

MiR-21, miR-34a, miR-125b, miR-181d and miR-648 levels inversely correlate with MGMT and TP53 expression in primary glioblastoma patients

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Abstract

Introduction: *TP53* and *MGMT* alterations play a crucial role in glioblastoma (GB) pathogenesis. *TP53* and *MGMT* function is affected by several pathologic mechanisms, such as point mutations or promoter methylation, which are well characterized. Expression of both genes can be regulated by other mechanisms as well, e.g., microRNAs (miRNAs). Moreover, cross-talk among various pathologic processes may occur, further affecting *MGMT* and *TP53* functionality.

Material and methods: In 49 GB patients, we analyzed the possible associations between *TP53* and its miRNA regulators *miR-125b*, *miR-21*, and *miR-34a*, as well as *MGMT* and its miRNA regulators *miR-181d* and *miR-648*. We evaluated the possible influence of mutational and methylation status on the pre-identified associations.

Results: In patients with immunohistochemistry-detected *TP53* overexpression, expression levels of *miR-34a* and *TP53* were negatively correlated ($r = -0.56$, $p = 0.0195$), and in patients with *TP53* mutations, expression levels of *TP53* and *miR-21* were negatively correlated ($r = -0.67$, $p = 0.0330$). In patients with *MGMT* methylation, expression levels of *MGMT* were negatively correlated with *miR-648* and *miR-125b* expression levels ($r = -0.61$, $p = 0.0269$ and $r = -0.34$, $p = 0.0727$, respectively).

Conclusions: Our findings demonstrate that selected miRNAs are significantly correlated with *MGMT* and *TP53* levels, but the extent of this correlation differs regarding the *TP53* and *MGMT* mutational and promoter methylation status.

Key words: GB, *MGMT*, *TP53*, O6-methylguanine-DNA methyltransferase, microRNA.

Introduction

Glioblastoma (GB, World Health Organization grade IV) is the most common malignant primary brain tumor in adults [1, 2]. Due to its rapid growth and inherent tendency to recur, GB is inevitably fatal within a short time after diagnosis, despite an extensive search for novel treatment modalities [3–5]. The current standard of care (called “Stupp’s regimen”), i.e., maximal extent of surgical resection followed by radiotherapy with concomitant and adjuvant temozolomide chemotherapy, results in a median survival of ~15 months [3, 4, 6]. *MGMT* methylation is associated with a better response to adjuvant temozolomide (TMZ) and a median overall survival of 22 months [7]. Treatment of refractory tumors is particularly difficult as secondary chemoresistance frequently develops, and no alternative treatment has proven effective thus far [8].

The huge majority of GBs arise de novo and are termed primary, *IDH*-wild type, which differ from secondary GB having a mutation within *IDH1* or *IDH2* and resulting from the progression of a lower grade glioma [9]. Almost all GBs feature alterations in three molecular pathways: the *RTK/RAS/PI-3K*, *TP53*, and *RB1* pathways in 88%, 87%, and 78%, respectively [10]. *TP53* mutations are reported in 28% to 37.5% of primary GBs [11, 12]. *TP53* promoter methylation, *TP53* mRNA level, and *TP53* protein overexpression are not related, suggesting that other mechanisms are involved, probably including microRNAs (miRNAs) [13, 14]. *TP53* activity is closely associated with miRNAs as it regulates the expression and maturation of several miRNAs, while its expression and function are modified by other miRNAs [15]. *MGMT* is a key DNA repair enzyme that specifically removes the promutagenic methyl/alkyl group from the O6 position of guanine by transferring it to the cysteine acceptor site on the protein itself and restores the guanine to its normal form without causing DNA strand breaks [8, 16]. Methylation of the O6 position of guanine is the most clinically relevant effect of alkylating agents (as it cannot be repaired by mismatch repair mechanisms), which is directly repaired by *MGMT* and, therefore, reduced *MGMT* expression is associated with a better response to alkylating chemotherapy [8, 16]. Methylation of the O6 position of guanine commonly results from *MGMT* promoter methylation, which is reported in 45% to 70% of GBs [7, 17, 18]. Additionally, Kreth *et al.* showed that GBs may downregulate *MGMT* expression independently of promoter methylation by elongation of the 3'-untranslated region of the mRNA, rendering the alternatively polyadenylated transcript susceptible to miRNA-mediated regulation [19].

In the present study, we investigated whether selected miRNAs levels associate with *TP53* and *MGMT* levels in primary glioblastoma patients with special reference to specific alterations in these genes. We analyzed *miR-21*, *miR-34a*, and *miR-125b* as potential regulators of *TP53* and *miR-648*, and *miR-181d* and *miR-125b* as potential regulators of *MGMT* [15, 19–23]. *MiR-21* is one of the most frequently overexpressed miRNAs in human GB cell lines [23–25]. Expression of *miR-34* family members directly regulates *TP53* [26–28]. Expression of *miR-34a* promotes *TP53*-mediated apoptosis, cell cycle arrest, and cell senescence, whereas inactivation of endogenous *miR-34a* strongly inhibits *TP53*-dependent apoptosis [15]. *miR-125b* is an important negative regulator of *TP53* and *TP53*-induced apoptosis during development and during the stress response [29]. Additionally, *miR-125b* confers TMZ resistance by targeting tumor necrosis factor α -induced protein 3 (*TNFAIP3*) and *NF- κ B* inhibitor interacting RAS-like 2 (*NKIRAS2*) independently of *TP53* and *MGMT*, suggesting a potential predictive value of this microRNA [30]. *miR-181d* and *miR-648* were identified as the most important post-transcriptional regulators of *MGMT* in GBs, as *miR-181d* induces *MGMT* mRNA degradation, while *miR-648* affects *MGMT* protein translation [19, 31].

Material and methods

Study cohort

We recruited 49 patients (31 males, 18 females) with newly diagnosed glioblastoma multiforme (GBM) from 2006 to 2011. All tumors were histopathologically examined and classified according to the World Health Organization classification of tumors of the central nervous system [32]. Median age at surgery was 63.00 (interquartile range (IQR): 58.00–68.00) years. Formalin-fixed paraffin-embedded (FFPE) samples were collected at the Department of Pathology, Chair of Oncology, Medical University of Lodz, Poland. For details, see Table I. Ethics committee approval was obtained from the Institutional Review Board of the Medical University of Lodz (Number RNN/226/11/KE).

RNA isolation

Total RNA was extracted from FFPE tissue using the miRNeasy FFPE Kit (Qiagen, Germany) according to the manufacturer's instructions. The yield and quality (260/280 ratio) of the RNA were measured using a Picodrop spectrophotometer (Picodrop Limited, UK). Purified total RNA was immediately used for cDNA synthesis or stored at -80°C until use.

Table I. Study group characteristics. Nominal variables are presented as number followed by percentages and continuous variables are presented as medians followed by interquartile ranges

Variable	Number (%) or median (IQR)
Sex (males)	32 (65.31)
Race (Caucasian)	49 (100.00)
<i>MGMT</i> methylation	19 (38.78)
<i>MGMT</i> overexpression (IHC)	17 (34.39)
<i>TP53</i> overexpression (IHC)	20 (40.82)
<i>TP53</i> mutations	12 (24.49)
<i>TP53</i> methylation	4 (9.52)
<i>MGMT</i> [$2^{-\Delta Ct}$]	0.10 (0.07–0.18)
<i>TP53</i> [$2^{-\Delta Ct}$]	0.08 (0.05–0.20)
Age at diagnosis [years]	63.0 (58.0–68.0)
Overall survival [months]	9.0 (3.0–14.0)

MGMT and *TP53* mRNA quantification

Total RNA (500 ng) was used for cDNA synthesis. We used a QuantiTect Reverse Transcription Kit (Qiagen) and followed the manufacturer's protocol. The cDNA samples were kept frozen at -20°C .

mRNA expression levels were measured using standard TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA): tumor protein *TP53* (*TP53*, Hs01034249_m1), methylguanine-DNA methyltransferase (*MGMT*, Hs01037698_m1), and glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*, Hs99999905_m1) as the endogenous control. TaqMan PCR assays were performed in 10- μl reactions using 50 ng cDNA, 5 μl KAPA PROBE FAST qPCR Kit Master Mix ABI Prism (Kapa Biosystems), and 0.5 μl of the appropriate TaqMan Gene Expression Assay. All reactions were run in duplicate on a Rotor Gene 3000 Real-Time PCR System (Corbett Life Science) according to the following thermal cycling conditions: 10 min at 95°C and 40 cycles each of 10 s at 95°C and 60 s at 60°C .

TP53 and *MGMT* expression levels were calculated using the $2^{-\Delta Ct}$ method. ΔCt was calculated by subtracting the *Ct* of the investigated gene from the *Ct* of the endogenous control. Samples were excluded from further analysis if the *Ct* difference between duplicates was greater than 1 (treated as experiment error, e.g., degraded RNA). All patients with good quality RNA available ($n = 46$, 3 patients were found to have degraded RNA) were included in this analysis.

miRNA quantification

Reverse transcription was carried out on 10 ng of total RNA in 15- μl reactions using the TaqMan

MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instruction. miRNA was quantified using standard TaqMan MicroRNA Assays (Applied Biosystems): *hsa-miR-21* (Assay ID: 000397), *hsa-miR-34a* (Assay ID: 000426), *hsa-miR-125b* (Assay ID: 000449), *hsa-miR-181d* (Assay ID: 466352_mat), *hsa-miR-648* (Assay ID: 001601), and *hsa-miR-103* (Assay ID: 000439) as a control. The 20- μl qPCR included 1.33 μl RT product, 10 μl TaqMan Universal PCR Master Mix and 1 μl TaqMan miRNA Assay (20 \times). The reactions were incubated in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were run in duplicate.

TaqMan PCR assays were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) and analyzed using Sequence Detection System 2.3 software. Fold induction values (RQ) were calculated according to the equation $2^{-\Delta\Delta Ct}$, where ΔCt represents the differences in the cycle threshold numbers between the target gene and spike-in control, and $\Delta\Delta Ct$ represents the relative change in these differences between examined and control groups.

DNA isolation

Genomic DNA was extracted from FFPE tissue using the QIAamp DNA FFPE Tissue Kit (Qiagen) in accordance with the manufacturer's protocol. The concentration (A260) and purity (A260/A280 ratio) of isolated DNA were determined using a Picodrop spectrophotometer (Picodrop Limited). The DNA was stored at -20°C .

Polymerase chain reaction amplification

Exons 5–8 of the *TP53* gene were amplified by polymerase chain reaction (PCR). The purified DNA was used as a template for PCR amplification. The reaction mixture in 20 μl volume contained 50 ng of DNA, 1 U Taq DNA polymerase (Promega, Fitchburg, WI, USA), 1.5 mM MgCl_2 , 0.25 mM each of dATP, dCTP, dGTP, and dTTP, and 0.5 μM of the primers. The amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems).

The primer sequences were as follows: for *TP53*Ex5_6, forward 5'-CACTTGTGCCCTGACTTTCA-3' and reverse 5'-CTTAACCCCTCCTCCAGAG-3' (product of 464 bp); for *TP53*Ex7, forward 5'-TCATCTTG-GCCCTGTATTATCTC-3' and reverse 5'-GTGCAG-GGTGGCAAGTGG-3' (product of 163 bp); and for *TP53*Ex8, forward 5'-CAAGGGTGGTTGGGAGTAGA-3' and reverse 5'-TGCTAGGAAAGAGGCAAGGA-3' (product of 331 bp). Thermal cycling conditions for PCR reactions were: "touchdown" protocol for *TP53*Ex5_6 and *TP53*Ex8: 94°C for 5 min, 63°C for 45 s, 72°C for 1 min, 1 cycle 94°C

for 1 min, 61°C for 45 s, 72°C for 1 min, 34 cycles 94°C for 1 min, 60°C for 45 s, 72°C for 1 min, and 72°C for 10 min; conditions for *TP53*Ex7 were 94°C for 5 min, 40 cycles 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 72°C for 7 min.

Microchip electrophoresis of DNA fragments

The PCR amplification products were separated and analyzed on the automated microchip electrophoresis system MCE-202 MultiNa (Shimadzu, Japan) using the DNA-1000 kit according to the manufacturer's protocol. A SYBR Gold fluorescent dye for DNA staining (Invitrogen, Carlsbad, CA, USA) and PhiX174 DNA/Hae III Markers (Promega) were used to determine the sizes of the PCR products.

TP53 sequencing analysis

Amplification products were purified using the MiniElute PCR Purification Kit (Qiagen). The sequencing reactions were run using a Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, USA) on the GeneAmp PCR System 9700 using standard sequencing protocols. Sequence analysis was performed using the same primers used in the preamplification step.

The PCR products were purified to eliminate unincorporated primers and dNTPs using the Big-Dye XTerminator Purification Kit and then subjected to separation by the 3130xl GeneGenetic Analyzer (Applied Biosystems).

IDH1 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'- GGCAC-CCATCTTCTGTGTTT-3' (sense) and 5'-ATATG-CATTTCTCAATTTCA-3' (antisense). The sequencing primers used were *IDH1*-exon 4 – 5'-CGGTCTCA GAGAAGCCATT-3' (sense) and *IDH1* exon 4 – 5'-CA CATTATTGCCAACATGAC-3' (antisense). A Li-Cor automatic sequencer system was used for the separation and analysis of PCR-sequencing products.

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, USA) according to the manufacturer's protocol. The bisulfite-treated DNA was stored at –80°C until use. CpGenome Universal Methylated DNA 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 for *TP53* promoter methylation was performed as previously described. Methylation-specific PCR for *MGMT* promoter methylation was performed in a two-step approach as previously reported [13, 17]. 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 Doc 1000 Bio Rad Image System. Repeat testing was performed to confirm the results.

Immunohistochemistry for *TP53* and *MGMT* protein expression

Immunohistochemical overexpression of *TP53* was studied using monoclonal antibody anti-*TP53* (clone DO-7, 1 : 100 dilution; DAKO, Glostrup, Denmark) and that of *MGMT* was evaluated using anti-*MGMT* antibody (clone MT23.2, dilution 1 : 50; Zymed), processed with the EnVision (DAKO) system. The *TP53* antibody labels wild-type, which has a very short half-life and is present in small amounts in normal cells, and mutant-type *TP53* protein that significantly prolongs the half-life of the protein and is detected by positive staining as product of a point mutation in the *TP53* gene. Tumor sections were examined for *TP53* immunoreactivity under a microscope at 20× and 40× magnifications. Expression of *TP53* was considered positive when the proportion of positive cells was greater than 10% (Figure 1 A) [33]. Negative *TP53* staining in tumor sections is presented in Figure 1 B.

We used MT23.2 antibody due to the best agreement between methylation-specific PCR and immunohistochemistry (IHC) results reported previously [34]. For the semiquantitative immunohistochemical *MGMT* scoring we applied a cut-off of 15% immunolabeled cells for GBM, based on a prior publication (Figures 1 C, D) [35]. Only nuclear staining was considered for the evaluation. Positive endothelial and lymphocytes cells were considered as internal positive controls.

The IHC results were validated using positive and negative tissue controls in all series of immunostained slides. The positive controls (with tissues containing the target antigen at a known and stable expression level) were performed for validation of *TP53* and *MGMT* on ovarian carcinoma and additionally for *MGMT* in breast carcinoma. Positive endothelial and lymphocytes cells were considered as internal positive controls. To examine negative control staining, neoplastic tissue slides were evaluated using mouse isotype anti-

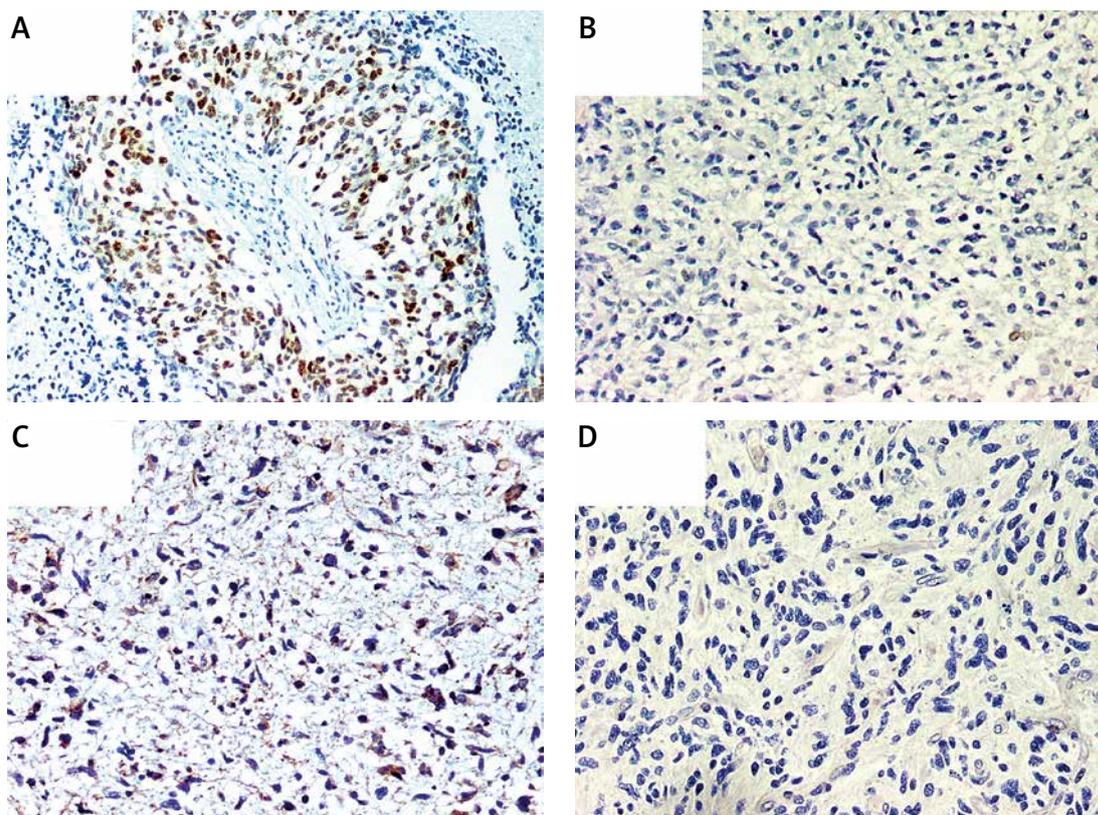


Figure 1. A – Immunohistochemically detected TP53 protein overexpression. B – Sample negative for TP53 overexpression. C – Immunohistochemically detected MGMT protein overexpression. D – Sample negative for MGMT overexpression. 200× magnification was used for presentation of all images

body Ready-to-Use FLEX Negative Control Mouse (Cocktail of mouse IgG1, IgG2a, IgG2b, IgG3 and IgM, IR750, DAKO, Denmark).

Statistical analysis

Continuous variables are presented as medians followed by IQR, while nominal variables are presented as numbers followed by percentages in brackets. The Shapiro-Wilk test was used for the distribution assessment. Grubbs' test was used to detect outliers. Continuous variables were compared using the Mann Whitney *U*-test due to the non-normal distribution. Spearman's rank test was used for correlation assessment. Differences between categorical variables were evaluated using the χ^2 or two-tailed Fisher's exact test. The statistical analysis was exploratory, which is why we did not perform post-hoc corrections for multiple testing. For the outcome analyses, overall survival was defined as the time period from diagnosis to last follow-up, with censoring of live patients at the last follow-up. Overall survival data are presented as Kaplan-Meier survival curves and compared within subgroups using the log-rank test. Cox hazards regression analyses of overall survival adjusted for age were performed for each variable. The Statistica 12.5

PL package (StatSoft, Tulsa, OK, USA) was used for the analysis. *P*-values < 0.05 were considered statistically significant.

Results

Molecular characteristics of the study group

Twelve (24.49%) patients carried *TP53* mutations, of which 5 were detected in exon 5, 2 in exon 6, another 2 in exon 7, and 3 in exon 8 of *TP53*. *TP53* promoter methylation was detected in 4 (8.16%) patients. No association between *TP53* mutations and *TP53* promoter methylation was detected ($p = 1.00$). *TP53* protein was overexpressed in 20 (40.82%) patients. Quantification of *TP53* mRNA was successful among 41 (83.67%) patients. *TP53* mRNA levels were lower in tumor samples than in samples from normal brain. The *TP53* mRNA level tended to be higher in patients with IHC-detected *TP53* overexpression than in patients without *TP53* protein overexpression ($p = 0.048$, Figure 2 A). Neither *TP53* mutations nor promoter methylation affected the *TP53* expression levels ($p = 0.846$ and $p = 0.9384$).

MGMT was methylated in 19 (38.78%) cases. IHC-detected *MGMT* overexpression was found in 17 (34.69%) patients. *MGMT* mRNA was quanti-

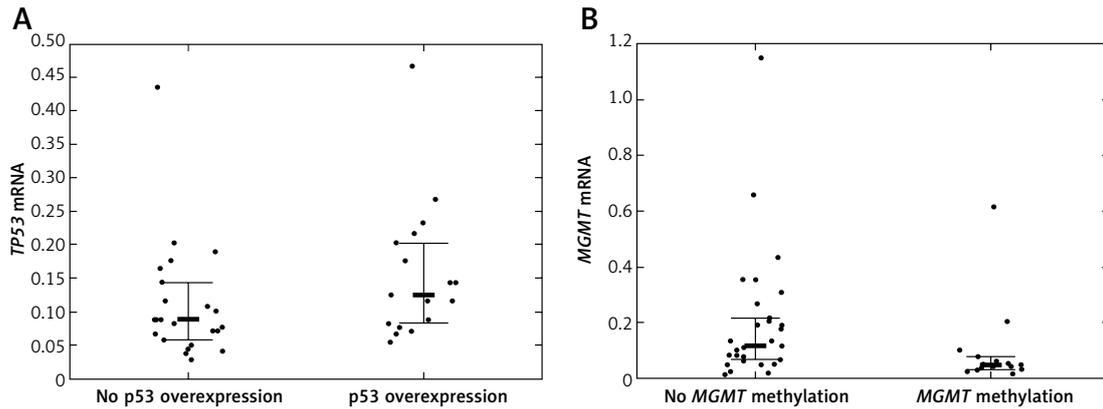


Figure 2. A – *TP53* mRNA level according to *TP53* immunohistochemically detected overexpression status; B – *MGMT* mRNA level according to *MGMT* promoter methylation status

Bolded bars represent median and whiskers indicate interquartile ranges. Dots represent row data.

fiable in 45 (91.84%) patients. *MGMT* expression tended to be lower in patients with *MGMT* methylation than in patients without *MGMT* methylation ($p = 0.0242$, Figure 2 B). IHC-detected *MGMT* overexpression was not associated with elevated *MGMT* mRNA levels ($p = 0.0645$). *MGMT* mRNA levels were higher in tumor tissue samples than in normal tissue samples.

We detected no *IDH1* mutations. Nine (18.37%) patients carried none of the investigated molecular lesions.

Association between selected miRNAs and mRNA levels of *TP53* and *MGMT*

MiRNA quantification was applicable among 48 (97.96%) patients included in the analyses. The levels of all but *miR-125b* were higher in tumor samples than in control normal brain tissue.

There were no significant correlations between miRNAs and *TP53* mRNA levels in the whole group ($p > 0.05$ for all comparisons). *miR-34a* and *TP53* expression levels were negatively correlated in patients with IHC-detected *TP53* overexpression ($r = -0.56$, $p = 0.0195$). The levels of *miR-34a* were higher in patients without *TP53* mutations than in patients with *TP53* mutations ($p = 0.0051$). In addition, *TP53* and *miR-21* expression levels were negatively correlated in patients with *TP53* mutations ($r = -0.67$, $p = 0.0330$).

We found no significant correlations between *MGMT* mRNA and selected miRNA levels in the whole study group ($p > 0.05$ for all comparisons). *MGMT* and *miR-648* expression levels were, however, negatively correlated in patients with *MGMT* methylation ($r = -0.61$, $p = 0.0269$). Additionally, *MGMT* and *miR-125b* were negatively correlated in patients without *MGMT* methylation ($r = -0.34$, $p = 0.0727$). The negative correlation between *MGMT* mRNA and *miR-181d* was close to significant ($r = -0.27$, $p = 0.0760$). The *miR-21* level was

lower in patients with *MGMT* methylation than in patients without *MGMT* methylation ($p = 0.0391$).

Survival analyses

We recorded 47 deaths and median (IQR) overall survival was 9.00 (3.00–14.00) months (Table I). None of the investigated molecular alterations affected overall survival in the univariate analysis and only age at diagnosis affected the prognosis ($p = 0.0295$). The Cox proportional hazards regression analyses of each molecular variable adjusted for age at diagnosis showed no prognostic effect.

Discussion

In the present study, we investigated whether *TP53* and *MGMT* expression levels were associated with the levels of preselected miRNAs, and found that the associations may be revealed in a specific molecular setting (i.e., mutation or methylation).

This analysis was performed in a representative group of 49 adult GB patients undergoing standard treatment and close follow-up. The *IDH1*^{R132H} mutation was excluded by sequencing all patients, confirming that all tumors could be considered primary GBs [1]. We investigated the associations between selected miRNAs and *TP53* and *MGMT* levels at both the mRNA and protein levels.

We observed lower expression of *miR-34a* in GB patients with a *TP53* mutation than in those without a mutation, consistent with previous reports [26, 36, 37]. Furthermore, in patients with a nuclear accumulation of *TP53*, which is usually associated with a mutation [38, 39], *miR-34a* expression was negatively correlated with *TP53* mRNA levels. In contrast with previous reports, however, we detected higher *miR-34a* expression in tumor tissue than in normal tissue [36, 37]. Gao *et al.* and Ren *et al.* indicated a potential prognostic role of *miR-34a* in GB, but such an association was not observed

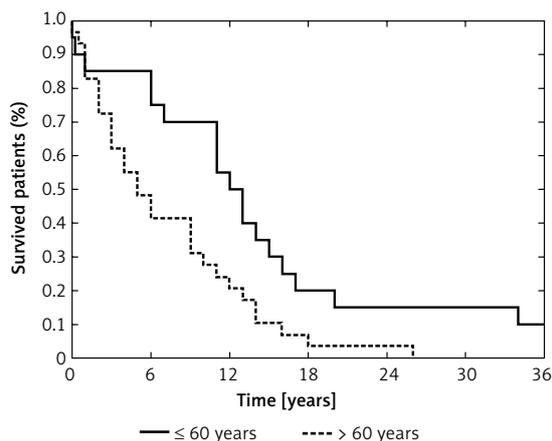


Figure 3. Probability of overall survival in the study group according to age at diagnosis (≤ 60 years vs. > 60 years) ($p = 0.0099$)

in this group [37, 40]. Additionally, we detected a negative correlation between *miR-21* and *TP53* mRNA levels in patients with *TP53* mutations, in accordance with the report by Mraz *et al.* [41]. We observed higher expression of this miRNA in tumor tissues than in normal tissues, which has been reported for many solid tumors, including GB [42–46]. Some authors postulate *miR-21* as a negative prognostic marker, but this was not confirmed in the present study [46, 47].

MGMT can be cooperatively downregulated by *miR-181d* and *miR-648* at the post-transcriptional or translational level [19, 31, 48]. We detected a negative correlation between *MGMT* expression and *miR-648* in patients with *MGMT* promoter methylation. Similarly, Kushwaha *et al.* reported that transfection with *miR-648* did not consistently affect *MGMT* expression [48]. Additionally, we observed a negative correlation between *MGMT* mRNA and *miR-181d*. Although this finding was not statistically significant, it supports the previous report that *miR-181d* may induce *MGMT* mRNA degradation [19]. This miRNA was also suggested by Zhang *et al.* to be a potential negative prognostic factor in GB, but we did not observe such an association in this group [31].

Age at diagnosis is considered a key prognostic factor in GB patients, which was also apparent in our study – patients older than 60 years had worsened overall survival compared with younger patients ($p = 0.0099$, Figure 3) [1, 49, 50]. We also analyzed the effect of molecular lesions inactivating *TP53* and *MGMT* on overall survival, but those analyses showed no prognostic value of any of the examined variables, including the levels of selected miRNAs. Taking into consideration that previous reports indicated the prognostic impact of the molecular characteristics of GBs, we think that our analysis could have been underpowered and plan to extend our study group in the future.

In this paper, we also corroborated the previous findings of no relation between *TP53* promoter methylation and *TP53* mRNA expression or nuclear protein accumulation [14]. Additionally, *TP53* expression was not associated with *TP53* mutations.

When conducting the study and analyzing the data, we encountered two problems that could have influenced our final conclusions. First, we did not have fresh frozen tissue for the DNA and RNA isolation and in 4 patients the FFPE material was degraded. Nevertheless, we consider the expression levels detected in the other patients robust, as we used the standard methodology, which was found to be optimal previously [51, 52]. Second, the study group consisted of 49 GB patients and some analyses (especially the survival analyses) could have been underpowered. Additional investigation in a larger group is intended.

In conclusion, the *MGMT* and *TP53* alterations are major features in GB pathogenesis that affect the survival of patients with primary GB. We demonstrated that *miR-21*, *miR-34a*, *miR-125b*, *miR-181d*, and *miR-648* can be considered good indicators of *TP53* and *MGMT* function in GB patients; however, other mechanisms of inactivation should be taken into consideration in subsequent studies investigating this important issue.

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Conflict of interest

The authors declare no conflict of interest.

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