was evaluated using the Cox hazards regression analysis. For the Cox regression analysis, proportional hazards were considered. Proportionality was tested by the method of Grambsch and Therneau. We estimated the univariate effect on methylation for each single gene and then we have included in the models major clinical predictors of outcome such as sex, gender and histology, which allowed us control for the potential confounding effects of these late variables. The final multivariate model included as covariates age, gender and histology. Likelihood ratio tests were used to compare candidate models. A value of $P < 0.05$ was considered to be significant. Analyses were performed with the SPSS software (version 10, SPSS, Chicago, IL).

Results

Clinico-pathological characteristics of the patients

The male to female ratio was 1:1. The median age at diagnosis of primary tumor was 56.5 years (range: 23–70 years, SD: 13.1). Patients with WHO grade II astrocytomas were, as expected, significantly younger at diagnosis ($P = 0.039$, *t*-test). The overall median TTP was 8.5 months (range: 1–72 months, SD: 14.4, 95% confidence interval: 5.07–13.66 months); when considering only cases with primary GBM, the median TTP was 8 months (range: 1–15 months, SD: 4.2). An association between age and TTP was not evidenced in our collective ($P = 0.35$, rho = 0.30, Spearman correlation test). Regarding tumor location, TTP was longer in patients with GBM located in temporal lobe ($P = 0.052$, Mann–Whitney *U*-test), suggesting a trend to better outcome.

CpG island promoter methylation analysis of the investigated genes

In 6 of 16 patients (37.5%), the CpG islands of the promoter regions of all 13 evaluated genes of the relapsed GBM had identical methylation status as the primary tumor. In 10 of 16 patients (62.5%), the DNA methylation pattern was different between the primary tumor and the relapsed GBM (Figure 1). In particular, a change in *CASP8* methylation was associated with relapsed GBM (7 of 16 patients, 43.8%, $P = 0.031$, sign test). Illustrative examples of the methylation analysis are shown in Figure 2. Significant differences of DNA methylation profile between primary (*de novo*) and secondary (evolving from low-grade astrocytoma) GBMs were not observed.

Evaluating the relationship between gene methylation and TTP, we noted that patients with methylated *BLU* in the primary GBM showed a significant longer TTP (median TTP: 17 months) compared with those patients who did not (median TTP: 7 months, $P = 0.002$, Mann–Whitney *U*-test). These findings were confirmed by Kaplan–Meier analysis ($P = 0.002$, Figure 3).

By multivariate Cox hazard analysis, an association between TTP and promoter methylation was observed for *CASP8* and *BLU*. In the final multivariate analysis, we observed that unmethylated *CASP8* associated with methylated *BLU* correlated with a longer TTP (median TTP: 17 months, $P = 0.003$). Additionally, methylated *CASP8*-together with unmethylated *BLU* was related with a shorter TTP (median TTP: 6 months, $P = 0.006$, Table I).

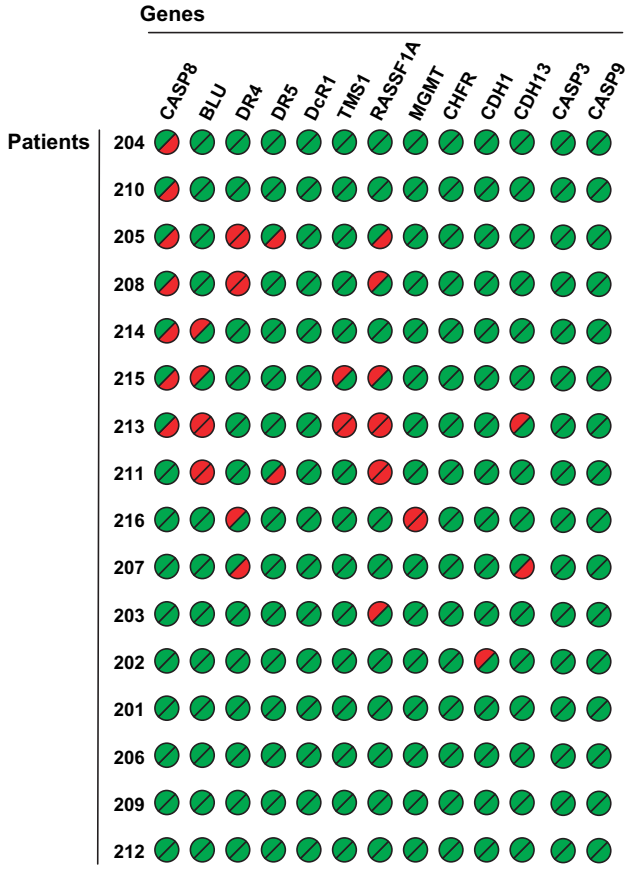

Fig. 1. Graph showing the promoter methylation status of 13 analyzed genes in the investigated neoplasms. Lower and upper semicircles represent GBM relapses and their corresponding primary tumors, respectively. The green color indicates unmethylated gene promoter and red color indicates hypermethylated promoter.

Methylated *CASP8* was observed in 83.3% of GBM with methylated *RASSF1A*. Additionally, methylated *CASP8* was observed in only 33.3% of GBM with unmethylated *RASSF1A*. Statistically, the co-occurrence of methylation of both genes was significant for both the primary and the relapsed GBM (both $P = 0.039$, Kendall rank correlation test).

Discussion

GBM, as an essentially incurable malignancy, still represents an enormous therapeutical challenge. Many efforts have been made to identify the mechanisms associated with recurrence and progression in order to design molecular tailored therapies. In a previous analysis, a significant proportion of relapsed GBM was observed to display a different genetic pattern compared with their primary neoplasms regarding TSGs, oncogenes and regulator genes implicated in cell adherence and invasion (14). Microarray studies of gene expression was added for this purpose and demonstrated that the malignant progression of grade II and III gliomas to GBM was associated with a distinct expression pattern of genes involved in cell proliferation, migration and aberrant neo-angiogenesis (15). GBMs typically show accumulation of alterations during the clonal expansion of transformated glial cells, which underlies the genetic and cytogenetic heterogeneity of intratumoral GBM regions, synchronous multilocular GBM and metachronous GBM (primary and corresponding relapse) (16–19),

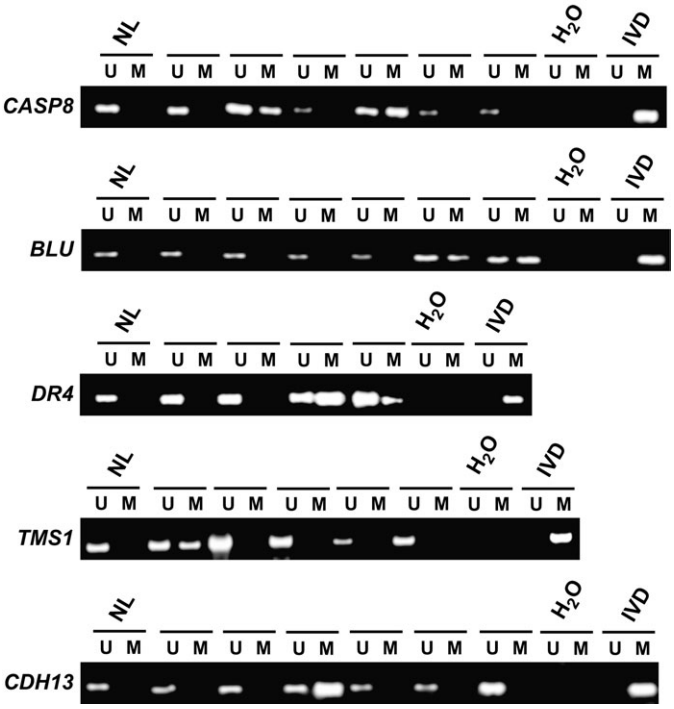

Fig. 2. Analysis of CpG island promoter methylation status in GBMs by the methylation-specific polymerase chain reaction assay. Molecular weight markers are shown in the left. The presence of a visible polymerase chain reaction product in those lanes marked 'U' indicates the presence of unmethylated genes; the presence of product in those lanes marked 'M' indicates the presence of methylated genes. Water (H_2O) was used as negative polymerase chain reaction control. *In vitro* methylated placental DNA (IVD) was used as positive control for methylated genes. DNA from normal lymphocytes (NLs) was used as negative control for methylated alleles.

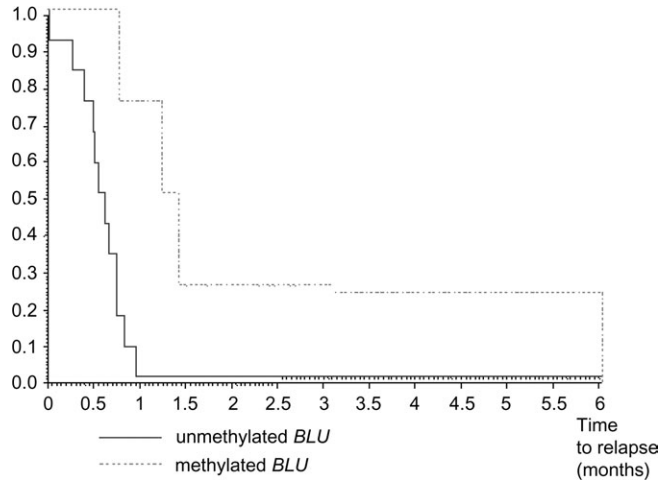

Fig. 3. Kaplan–Meier analysis of TTP and methylation status of the promoter of *BLU*. A significant correlation between *BLU* hypermethylation (dotted line) and TTP was observed ($P = 0.002$).

and contribute to explain the increasing genetic aberrations observed during the disease progression in the aforementioned studies.

Epigenetic fingerprints of specific human cancers were previously contoured (20) and a link between epigenetic changes and outcome of patients was proposed (21–24). In our study, we have observed that the former might also be applicable to human relapsed glioblastoma

Table I. Multivariate analysis of prognostic factors (Cox regression for TTP)

Variable	HR	95% CI	*P* (chi-square)
Methylation of *BLU* in primary tumor (yes/no)	0.004	0.001–0.025	0.003
Methylation of *BLU* in relapsed GBM (yes/no)	15.912	3.69–68.46	0.050
Methylation of *CASP8* in relapsed GBM (yes/no)	8.001	3.37–18.98	0.006

For this multivariate analysis, only methylation status of *CASP8* and *BLU* was significant predictor of TTP. HR, hazard ratio; CI, confidence interval.

since hypermethylation of genes implicated in key cell functions changed in 62.5% of the patients. A significant epigenetic silencing of the pro-apoptotic gene *CASP8* was observed during progression of primary to recurrent GBM ($P = 0.031$, sign test), probably conferring tumor cells a relevant further growth advantage. In brain tumors, *CASP8* is hypermethylated in 40% of neuroblastomas and medulloblastomas (25,26). Moreover, a high concordance between gene methylation, decreased mRNA levels and protein expression was observed as well (25). *CASP8* encodes a protein at the top of the mitochondria-independent apoptosis cascade (27). This pathway is triggered by ligation of death receptors, such as Fas- and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors (DR4 and DR5), by their cognate ligands (28). The ligation leads to activation of *CASP8* through an intracellular intermediate protein, Fas-associated protein with death domain. The activation of *CASP8* directly activates the executioner *CASP3*, which leads to dismantling of the cell. *CASP8* can also promote the activation of downstream caspases indirectly through proteolysis of BID (BH3 interacting domain death agonist protein), which then triggers the mitochondria-dependent apoptosis pathway (for review see ref. 29). TRAIL (like tumor necrosis factor and Fas ligand) is a potent inducer of apoptosis in different cell lines (30). The pro-apoptotic effect of TRAIL is counteracted by the TRAIL decoy receptors DcR1 and DcR2, which are homologues of DR4 and DR5 but defective in their death domains. Because of the preferential expression of these antagonistic receptors in normal tissues, it was postulated that both protect from TRAIL-induced apoptosis, competing with the DR for the ligand (31). Hypermethylation of *DR4* and *DR5* (up to 66.7%) as well as *DcR* (up to 25%) was observed in neuroblastomas (32). In non-central nervous system malignancies, higher methylation rates of *DcR1* and *DcR2* were reported (up to 100%), whereas *DR4* and *DR5* were rarely hypermethylated (33).

In contrast to the findings reported by van Noesel *et al.* (32) and Shivapurkar *et al.* (33), we did not observe CpG island hypermethylation of the potentially anti-apoptotic *DcR1* in any GBM as well as lower rates of hypermethylation of *DR4* and *DR5* (25 and 12.5%, respectively). Moreover, the executioner *CASP3* and the mitochondria-dependent *CASP9* remained unmethylated in primary and relapsed GBM. This scenario led us to suggest that the point of no return could be out of the mitochondrial cascade and that epigenetic deregulation of the extrinsic apoptosis involves *CASP8* and also, but less frequently, the death receptors. In this context, inactivation of the Fas-apoptotic pathway through hypermethylation of *CASP8*/*DRs* accompanied by unmethylated *DcRs* may favor the survival of remaining tumor cells after current treatment modalities, for instance, through extension of the time required for cellular dismantling. This is supported by the clinical observation that patients with methylated *CASP8* GBM showed a significantly shorter TTP, remarking the relevance of DNA methylation pattern in tumor behavior.

Concerning *MGMT*, epigenetic silencing of this DNA repair gene in GBM was previously observed to be associated with a better response to alkylating drugs such as BCNU [carmustine, 1,3-bis(2-chloroethyl)-1-nitrosourea] or temozolomide (5,34,35). Nevertheless, *MGMT* hypermethylation without associated alkylating chemotherapy was linked to a significant poor prognosis in different human tumors

(36) including grade II astrocytomas (37). In our study, *MGMT* hypermethylation was only found in one case, a grade II astrocytoma which locally progressed post-operatively to GBM after a very short time of 3 months, which agrees with the former study. Our results are also concordant with the observation that, in the pathway leading to GBM evolving from grade II astrocytomas, *MGMT* becomes hypermethylated at an early stage (38) and remains hypermethylated through the progression to the most malignant form, a situation which was also described in small adenomas and carcinomas of colorectal origin (39).

BLU, a putative TSG located at 3p21.3, just centromeric to *RASFF1A* in a gene-rich critical deleted region, was also objective of our attention, since its hypermethylation was described in both glioma cell lines and astrocytomas (40). Remarkably, we found that 75% of patients with methylated *BLU* also showed hypermethylation of *RASFF1A*. Whether methylation of both genes are independent events or, alternatively, they are the result of a region-wide DNA methylation cannot be elucidated. When relating clinical data and molecular findings, we observed that hypermethylation of *BLU* in primary GBM was significantly associated with a longer TTP, as suggested for neuroblastomas (41). Together with the results of *CASP8* methylation, one may speculate the existence of a subgroup of GBM showing methylated *BLU* and unmethylated *CASP8* with longer TTP, thus providing a molecular tool to identify GBM with better response to current radiochemotherapy. This point is currently under investigation.

We have provided evidence that hypermethylation of *CASP8* is a frequent feature of relapsed GBM compared with the corresponding primary tumors. This characteristic, together with the results of *CASP9* methylation analysis, strongly suggests that epigenetic deregulation of the mitochondria-independent apoptosis is a relevant characteristic in recurrent GBM. The development of targeted therapies restoring functional extrinsic apoptosis, as recently shown *in vivo* with the synergistic combination of the DNA demethylating agent decitabine and TRAIL (42), may provide a useful tool to overcome the resistance of GBM to contemporary treatment modalities.

Supplementary material

Supplementary table 1 can be found at http://carcin.oxfordjournals.org/.

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