TP53 Promoter Methylation in Primary Glioblastoma: Relationship with *TP53* mRNA and Protein Expression and Mutation Status

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Reduced expression of *TP53* by promoter methylation has been reported in several neoplasms. It remains unclear whether *TP53* promoter methylation is associated with reduced transcriptional and protein expression in glioblastoma (GB). The aim of our work was to study the impact of *TP53* methylation and mutations on *TP53* mRNA level and protein expression in 42 molecularly characterized primary GB tumors. We also evaluate the impact of all molecular alterations on the overall patient survival. The frequency of *TP53* promoter methylation was found in 21.4%. To the best of our knowledge, this is the first report showing such high frequency of *TP53* promoter methylation in primary GB. There was no relation between *TP53* promoter methylation and *TP53* mRNA level ($p=0.5722$) and between *TP53* promoter methylation and *TP53* protein expression ($p=0.2045$). No significant associations were found between *TP53* mRNA expression and mutation of *TP53* gene ($p=0.9076$). However, significant association between *TP53* mutation and *TP53* protein expression was found ($p=0.0016$). Our data suggest that in primary GB *TP53* promoter methylation does not play a role in silencing of *TP53* transcriptional and protein expression and is probably regulated by other genetic and epigenetic mechanisms associated with genes involved in the *TP53* pathway.

Introduction

GLIOMASTOMA (GB) (World Health Organization [WHO], G IV) is the most common primary tumor of the central nervous system in adults, with the most aggressive clinical course and fatal prognosis. Despite a better understanding of genetic alterations linked to GB, there has been little improvement in patient survival over the past decades. Before the era of first-line radio- and chemotherapy with alkylating agents, especially temozolomide (TMZ), median survival was below 1 year (Burger and Green, 1987). At present, by a combination of radiotherapy (RT) with the alkylating cytostatic drug TMZ median overall survival has been increased to 15 months (Stupp *et al.*, 2009).

There are two types of GBs: primary that developed *de novo* and secondary that developed by progression from low-grade astrocytomas. In primary GB, the most common molecular alterations are LOH 10q (over 70%), *EGFR* amplification (about 40%), *MDM2* amplification, LOH 10p and 10q, and *p16^{INK4a}* and *PTEN* mutation. In secondary GB, the first common molecular event in multistep carcinogenesis is the mutation of *IDH1*, *TP53*, and LOH on 17p, 10q, and 19q (Lang *et al.*, 1994; Ohgaki *et al.*, 2004; Ohgaki and Kleihus, 2009).

The *TP53* mutation occurs in about 30% of cases of primary GB. However, this alteration is found in 65%–90% of cases of secondary GB, making it the most important molecular indicator, and occurs early in tumorigenesis (59% in low-grade astrocytoma and 53% in anaplastic

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astrocytoma) (Ohgaki *et al.*, 2005). The *TP53* gene encodes a protein playing key roles in the cell cycle, response to DNA damage, cell differentiation, and cell death, and thus maintains genomic stability (Bögler *et al.*, 1995). Mutations of the *TP53* gene often cause changes in the conformation of the protein and lead to its inactivation and accumulation of mutated protein in the nuclei of tumor cells (Hainaut *et al.*, 1995).

In primary GB, *TP53* point mutations are equally distributed through the 5–8 exons (*hot-spot* mutations), whereas in secondary GB most frequent mutation point concerns codon 248 and 273 (exon 7 and 8), and in CpG sites (methylation region) (Ohgaki *et al.*, 2004). Interestingly, a mutation at codon 175 has been related to a shorter survival time of the patient (Ohgaki *et al.*, 2004). The altered function of *TP53* may result not only in mutations, but also from alterations in *MDM2*, *MDM4*, or *CDKN2A/p14^{ARF}*, which binds to *MDM2* and inhibits *MDM2*-mediated *TP53* degradation. The Cancer Genome Atlas (TCGA) Research Network project study has reported alterations in the *TP53/MDM2/CDKN2A* pathway in about 87% of investigated GBs (Cancer Genome Atlas Research Network, 2008). The majority of *TP53* mutations and examples of *TP53* loss of heterozygosity (54%) are found in the proneural type of GB that shows high expression of oligodendrocytic genes, and they were associated with a better prognosis and younger age (Verhaak *et al.*, 2010).

Primary GB, in contrast with secondary, has been associated with the presence of *EGFR* amplification and absence of *TP53* mutation (is considered mutually exclusive) (Ohgaki *et al.*, 2004). It is not known whether changes in *TP53* methylation status and expression is also mutually exclusive in GB.

Molecular markers that are related to better outcome of glioblastoma are *MGMT* promoter methylation and *IDH1* mutations. *MGMT* promoter methylation is associated with a stronger benefit of radiochemotherapy in GBs and *IDH1* mutations are a strong and independent predictor of survival in secondary GB (Stupp *et al.*, 2009; Yan *et al.*, 2009). Promoter methylation of *MGMT* was linked to the presence of G:C to A:T transition mutations in *TP53* in many types of cancer, including GB (Bello *et al.*, 2004). *IDH1* mutations mainly occurred in secondary GBs (>70%), associated with young age of patients and with an increase in overall survival, but very rare in primary GBs (<5%) and *IDH1* mutations is considered as a molecular marker discriminating secondary GBs from primary GBs (Nobusawa *et al.*, 2009).

Methylation in cancer often occurs in the promoter regions of tumor suppressor genes and may lead to inactivation of gene expression (Esteller and Herman, 2002). Hypermethylation of promoters usually occurs at CpG island regions in about 60% of human genes with specific tumor types (Bird, 1996). The association of *TP53* methylation and expression in GB is still not well known. Interestingly, the promoter of *TP53* does not contain CpG islands in the 5' region, and a basal promoter region of 85 bp (nt 760–844) is essential for enabling full promoter activity of *TP53*, either by directly blocking the binding transcription factor or by directing the binding of repressor proteins (Bienz-Tadmor *et al.*, 1985; Tuck and Crawford, 1989). Further, this region has been shown to be methylated in several cancers (Pogribny and James, 2002; Agirre *et al.*,

2003; Hurt *et al.*, 2006). Experimental evidence suggests that the regulation of *TP53* expression is complex and involves both translational (mainly in the case of post chronic genotoxic stress) (Reisman and Rotter, 1993), and post-translational mechanisms to control the intracellular levels of the p53 protein after acute DNA damage (Kastan *et al.*, 1991).

Treatment with 5-aza-2'-deoxycytidine (5-aza-dC) has been experimentally shown to lead to upregulated expression of *TP53* mRNA and protein in some, but not all, glioma cell lines, suggesting that promoter methylation is associated with reduced expression in some malignant glioma cell lines (Amatya *et al.*, 2005). In addition, in another study, 5-aza-dC may have induced a p53-dependent DNA damage-response pathway independent of *TP53* promoter methylation status without increasing transcript levels of the gene (Karpf *et al.*, 2001). Downregulation of p53 transcriptional level in cultured cells transfected with a plasmid incorporating a p53 promoter containing methylated CpG dinucleotides has been shown (Schroeder and Mass, 1997). Moreover, alterations in single-site CpG methylation in the *TP53* promoter region have been found to lead to initiation of *de novo* methylation and progressive spreading of methylation associated with transcriptional inactivation of the *TP53* gene, and reduced p53 expression in rats (Pogribny *et al.*, 2000).

Reduced level of *TP53* by methylation of promoter CpG dinucleotides has been reported in some neoplasms (Pogribny *et al.*, 2000; Kang *et al.*, 2001; Agirre *et al.*, 2003). However, whether *TP53* promoter methylation is associated with reduced transcriptional and translational expression in GB patients remains unclear.

This present study evaluates the status of the *TP53* gene, particularly the impact of methylation and mutation on mRNA and protein expression. The search was focussed on any mutual relationship existing between *TP53* alterations (methylation, abnormal mRNA level, mutations, and protein overexpression) and other molecular alterations (*MGMT* methylation and *EGFR* amplification). We also evaluate the impact of all molecular alterations on the overall survival.

Materials and Methods

Tumor samples and DNA extractions

Tumor brain tissue samples were obtained from 42 patients with primary GB (21 men and 21 women) who were treated in the Departments of Neurosurgery of "Copernicus" Memory Hospital in Lodz, Barlicki Clinical Hospital of Medical University of Lodz, Voivodal Specialistic Hospital in Olsztyn, and Perzyna Memorial Hospital in Kalisz, Poland from 2002 to 2005. Informed consent from patients was obtained in every case (agreement of Bioethical Committee of Medical University of Lodz, Poland RNN/192/03/KE). The age of patients ranged from 23 to 76 years, mean age was 59.1 ± 11.8 . All tumors were histopathologically examined and classified according to the WHO classification of tumors of the Central Nervous System (CNS) (Kleihus *et al.*, 2007).

The following criteria to determine primary GB has been applied: a short clinical history of less than 3 months and the presence of histopathological features of GB at the first biopsy without any evidence of precursor low-grade astrocytomas (Watanabe *et al.*, 1997). The molecular criteria confirming the

primary GB were frequency of *EGFR* amplification (37.5%), *IDH1* mutation (2.44%), and *TP53* mutation (26.2%). The results for *EGFR* amplification, *IDH1*, and *TP53* were partly published before (Jesionek-Kupnicka *et al.*, 2013).

All patients underwent total or partial surgery and RT and seven underwent chemotherapy (TMZ). TMZ was not used in standard protocol therapy in the years 2002–2005 in Poland.

DNA was isolated by standard proteinase K digestion and phenol/chloroform extraction from frozen tumor tissue samples were taken before radio- and/or chemotherapy. Histologically all specimens consisted of at least 80% tumor cells.

TP53 gene sequencing analysis

Four genomic regions of the *TP53* gene (exons 5–8) were amplified by polymerase chain reaction (PCR), as described previously (Wojcik *et al.*, 2005). Sequence analysis was performed by the dideoxy termination method using the SequiTherm Excel DNA Sequencing Kit (Epicentre Technologies, Madison, WI) and fluorescent-labeled primers as described previously (Jesien-Lewandowicz *et al.*, 2009). Products of the sequencing reaction were visualized and analyzed using a LiCor automated laser fluorescence sequencer.

IDH1 gene sequencing analysis

Exon 4, including codon 132 of the *IDH1* gene, was amplified by PCR and sequenced using the dideoxy termination method and SequiTherm Excel DNA Sequencing Kit (Epicentre Technologies). The primers used for PCR amplification of the DNA sequences were IDH1-5'-GGCACCCATCTTCTGTGTTT-3' (sense) and 5'-ATATATGCATTTCTCAATTTCA-3' (antisense). The sequencing primers used were IDH1 exon 4-5'-CGGTCTTCA GAGAAGCCATT-3' (sense) and IDH1 exon 4-5'-CA CATTATTGCCAACATGAC-3' (antisense). A Li-Cor automatic sequencer system was used for separation and analysis of PCR-sequencing products.

EGFR amplification analysis

Multiplex PCR was performed for evaluation of *EGFR* amplification with superoxide dismutase 1 (*SOD1*) used as a reference gene. PCR conditions and primer sequences were used as previously reported (Rieske *et al.*, 2009).

Methylation-specific PCR for TP53 and MGMT promoter methylation analysis

Sodium bisulfite modification of isolated genomic DNA was performed using the CpGenome DNA kit (Chemicon International, Inc., Temecula, CA) according to the manufacturer's protocol. The bisulfite-treated DNA was stored at -80°C until use. CpGenome Universal Methylated DNA (Chemicon International) was used as a methylation-positive control for the methylated alleles, and DNA from peripheral blood leukocytes was used as the control for unmethylated alleles. The methylation-specific PCR (MSP) for *TP53* promoter methylation was performed as previously described (Amatya *et al.*, 2005). MSP for *MGMT* promoter methylation was performed in a two-step approach as previously reported (Jesien-Lewandowicz *et al.*, 2009). For each PCR, methylated and unmethylated DNA was included as positive and negative controls, and water was used as a control for the PCR reaction. PCR products were separated on

3% agarose gels containing ethidium bromide and documented using the Gel Doc1000 Bio Rad Image System. Repeat testing was performed to confirm results.

RNA isolation

RNA was extracted by means of RNeasy Mini Kit (QIAGEN, Venlo, Netherlands) from tumor tissues and three normal brain tissues used as a reference. RNA samples were treated with DNAase. One hundred nanograms of total RNA was reverse-transcribed into single-stranded cDNA in a final volume of 40 μL containing 50 mM DTT, 1.5 μg oligo(dT), 0.5 mM dNTP, 40 U RNase inhibitor, and 200 U M-MLV reverse transcriptase (Promega Corporation, Madison, WI).

Real-time RT-PCR for TP53 mRNA expression

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed on a Rotor Gene 6000 instrument (Corbett, Life Sciences, Australia) for the *TP53* gene (TaqMan[®] Gene Expression Assays no Hs00153340_m1 and no Hs00153349_m1) and *GAPDH* (TaqMan Gene Expression Assays no Hs99999905_m1) was used as a reference gene for normalization of the target gene expression levels. All experiments were performed in duplicate and gene expression was analyzed using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmitteng, 2001).

The expression of the *TP53* was determined in 38 samples of primary GB (for four cases *TP53* expression was not determined). The threshold cycle (CT) indicates the fractional number at which the amount of amplified target reaches a fixed threshold. CT values for particular analyzed genes were normalized to *GAPDH* and related to control normal brain tissues. The level of *TP53* gene expression was increased in 34.2% (13 cases) of GBs considered as fold change greater than two. Diminished expression was defined as fold change lower than 0.5.

Immunohistochemistry for TP53 protein expression

The standard IHC technique was performed to determine the expression of TP53 in tumor samples using monoclonal antibody anti-p53 (clone DO-7, 1:100 dilution; DAKO, Glostrup, Denmark), and processed with EnVision (DAKO) system. The method was previously described in detail (Szybka *et al.*, 2009). The antibody labels wild-type and mutant-type p53 protein and is a useful tool for the identification of TP53 accumulation in human neoplasias. The normal TP53 protein has a very short half-life and is present in small amounts in normal cells (Fig. 1A). In contrast, mutant TP53 protein produced by malignant cells is usually a product of a point mutation in the *TP53* gene that significantly prolongs the half-life of the protein and is detected as positive staining (Fig. 1B, C). Tumor sections were examined for TP53 immunoreactivity under the microscope at 20 $\times$ and 40 $\times$ magnifications. The expression of TP53 was considered as positive when the proportion of positive cells was more than 10% (Fig. 1B, C) (Nagpal *et al.*, 2006). The negative TP53 protein staining in tumor section is presented on Figure 1D.

Statistical analysis

Continuous variables were compared using the Mann-Whitney's *U* test. Factorial analysis of variance (ANOVA)

FIG. 1. (A) The lack of immunoreactivity in normal non-neoplastic white matter of brain outside the tumor (hematoxylin counterstaining, anti-TP53, DAKO, $\times 200$) scale bar, 10 μm ; (B, C) Strong nuclear immunohistochemical overexpression of TP53 protein in GB (hematoxylin counterstaining, anti-TP53, DAKO, $\times 400$ and 200) scale bars, 50 μm and 10 μm ; (D) The negative immunoreaction in tumor GB tissue (hematoxylin counterstaining, anti-TP53, DAKO, $\times 200$) scale bar, 10 μm . GB, glioblastoma.

was harnessed to evaluate influence of more than one nominal variable. Normal distribution fitting was assessed with usage of Shapiro–Wilk test and homoscedasticity was assessed with Levene’s test. Fisher’s exact test was used to test the associations between molecular alterations. Spearman rank test was used for correlations assessments. All results were considered statistically significant when two-sided p was <0.050 . STATISTICA 10.0 (Statsoft, Tulsa, OK) software and the VassarStats online calculator (www.vassarstats.net/) were used for statistical analysis.

Results

Associations between molecular alterations

TP53 alterations. The *TP53* mutations were identified in 26.2% (11/42) of cases within exons 5, 6, 7, and 8. All the mutations of *TP53* were missense. *TP53* promoter methylation was detected in 9/42 cases (21.4%). An increased transcriptional level of *TP53* was detected in 13/38 cases (34.2%) and a decreased level in 6/38 (15.8%). The remaining samples were classified as neutral expression (Table 1). Four cases were not analyzed for *TP53* expression.

There was no relation between *TP53* mRNA level and promoter methylation (Median for group with methylation (Me) 1.41 (interquartile range [IQR] 0.64–16.00) vs. Me for group without methylation 1.19 (IQR 0.78–2.55); (Fig. 2; Mann–Whitney’s U test $p=0.5722$).

Moreover, there was no significant relation between *TP53* mRNA expression and any mutation in *TP53* (Me for group with mutation 1.49 [IQR 1.04–2.30] vs. Me 1.09 [IQR 0.75–3.04]; Mann–Whitney’s U test $p=0.9076$) or transition mutation in *TP53* (Me for group with transition 1.30 [IQR 0.68–7.65] vs. Me 1.34 [IQR 0.78–2.83]; Mann–Whitney’s U test $p=0.9857$). Relation between *TP53* promoter methylation occurs irrespective of the *TP53* mutation status (Fisher’s exact test $p=0.2086$).

There was no significant interaction between methylation and mutation status and *TP53* level (Fig. 3; p ANOVA=0.7867).

Protein of *TP53* gene was present in 12 cases (37.5%). No significant association between mRNA *TP53* level and protein expression were found (Me for group with present protein 1.41 [IQR 1.04–13.00] vs. Me 1.07 [IQR 0.64–3.25]; Mann–Whitney’s U test $p=0.5612$). Significant association between *TP53* mutation and TP53 protein expression was found (21.88% of total had both mutation and protein expression vs. 59.38% had none of them; Fisher’s exact test $p=0.0016$) (Fig. 4). The same pattern was observed for group with *TP53* transition (15.63% of total had both transition and protein expression vs. 59.38% had none of them; Fisher’s exact test $p=0.0185$) and for *TP53* methylation (15.63% of total had both methylation and protein expression vs. 53.13% had none of them; Fisher’s exact test $p=0.2045$) even though the latter was not significant. The results of all alterations are presented in Table 1.

EGFR amplification

There was no relation between *EGFR* amplification and TP53 expression (Me for group with amplification 1.02 [IQR 0.78–1.93] vs. Me 1.49 [IQR 0.71–2.83]; Mann–Whitney’s U test $p=0.5812$), *TP53* mutation (5.0% of total had both abnormalities vs. 40.0% had none of the regarded abnormalities; Fisher’s exact test $p=0.1582$), *TP53* transition (5.0% of total had both abnormalities vs. 47.5% had none of the regarded abnormalities; Fisher’s exact test $p=0.6857$) or *TP53* methylation (2.5% of total had both abnormalities vs. 42.5% had none of the regarded abnormalities; Fisher’s exact test $p=0.1166$). Relation between *EGFR* amplification and TP53 expression occurs irrespective of the *TP53* mutation status (Fisher’s exact test $p=1.0$).

TABLE 1. CLINICAL DATA AND RESULTS OF MOLECULAR ANALYSIS

	Valid N	Median (interquartile range)		Missing data (N)
Age (years)	42	61 (51–68)		0
Overall survival (months)	42	11.5 (8–16)		0
qRT-PCR Fold Change of <i>TP53</i>	38	1.3 (0.78–2.83)		4
Sex	Male (N) 21 (50.00%)	Female (N) 21 (50.00%)		0
<i>TP53</i> expression	Down 6 (15.79%)	Up 13 (34.21%)	Equal 19 (50.00%)	4
All types of <i>TP53</i> mutation	Absent 31 (73.81%)	Present 11 (26.19%)		0
<i>TP53</i> transition GC>AT	Absent 34 (80.95%)	Present 8 (19.05%)		0
<i>TP53</i> methylation	Absent 33 (78.57%)	Present 9 (21.43%)		0
<i>TP53</i> protein presence	Absent 20 (62.50%)	Present 12 (37.50%)		10
<i>MGMT</i> methylation	Absent 12 (28.57%)	Present 30 (71.43%)		0
<i>EGFR</i> amplification	Absent 25 (62.50%)	Present 15 (37.50%)		2

qRT-PCR, quantitative reverse transcription polymerase chain reaction.

MGMT methylation

There was no relation between *MGMT* methylation and *TP53* methylation (Fisher's exact test $p=0.6987$) or *TP53* mRNA expression (Me for group with *MGMT* methylation 1.57 [IQR 0.81–7.21] vs. Me 1.07 [IQR 0.59–2.55]; Mann–Whitney's U test $p=0.3424$). Also no relation was found significant between *MGMT* methylation and *TP53* mutation (23.81% of total had both abnormalities vs. 26.2% had none of the regarded abnormalities; Fisher's exact test $p=0.1333$), and between *MGMT* methylation and transition (16.7% of total had both abnormalities vs. 26.2% had none

of the regarded abnormalities; Fisher's exact test $p=0.4024$) or *EGFR* amplification (25.0% of total had both abnormalities vs. 15.0% had none of the regarded abnormalities; Fisher's exact test $p=0.7162$).

The TP53 alterations and influence on survival

The *TP53* mutation. There was no difference in survival between patients with *TP53* mutation ($n=11$) (median survival 15.0 months; IQR 7–18 months) and without *TP53* mutation ($n=31$) (median survival 12 months; IQR 7–16 months; Mann–Whitney's U test $p=0.8976$).

FIG. 2. No significant relation between *TP53* mRNA level and promoter methylation ($p=0.5722$). Horizontal lines represent medians with lower and upper quartile boundaries. Each individual data-point is represented on the figure.

FIG. 3. No relation between *TP53* mRNA level depending on *TP53* mutation status was found ($p=0.7867$). Horizontal lines represent medians with lower and upper quartile boundaries. Each individual datapoint is represented on the figure.

TP53 methylation

Median survival in patients with *TP53* methylation ($n=9$) was 11.0 months (IQR 9–15 months). Median survival in patients without *TP53* methylation ($n=33$) was 12 months (IQR 9–17 months). There was no significant difference in median survival between patients with different *TP53* methylation status (Mann–Whitney's U test $p=0.4079$).

Expression of mRNA *TP53*

Median survival for patients with decreased *TP53* mRNA expression ($n=6$) was 11 months (IQR 8–20 months), for

patients with increased mRNA *TP53* expression ($n=13$) was 12 months (IQR 9–12 months) and with unaltered expression ($n=19$) was 12 months (IQR 8–18 months). The difference between three groups were not significant (Kruskal–Wallis' test, factorial ANOVA $p=0.5943$).

P53 protein expression

No difference in overall survival between patient with present ($n=12$) and absent ($n=20$) *TP53* protein was found (Me for group with protein expression 11 months [IQR 5.5–13.0 months] vs. Me 12 months [IQR 8.5–16.5 months]; Mann–Whitney's U test $p=0.3403$). Relation between *TP53*

FIG. 4. Relation between *TP53* mutation status and *TP53* protein presence ($p=0.0016$).

protein expression and *EGFR* amplification was not significant (13.33% of total had both *EGFR* amplification and protein expression vs. 30.00% had none of them; Fisher's exact test $p=0.4651$).

EGFR amplification

EGFR amplification was identified in 37.5% (15/40) (Table 1). Median survival in patients with *EGFR* amplification ($n=15$) was 12.0 months (IQR 5–14 months), while in patients without *EGFR* amplification ($n=25$), it was 11.0 months (IQR 8–18 months). The difference in survival time was not significant (Mann–Whitney's U test $p=0.5388$). No significant relation between *MGMT* expression and *EGFR* amplification was found (Me for group with amplification 0.018 [IQR 0.008–0.149] vs. Me 0.026 [IQR 0.004–0.074]; Mann–Whitney's U test $p=0.4854$).

MGMT methylation

Methylation of *MGMT* promoter gene was detected in 30/42 (71.43%). Median survival in patients with *MGMT* promoter methylation ($n=30$) was 12.0 months (IQR 8–14 months), while median survival without *MGMT* promoter methylation ($n=12$) was 10 months (IQR 6.5–17 months). The difference between two groups was not significant (Mann–Whitney's U test $p=0.6967$).

The IDH1 mutation

IDH1 mutation was found only in one of 41 examined cases (2.44%). One case was not available for *IDH1* mutation study.

Discussion

It has been experimentally demonstrated that a reduction in the dosage of wild-type *TP53* genes without mutation is sufficient to promote tumorigenesis (Venkatachalam *et al.*, 1998), and this is congruent with the possibility that *TP53* promoter region methylation and reduced *TP53* gene expression contribute to the selection and expansion of pre-neoplastic cells. Although, a reduced level of *TP53* by methylation of promoter CpG dinucleotides has been reported in acute lymphoblastic lymphoma (Agirre *et al.*, 2003) and in hepatocellular carcinoma (Pogribny and James, 2002) such a relationship is not clear in GB.

In our study, the frequency of *TP53* promoter methylation in primary GB was found in 21.4%. This report shows the high frequency of *TP53* promoter methylation in primary GB. There was no relation between *TP53* promoter methylation and *TP53* protein and mRNA level. As far as we are concerned there are no other publications concerning the relationship between *TP53* mRNA and protein expression and methylation in primary GB patients. These observations suggest that in primary GB *TP53* promoter methylation does not play a role in regulation of *TP53* expression. These results indicate that in primary GB the transcriptional and translational *TP53* level is probably controlled by other genetic and epigenetic mechanisms associated with genes involved in the *TP53* pathway. Our finding that *TP53* promoter methylation occurs irrespective of the *TP53* mutation status is consistent with previous observations (Amatya *et al.*, 2005). It can be assumed that the mechanism of *TP53*

level regulation is more complex and *TP53* promoter methylation plays only an adjective epigenetic role in GB. Indeed, in whole-genome integrative analysis in GB, other genes with concordant CpG sites have been found to display an inverse association between both promoter methylation and expression level: *B3GNT5*, *FABP7*, *ZNF217*, *BST2*, *OAS1*, *SLC13A5*, *GSTM5*, *ME1*, *UBXD3*, *TSPYL5*, *FAAH*, *C7orf13*, and *C3orf14* (Etcheverry *et al.*, 2010). Silencing by DNA hypermethylation of other genes involved in key cellular functions like cell cycle (*p16^{INK4a}* and *p15^{INK4b}*), DNA repair and genome integrity (*MGMT* and *MLH1*), and tumor suppression (*RB1*, *VHL*, *EMP3*, *RASSF1A*, *BLU*), in addition to tumor invasion and apoptosis (*DAPK*, *TIMP3*, *CDH1*, *PCDH-gamma-A11*, *TMS1/ASC*) has been involved in gliomagenesis [rev. in Martinez *et al.* (2009)]. Interestingly, a large cDNA-array study did not reveal any gene cluster separating groups of gliomas on the basis of their *TP53* status (Godard *et al.*, 2003); this may be due to the fact that in >70% of all astrocytic gliomas, the *TP53* pathway has been found to be inactivated not only by mutational alteration of *TP53*, but also by a variety of alternative mechanisms: overexpression of *MDM2*, (a negative regulator of *TP53*) or inactivation of *p14^{ARF}* (a negative regulator of *MDM2*) or by deletion or promoter methylation of these genes (Ichimura *et al.*, 2000). *p14^{ARF}* was found to induce stabilization of the *TP53* transcription factor, leading to the expression of critical *TP53* target genes, which can mediate cell cycle arrest or induce apoptosis [rev. in Zerrouqi *et al.* (2012)]. This confirms a recent study of TCGA Research Network, which revealed a cluster of genes whose drastic over- and under-expression is associated with *TP53* mutation status (Cancer Genome Atlas Research Network, 2008). Among other things, *MDM2* is overexpressed when *TP53* is wild type: that is, *MDM2* overexpression is mutually exclusive with *TP53* mutation (Masica and Karchin, 2011).

The frequency of *TP53* methylation in gliomas depends on the used primers. *TP53* promoter methylation is frequent in low-grade gliomas but is not well known in GB. In one study, *TP53* presented CpG island hypermethylation constituted only 2% in GB, and 8% in diffuse astrocytomas and anaplastic astrocytomas (Gonzalez-Gomez *et al.*, 2003). Using other MSP primers (nt 632–894 and nt 636–915) in Amatya *et al.* (2005) study, *TP53* promoter methylation frequency reached 60% of low-grade astrocytomas, 61% of oligoastrocytomas, and 73% of oligodendrogliomas, but GBs have not been studied. In the present study, using the same primers for the region as mentioned above 21.4% of *TP53* promoter methylation was detected. Such high frequency of *TP53* promoter methylation in primary GB was not previously mentioned in literature. Although, there was also no significant relation between *TP53* mRNA expression and any mutation in *TP53* gene, a significant association between *TP53* mutation and *TP53* protein expression was found, which is consistent with other studies. A correlation between *TP53* mutations and the accumulation of *TP53* in the nucleus has been observed in many types of neoplasms, including GB (Newcomb *et al.*, 1993; Soong *et al.*, 1996). Stabilization of *TP53* is tightly regulated by ubiquitination. *TP53* is mainly ubiquitinated by *MDM2* ubiquitin but other ubiquitin ligases such as *ARF-BP1* and *MdmX* are also involved in the regulation leading to *TP53* proteasomal degradation (Chen *et al.*, 2005; Wang *et al.*, 2011). *MDM2*

is a key negative regulator of TP53 function. The TP53-MDM2 autoregulatory feedback loop regulates activity of the TP53 protein and expression of MDM2; wild-type TP53 protein induces transcription of the *MDM2* gene and MDM2 protein inhibits the ability of TP53 to activate transcription (Momand *et al.*, 1992). This tight control is lost in tumors harboring mutant TP53, which exhibits a significant TP53 stabilization compared with wild-type TP53 tumors. In human cancer cells mutant TP53, despite its ability to interact with MDM2, suffers from lack of ubiquitination and thus diminished TP53 degradation (Li *et al.*, 2011).

TP53 mutations and *EGFR* amplification were considered as mutually exclusive (Ohgaki *et al.*, 2004). However, in the present study there were two cases with coincidental *EGFR* amplification and TP53 mutations; these results have been partly published previously (Jesionek-Kupnicka *et al.*, 2007). Indeed, the copresentation of TP53 mutations and *EGFR* amplification was sporadically noticed in single cases of GB (Ueki *et al.*, 2002; Okada *et al.*, 2003; Gil-Benso *et al.*, 2007). Simultaneous *EGFR/TP53* alterations and the *CDK4* amplification had the adverse prognostic value in the overall survival along with age, and RT in patients with primary GB (Ruano *et al.*, 2009). In this study there was no relation between *EGFR* amplification and TP53 expression and methylation status. In this study the frequency of *IDH1* mutation was low, which is typical for primary glioblastoma and correspond to other studies (Ichimura *et al.*, 2009; Yan *et al.*, 2009).

In our study, the most common molecular alteration in primary GB was *MGMT* methylation (71.4%), which is consistent with our previous study (Jesien-Lewandowicz *et al.*, 2009). There was no relation between *MGMT* methylation and TP53 mutation (including transitions G:C>A:T) in contrast to other studies (Nakamura *et al.*, 2000; Shamsara *et al.*, 2009).

In terms of clinical outcome, there was no impact of mRNA TP53 levels on survival in patients. Interestingly, Blough *et al.* demonstrated that TP53 status may influence response to TMZ in differentiated cells in a GB. Glioma cell lines that did not express a functional p53 were significantly more sensitive to TMZ than cell lines with functionally intact wild-type p53 expression, an effect that is independent of *MGMT* status (Blough *et al.*, 2011).

In conclusion, our data suggest that in GB TP53 promoter methylation does not play a role in silencing of TP53 transcriptional and translational expression and is probably regulated by other genetic and epigenetic mechanisms associated with genes involved in the TP53 pathway.

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Disclosure Statement

No competing financial interests exist.

References

Agirre, X., Vizmanos, J.L., Calasanz, M.J., García-Delgado, M., Larráyo, M.J., and Novo, F.J. (2003). Methylation of CpG

- dinucleotides and/or CCWGG motifs at the promoter of *TP53* correlates with decreased gene expression in a subset of acute lymphoblastic leukemia patients. *Oncogene* **22**, 1070–1072.
- Amatya, V.J., Naumann, U., Weller, M., and Ohgaki, H. (2005). TP53 promoter methylation in human gliomas. *Acta Neuropathol* **110**, 178–184.
- Bello, M.J., Alonso, M.E., Amiñoso, C., Anselmo, N.P., Arjona, D., Gonzalez-Gomez, P., *et al.* (2004). Hypermethylation of the DNA repair gene *MGMT*: association with TP53 G:C to A:T transitions in a series of 469 nervous system tumors. *Mutat Res* **554**, 23–32.
- Bienz-Tadmor, B., Zakut-Houri, R., Libresco, S., Givol, D., and Oren, M. (1985). The 5' region of the p53 gene: evolutionary conservation and evidence for a negative regulatory element. *EMBO J* **4**, 3209–3213.
- Bird, A.P. (1996). The relationship of DNA methylation to cancer. *Cancer Surv* **28**, 87–101.
- Blough, M.D., Beauchamp, D.C., Westgate, M.R., Kelly, J.J., and Cairncross, J.G. (2011). Effect of aberrant p53 function on temozolomide sensitivity of glioma cell lines and brain tumor initiating cells from glioblastoma. *J Neurooncol* **102**, 1–7.
- Bögler, O., Huang, H.J., Kleihues, P., and Cavenee, W.K. (1995). The p53 gene and its role in human brain tumors. *Glia* **15**, 308–327. Review.
- Burger, P.C., and Green, S.B. (1987). Patient age, histological features, and length of survival in patients with glioblastoma multiforme. *Cancer (Phila.)* **59**, 1617–1625.
- Cancer Genome Atlas Research Network. (2008). Comprehensive genomic characterization defines human glioblastoma genes and core pathways. *Nature* **455**, 1061–1068.
- Chen, D., Kon, N., Li, M., Zhang, W., Qin, J., and Gu, W. (2005). ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor. *Cell* **121**, 1071–1083.
- Esteller, M., and Herman, J.G. (2002). Cancer as an epigenetic disease: DNA methylation and chromatin alterations in human tumours. *J Pathol* **196**, 1–7.
- Etcheverry, A., Aubry, M., de Tayrac, M., Vauleon, E., Boniface, R., Guenot, F., *et al.* (2010). DNA methylation in glioblastoma: impact on gene expression and clinical outcome. *BMC Genomics* **11**, 701.
- Gil-Benso, R., Lopez-Gines, C., Benito, R., López-Guerrero, J.A., Callaghan, R.C., Pellín, A., *et al.* (2007). Concurrent *EGFR* amplification and TP53 mutation in glioblastomas. *Clin Neuropathol* **26**, 224–231.
- Godard, S., Getz, G., Delorenzi, M., Farmer, P., Kobayashi, H., Desbaillets, I., *et al.* (2003). Classification of human astrocytic gliomas on the basis of gene expression: a correlated group of genes with angiogenic activity emerges as a strong predictor of subtypes. *Cancer Res* **63**, 6613–6625.
- Gonzalez-Gomez, P., Bello, M.J., Arjona, D., Lomas, J., Alonso, M.E., De Campos, J.M., *et al.* (2003). Promoter hypermethylation of multiple genes in astrocytic gliomas. *Int J Oncol* **22**, 601–608.
- Hainaut, P., Butcher, S., and Milner, J. (1995). Temperature sensitivity for conformation is an intrinsic property of wild-type p53. *Br J Cancer* **71**, 227–231.
- Hurt, E.M., Thomas, S.B., Peng, B., and Farrar, W.L. (2006). Reversal of p53 epigenetic silencing in multiple myeloma permits apoptosis by a p53 activator. *Cancer Biol Ther* **5**, 1154–1160.
- Ichimura, K., Bolin, M.B., Goike, H.M., Schmidt, E.E., Moshref, A., and Collins, V.P. (2000). Dereglulation of the p14ARF/MDM2/p53 pathway is a prerequisite for human

- astrocytic gliomas with G1-S transition control gene abnormalities. *Cancer Res* **60**, 417–424.
- Ichimura, K., Pearson, D.M., Kocialkowski, S., Bäcklund, L.M., Chan, R., Jones, D.T., *et al.* (2009). IDH1 mutations are present in the majority of common adult gliomas but rare in primary glioblastomas. *Neuro Oncol* **11**, 341–347.
- Jesien-Lewandowicz, E., Jesionek-Kupnicka, D., Zawlik, I., Szybka, M., Kulczycka-Wojdala, D., Rieske, P., *et al.* (2009). High incidence of MGMT promoter methylation in primary glioblastomas without correlation with TP53 gene mutations. *Cancer Genet Cytogenet* **188**, 77–82.
- Jesionek-Kupnicka, D., Kulczycka, D., Rieske, P., Szybka, M., Jabłonska, J., Potemski, P., *et al.* (2007). The infrequent simultaneous genetic alterations in glioblastoma multiforme (LOH 10, 17, 19q, TP53 mutation and EGFR amplification) with short clinical course. *Pol J Pathol* **58**, 79–85.
- Jesionek-Kupnicka, D., Szybka, M., Potemski, P., Kulczycka-Wojdala, D., Jaskólski, D., Bieńkowski, M., *et al.* (2013). Loss of heterozygosity (LOH) association with shorter survival in primary glioblastoma patients. *Pol J Pathol* **64**, 268–275.
- Kang, J.H., Kim, S.J., Noh, D.Y., Park, I.A., Choe, K.J., Yoo, O.J., *et al.* (2001). Methylation in the p53 promoter is a supplementary route to breast carcinogenesis: correlation between CpG methylation in the p53 promoter and the mutation of the p53 gene in the progression from ductal carcinoma *in situ* to invasive ductal carcinoma. *Lab Invest* **81**, 573–579.
- Karpf, A.R., Moore, B.C., Ririe, T.O., and Jones, D.A. (2001). Activation of the p53 DNA damage response pathway after inhibition of DNA methyltransferase by 5-aza-2'-deoxycytidine. *Mol Pharmacol* **59**, 751–757.
- Kastan, M., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R.W. (1991). Participation of p53 protein in the cellular response to DNA damage. *Cancer Res* **51**, 6304–6311.
- Kleihues, P., Burger, P.C., Aldape, K.D., Brat, D.J., Biernat, W., Bigner, D.D., *et al.* (2007). Glioblastoma. In *Classification of Tumours of the Central Nervous System*, D.N. Louis, H. Ohgaki, O.D. Wiestler, W.K. Cavenee, eds. (WHO IARC, Lyon), Chapter 1, pp. 33–46.
- Lang, F.F., Miller, D.C., Koslow, M., and Newcomb, E.W. (1994). Pathways leading to glioblastoma multiforme: a molecular analysis of genetic alteration in 65 astrocytic tumors. *J Neurosurg* **81**, 427–436.
- Li, D., Marchenko, N.D., Schulz, R., Fischer, V., Velasco-Hernandez, T., Talos, F., *et al.* (2011). Functional inactivation of endogenous MDM2 and CHIP by HSP90 causes aberrant stabilization of mutant p53 in human cancer cells. *Mol Cancer Res* **9**, 577–588.
- Livak, K.J., and Schmitteng, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–408.
- Martinez, R., Martin-Subero, J.I., Rohde, V., Kirsch, M., Alaminos, M., Fernandez, A.F., *et al.* (2009). A microarray-based DNA methylation study of glioblastoma multiforme. *Epigenetics* **4**, 255–264.
- Masica, D.L., and Karchin, R. (2011). Genes important in human glioblastoma progression and survival. *Cancer Res* **71**, 4550–4561.
- Momand, J., Zambetti, G.P., Olson, D.C., George, D., and Levine, A.J. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* **69**, 1237–1245.
- Nagpal, J., Jamoona, A., Gulati, N.D., Mohan, A., Braun, A., Murali, R., *et al.* (2006). Revisiting the role of p53 in primary and secondary glioblastomas. *Anticancer Res* **26**, 4633–4639.
- Nakamura, M., Yang, F., Fujisawa, H., Yonekawa, Y., Kleihues, P., and Ohgaki, H. (2000). Loss of heterozygosity on chromosome 19 in secondary glioblastomas. *J Neuropathol Exp Neurol* **59**, 539–554.
- Newcomb, E.W., Madonia, W.J., Pisharody, S., Lang, F.F., Koslow, M., and Miller, D.C. (1993). A correlative study of p53 protein alteration and p53 gene mutation in glioblastoma multiforme. *Brain Pathol* **3**, 229–235.
- Nobusawa, S., Watanabe, T., Kleihues, P., and Ohgaki, H. (2009). IDH1 mutations as molecular signature and predictive factor of secondary glioblastomas. *Clin Cancer Res* **15**, 6002–6007.
- Ohgaki, H., Dessen, P., Jourde, B., Horstmann, S., Nishikawa, T., Di Patre, P.L., *et al.* (2004). Genetic pathways to glioblastoma: a population-based study. *Cancer Res* **64**, 6892–6899.
- Ohgaki, H., and Kleihues, P. (2005). Population-based studies on incidence, survival rates, and genetic alterations in astrocytic and oligodendroglial gliomas. *J Neuropathol Exp Neurol* **64**, 479–89.
- Ohgaki, H., and Kleihues, P. (2009). Genetic alterations and signaling pathways in the evolution of gliomas. *Cancer Sci* **100**, 2235–2241.
- Okada, Y., Hurwitz, E.E., Esposito, J.M., Brower, M.A., Nutt, C.L., and Louis, D.N. (2003). Selection pressures of TP53 mutation and microenvironmental location influence epidermal growth factor receptor gene amplification in human gliomas. *Cancer Res* **63**, 413–416.
- Pogribny, I.P., and James, S.J. (2002). Reduction of p53 gene expression in human primary hepatocellular carcinoma is associated with promoter region methylation without coding region mutation. *Cancer Lett* **176**, 169–74.
- Pogribny, I.P., Pogribna, M., Christman, J.K., and James, S.J. (2000). Single-site methylation within the p53 promoter region reduces gene expression in a reporter gene construct: possible *in vivo* relevance during tumorigenesis. *Cancer Res* **60**, 588–594.
- Reisman, D., and Rotter V. (1993). The helix-loop-helix containing transcription factor USF binds to and transactivates the promoter of the p53 suppressor gene. *Nucleic Acids Res* **21**, 345–350.
- Rieske, P., Golanska, E., Zakrzewska, M., Piaskowski, S., Hulas-Bigoszewska, K., Wolańczyk, M., *et al.* (2009). Arrested neural and advanced mesenchymal differentiation of glioblastoma cells-comparative study with neural progenitors. *BMC Cancer* **9**, 54.
- Ruano, Y., Ribalta, T., Rodríguez de Lope, A., Campos-Martín, Y., Fiaño, C., Pérez-Magán, E., *et al.* (2009). Worse outcome in primary glioblastoma multiforme with concurrent epidermal growth factor receptor and p53 Alteration. *Am J Clin Pathol* **131**, 257–263.
- Schroeder, M., and Mass, M.J. (1997). CpG methylation inactivates the transcriptional activity of the promoter of the human p53 tumor suppressor gene. *Biochem Biophys Res Commun* **235**, 403–406.
- Shamsara, J., Sharif, S., Afsharnezhad, S., Lotfi, M., Raziee, H.R., Ghaffarzaghegan, K., *et al.* (2009). Association between MGMT promoter hypermethylation and p53 mutation in glioblastoma. *Cancer Invest* **27**, 825–829.
- Soong, R., Robbins, P.D., Dix, B.R., Griew, F., Lim, B., Knowles, S., *et al.* (1996). Concordance between p53 protein overexpression and gene mutation in a large series of common human carcinomas. *Hum Pathol* **27**, 1050–1055.
- Stupp, R., Hegi, M.E., Mason, W.P., van den Bent, M.J., Taphoorn, M.J., Janzer, R.C., *et al.* (2009). Effects of

- radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomized phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol* **10**, 459–466.
- Szybka, M., Zakrzewska, M., Rieske, P., Pasz-Walczak, G., Kulczycka-Wojdala, D., Zawlik, I., *et al.* (2009). cDNA sequencing improves the detection of P53 missense mutations in colorectal cancer. *BMC Cancer* **9**, 278.
- Tuck, S.P., and Crawford, L. (1989). Characterisation of the human p53 gene promoter. *Mol Cell Biol* **9**, 2163–2172.
- Ueki, K., Nishikawa, R., Nakazato, Y., Hirose, T., Hirato, J., Funada, N., *et al.* (2002). Correlation of histology and molecular genetic analysis of 1p, 19q, 10q, TP53, EGFR, CDK4, and CDKN2A in 91 astrocytic and oligodendroglial tumors. *Clin Cancer Res* **8**, 196–201.
- Venkatachalam, S., Shi, Y.P., Jones, S.N., Vogel, H., Bradley, A., Pinkel, D., *et al.* (1998). Retention of wild-type p53 in tumors from p53 heterozygous mice: reduction of p53 dosage can promote cancer formation. *EMBO J* **17**, 4657–4667.
- Verhaak, R.G., Hoadley, K.A., Purdom, E., Wang, V., Qi, Y., Wilkerson, M.D., *et al.* (2010). Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* **17**, 98–110.
- Wang, X., Wang, J., and Jiang, X. (2011). MdmX protein is essential for Mdm2 protein-mediated p53 polyubiquitination. *J Biol Chem* **286**, 23725–23734.
- Watanabe, K., Sato, K., Biernat, W., Tachibana, O., von Ammon, K., Ogata, N., *et al.* (1997). Incidence and timing of p53 mutations during astrocytoma progression in patients with multiple biopsies. *Clin Cancer Res* **3**, 523–530.
- Wojcik, I., Szybka, M., Golanska, E., Rieske, P., Blonski, J.Z., Robak, T., *et al.* (2005). Abnormalities of the P53, MDM2, BCL2 and BAX genes in acute leukemias. *Neoplasma* **52**, 318e24.
- Yan, H., Parsons, D.W., Jin, G., McLendon, R., Rasheed, B.A., Yuan, W., *et al.* (2009). IDH1 and IDH2 mutations in gliomas. *N Engl J Med* **360**, 765–773.
- Zerrouqi, A., Pyrzynska, B., Febbraio, M., Brat, D.J., and Van Meir, E.G. (2012). P14ARF inhibits human glioblastoma-induced angiogenesis by upregulating the expression of TIMP3. *Clin Invest* **122**, 1283–1295.

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