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ORIGINAL PAPER

Polycomb genes expression as a predictor of poor clinical outcome in children with medulloblastoma

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Abstract
Introduction Medulloblastoma is the most frequent type of embryonal tumor in the pediatric population, accounting for 20–25% of all brain tumors in children. Recently, the suspected contribution of the *Polycomb* group (PcG) genes in medulloblastoma development was described. PcG genes play an important role in developmental processes; they are also involved in the self-renewal of hematopoietic and neural stem cells as well as in malignant transformation.
Purpose In this study, we evaluated the expression of *BMI1*and *PCGF2*, members of family of PcG genes, and their potential target, *MYC* oncogene, and analyzed their association with demographic and clinical data.
Materials and methods Thirty-one children (18 males and 13 females, aged from 0.4 to 17 years) with medulloblastoma were included in this study. The gene's expression level was measured by quantitative real-time PCR, obtained using the two-color multiplexing technique.
Results We found that the higher expression levels of *BMI1* and *PCGF2* genes were associated with significantly decreased patient survival (p=0.02 and p=0.012, respectively). Significant differences between gender were found, with a higher expression level of the *PCGF2* gene observed among females (p=0.02).
Conclusion Our analysis showed correlation between *BMI1* and *PCGF2* gene's expression and survival in children with medulloblastoma.

Keywords *BMI1* · Clinical risk · Expression · Medulloblastoma · *MYC* · *PCGF2*

Magdalena Zakrzewska and Krzysztof Zakrzewski are equal contributors to this work.

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Introduction

Medulloblastoma (MB) is the most frequent type of highly malignant tumor occurring in the pediatric population, and despite significant advances in therapeutic modalities, it is still associated with a high mortality rate. Due to its frequency and aggressive behavior, a precise disease risk stratification of patients is essential. The current estimation of disease progression in patients with MB, based mainly on clinical factors, seems to be insufficient. Moreover, risk estimated on the neuropathological classification of this tumor is still controversial, inasmuch as no unambiguous criteria of characteristic morphological features are well-defined [11]. Until now, a number of molecular factors correlated with survival and predictors of outcome have

been proposed, but there is still a need to search for more appropriate classifiers for medulloblastoma.

Recently, the suspected contribution of the *Polycomb* group (PcG) of genes, *BMI1*and *PCGF2* (also known as *MEL18* or *ZNF144*), was described in MB pathogenesis [36]. Human PcG complexes were classified into polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2 subtypes. *BMI1* and *PCGF2* genes encode for highly homologous proteins of PRC1 and have overlapping functions in terms of development, self-renewal processes, and cancer growth, mostly through the modification of the chromatin structure [3, 36]. The significance of *BMI1* as a potential molecular diagnostic marker was described previously for breast and prostate cancer [10, 31]. In invasive ductal breast cancer, its overexpression was defined as the prognostic factor related to metastases and signaled a high possibility of a poor outcome [23]. Recently, the significance of *BMI1* in non-small cell lung cancer expansion was also reported, as well as its role in maintaining the function of bronchioalveolar stem cells [4, 8]. Similar mechanisms, including brain stem cells, were also well documented for glioblastoma [1]. In medulloblastoma, BMI1 was found to be overexpressed and their potential role in tumor pathogenesis via expansion of tumor stem cells was also strongly suggested, but studies into its probable link with patient outcomes have not yet been conducted thoroughly [13, 25, 27].

PCGF2 was described previously as the regulator of hematopoietic stem cell fate which downregulation intensify stem cell capability [21, 22]. The clinical value of that gene was confirmed in breast and prostate cancers, where negative expression of *PCGF2* was correlated with the proliferation of neoplastic cells and a worse prognosis of patients [16, 24, 34, 35].

In addition, an inverse correlation between *PCGF2* and *BMI1* expression in invasive breast cancer and breast cancer cell lines was observed [16, 24]. Interestingly, such a link was not reported for medulloblastoma cell lines and overlapping functions for both genes was strongly suggested in MB tumorigenesis [36]. Moreover, the negative correlation between *PCGF2* and *MYC*, as well as between *PCGF2* and *BMI1* expression and *PCGF2* downregulation of *BMI1* through *MYC*, was observed in human fibroblast cell lines [15]. The *MYC* gene was amplified in approximately 5–10% of medulloblastomas, while overexpressed in up to 50% of tumor samples. Such inconsistency could be caused by altered transcriptional processes dependent on transcriptional repressor proteins such as PcG family members *BMI1* and *PCGF2* [2, 14, 20].

In this work, we evaluated the expression of *BMI1*, *PCGF2*, and *MYC* genes to determine their potential connection with survival data of children with medulloblastoma.

Materials and methods

Patient samples

Thirty-one children with classic type medulloblastoma, operated on in the Department of Neurosurgery, Polish Mother's Memorial Hospital Research Institute, were included in this study. The group was comprised of 18 males and 13 females. The median age of patients at the time of diagnosis was 8 years (range, 0.4–17 years). All specimens were diagnosed at the Department of Molecular Pathology and Neuropathology, Chair of Oncology, Medical University of Lodz, according to the World Health Organization criteria [26].

Based on clinical data, the patients were divided into standard risk (SR) and high risk (HR) groups consist of 16 and 15 children, respectively. Patients under 3 years of age at the time of diagnosis, with more than 1.5 cm^3 of residual disease after surgery or with evidence of metastases, were classified to HR group, while children older than 3 years of age, with no evidence of metastatic disease and less than 1.5 cm^3 of residual disease, were stratified as SR group.

Seven patients under 3 years of age received chemotherapy (no radiotherapy) that consisted of alternate courses of vincristine, etoposide, and cisplatin and vincristine, etoposide, and cyclophosphamide during 18 months. HR patients were treated by preirradiation chemotherapy (four alternate courses) with vincristine, etoposide and carboplatin and etoposide, ifosfamide, and cisplatin and maintenance chemotherapy with vincristine, lomustine, and cisplatin (six to eight courses). SR patients received preirradiation chemotherapy (four alternate courses) consisting of vincristine, etoposide and carboplatin and vincristine, etoposide, and cyclophosphamide and maintenance chemotherapy with vincristine, lomustine, and cisplatin (six to eight courses). Radiotherapy was given with total doses of 35 Gy for brain and spine and 50–55 Gy on tumor site.

Survival periods were collected for all children. The mean follow-up time was 58 months (in a range from 4 to 156 months). The Bioethics Medical University Committee approved all analyses performed (no. RNN/154/06/KE).

RNA isolation

RNA was extracted using a commercially available RNeasy Mini Kit (cat. no. 74104; Qiagen) and treated with DNAase (cat. no. 79254; Qiagen). RNA concentrations were measured spectrophotometrically. One microgram of total RNA from all specimens and reference RNA (Human Cerebellum Brain Total RNA cat. no. 6820; Ambion, USA) was reverse-transcribed into single-stranded cDNA according to standard protocol (cat. no. 205313; Qiagen) in two independent reactions for each sample.

Amplification analysis

The expression levels of target and reference genes were measured by quantitative real-time PCR (Q-PCR), using the 2-color multiplexing technique. The Q-PCR experiment was designed using the free web-based ProbeFinder software version 2.41 for Human, which specifies a set of primers and probes from the Universal ProbeLibrary (cat. no. 04688627001, *BMI1*; cat. no. 04687671001, *MYC*; cat. no. 04687973001, *PCGF2*; Roche Diagnostic GmbH, Germany) as well as reference gene (cat. no. 05 046 157 001, *HPRT*; Roche Diagnostic GmbH, Germany). The sequences of the set of primers and numbers of probes used are listed in Table 1. The optimized Q-PCR assay was performed in a 20 μl reaction mixture containing water, 5 μl of template (cDNA), 10 μl FastStart TaqMan Probe Master (cat. no. 04 673 417 001; Roche Diagnostic GmbH, Germany), 500 nM of each of the primers, and 250 nM of each of the probe. Reactions were performed on a Rotor Gene 6000 instrument (Qiagene-Corbett Life Science, Sydney, Australia) under the following conditions: initial cDNA polymerase activation at 95°C for 15 min, followed by 45 cycles of 95°C for 15 s (denaturation) and 60°C for 45 s (annealing) with multi-channel detection. All samples were analyzed in triplicate to obtain replicates for statistical analyses.

Statistical analysis

A normalized relative expression level for a given target gene was calculated using the method described by Pfaffl et al. with pooled cDNA from all tumor samples used as a control, according to the equation,

$$\text{Ratio} = \frac{(E_{\text{TARGET}})^{\Delta \text{CP}_{\text{TARGET}}(\text{control}-\text{sample})}}{(E_{\text{REF}})^{\Delta \text{CP}_{\text{REF}}(\text{control}-\text{sample})}}$$

where E_{TARGET} and E_{REF} stand for the Q-PCR efficiency of target and reference gene amplification, respectively, and $\Delta\text{CP}_{\text{TARGET}}$(control − sample) and $\Delta\text{CP}_{\text{REF}}$(control − sample) denote the difference in crossing points (CP) between unknown and control samples for a given target and reference gene, respectively [28]. The differences in gene expression levels were evaluated by Mann–Whitney *U*. Statistical significance was assumed for p value ≤0.05. Kaplan–Meier curves were estimated from survival data for two groups based on the median expression level of each gene.

Results

The clinical data, including patient's age, sex, clinical risk, and follow-up data, are given in Table 2. Median levels of *BMI1*, *MYC*, and *PCGF2* mRNA expression were 1.94 (range, 1.21–3.10), 3.80 (range, 1.06–53.86), and 1.79 (range, 0.98–2.45), respectively (Table 2). Significant differences (p=0.02) between gender were found for the *PCGF2* gene, with higher expression level observed among females (Fig. 1). The survival analysis performed following the group stratification based on the median expression level of each gene revealed an association between higher expression levels of *BMI1* and *PCGF2* and a significantly decreased patient survival rate (p=0.02 and p=0.012, respectively). Estimation of outcomes, using the Kaplan–Meier method, demonstrate a prediction of 5-year survival rates of 35% and 65% for high and low expression levels of the *BMI1* gene, respectively (Fig. 2), and 37% and 70% for high and low expression levels of the *PCGF2* gene, respectively (Fig. 3). Kaplan–Meier survival analysis performed for female and male patients independently showed better outcome for female patients with low expression levels of the *PCGF2* gene (Figs. 4 and 5). Expression level of *MYC* gene was unrelated to clinical outcome (Fig. 6). Statistical analysis revealed significant differences in expression levels between two groups, obtained based on the clinical risk stratification (high risk vs. standard risk) for the *MYC* gene, but not for *BMI1* and *PCGF2* genes. There was no significant differences in

Table 1 Sequences of primers used for gene expression analysis, with numbers of probes used

Probe/primer name	Sequence (5′-3′)	Position	Amplicon size	Reference gene assay
Probe#63				HPRT
BMI1-F	TTCTTTGACCAGAACAGATTGG	888–909	112 bp	
BMI1-R	GCATCACAGTCATTGCTGCT	980–999		
Probe#34				
MYC-F	CACCAGCAGCGACTCTGA	741–758	102 bp	
MYC-R	GATCCAGACTCTGACCTTTTGC	821–842		
Probe#39				
PCGF2-F	TTCTCCGCAACAAGATGGAT	740–759	67 bp	
PCGF2-R	AGTGGCTCGTCCTCGTACA	788–806		

Table 2 Clinical data of medulloblastoma

Case	Age, yaers	Sex	Age category	Clinical risk	Follow-up, mo	Outcome	Gene relative expression level		
							BMI1	*MYC*	*PCGF2*
M1	8	F	>3	HR	24	A	2.21	3.80	1.64
M2	5	F	>3	HR	18	D	2.89	53.86	2.44
M3	12	F	>3	SR	37	A	2.21	2.93	1.82
M4	10	M	>3	HR	39	A	1.42	1.06	0.98
M5	7	M	>3	SR	40	A	1.21	2.45	1.48
M6	2	M	≤3	HR	4	D	2.16	19.52	2.34
M7	0.4	F	≤3	HR	8	D	2.22	6.97	2.26
M8	2	M	≤3	HR	15	D	2.02	3.77	1.71
M9	14	M	>3	HR	60	A	1.93	11.81	1.79
M10	5	F	>3	SR	16	D	2.38	2.65	2.12
M11	3	F	≤3	SR	60	A	2.38	3.52	2.11
M12	3	M	≤3	HR	30	D	2.06	10.42	1.59
M13	17	M	>3	SR	78	A	1.78	1.73	1.40
M14	7	M	>3	HR	81	D	1.52	2.65	1.87
M15	8	M	>3	HR	7	D	1.89	30.65	1.82
M16	8	M	>3	SR	81	A	1.39	1.69	1.44
M17	2	F	≤3	HR	107	A	1.50	2.68	1.52
M18	13	M	>3	SR	47	D	1.94	1.95	2.45
M19	15	F	>3	SR	120	A	1.78	13.54	1.88
M20	5	M	>3	SR	110	A	1.78	4.16	1.61
M21	8	F	>3	HR	42	D	1.71	20.94	2.24
M22	11	M	>3	SR	40	D	3.10	3.28	1.92
M23	13	M	>3	SR	3	D	1.26	1.11	1.27
M24	11	M	>3	SR	133	D	1.98	15.42	1.51
M25	10	M	>3	HR	134	D	2.66	35.46	1.80
M26	8	F	>3	SR	124	A	1.65	15.71	1.61
M27	6	M	>3	HR	30	D	2.86	11.36	1.41
M28	8	M	>3	SR	156	A	1.79	2.90	1.67
M29	7	F	>3	HR	29	D	1.99	33.33	2.16
M30	3	F	≤3	SR	2	D	1.80	1.40	1.45
M31	5	F	>3	SR	120	A	2.21	11.82	1.98
Reference gene (*HPRT*) relative expression level							1.65	1.65	1.70

Relative gene expression level evaluated by quantitative real-time PCR performed by 2-color multiplexing technique

A alive, *D* deceased, *F* female, *HR* high risk, *M* male, *SR* standard risk

expression levels of any of the genes studied between the two age groups (under 3 years vs. over 3 years). The analysis of correlation between genes reached the level of statistical significance, nevertheless it showed only low values of rank correlation coefficients obtained using Spearman's correlation test: *MYC* vs. *BMI1* (r^2=0.19, p=0.02), *MYC* vs. *PCGF2* (r^2=0.24, p=0.005), *BMI1* vs. *PCGF2* (r^2=0.26, p=0.003; Fig. 7). As indicated by obtained coefficient of determination, the correlation between *PCGF2* and *BMI1* accounted for only about 26% of common variability of these genes' expression (this correlation was excluded from consideration).

Discussion

Members of the PcG gene family act as epigenetic gene silencers and control biological processes, including self-renewal of both normal and neoplastic stem cells. Their role in tumorigenesis is related to the modulation of tumor suppressor pathways, crucial for the cell cycle and oncogenesis. A connection was observed between PcG genes overexpression and the INK4a/ARF, Rb, and TP53 pathways silencing [10, 24]. There was also some evidence of correlation between PcG transcriptional repressor proteins and developmental pathways such as Hedgehog, Wnt/

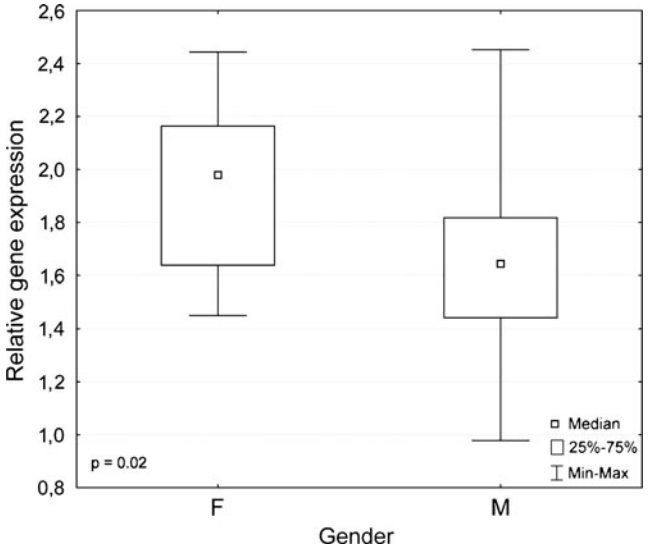

Fig. 1 *PCGF2* expression level according to gender. *F* female, *M* male. Data are presented as medians with respective interquartile ranges (IQR). Expression levels (median (IQR)) were 1.98 (1.63–2.16) and 1.64 (1.44–1.82) for females and males, respectively

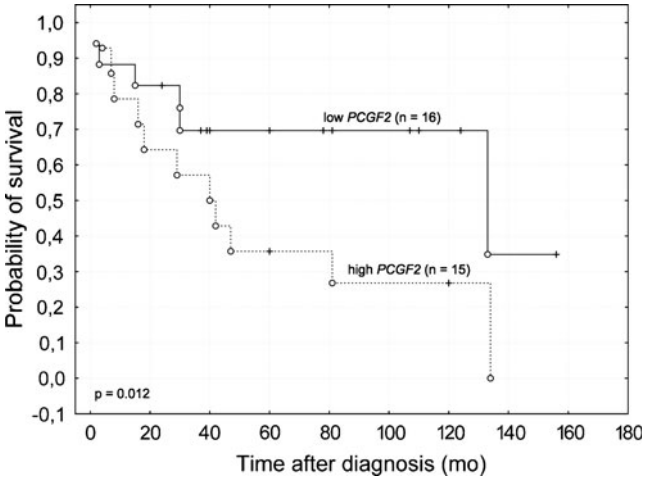

Fig. 3 Survival analysis of patients stratified by expression of *PCGF2*. Kaplan–Meier analysis of survival in 31 children with medulloblastoma. Patients were stratified into two groups based on median expression level. Follow-up is expressed as time from diagnosis, in months

beta-catenin, and Notch considered to be essential in medulloblastoma pathogenesis [27]. Via these pathways, PcG genes suppressed tumor development, progression, and expansion mainly by the limitation of putative cancer stem cells. Their expression and potential value as clinical biomarkers have been considered in selected types of cancers. The most often observed alteration has been upregulation of *BMI1*, which was related to a poor prognosis in epithelial cancers and leukemias [4, 8, 23, 29, 34]. In medulloblastoma, altered expression of *BMI1* was reported as well as correlation between BMI1 expression and the Hedgehog pathway activation [13, 27].

Leung et al. established the plausible significance of *BMI1* in medulloblastoma pathogenesis but its clinical value has not been well estimated until now [25]. Based on microarray analysis, Glinsky et al. suggested the usefulness of *BMI1* expression in prediction of outcomes in medulloblastoma [13]. In this work, we presented an association between a higher expression level of *BMI1* and decreased patient survival. Estimation of outcomes using the Kaplan–Meier method showed a trend towards a better outcome in the group with lower expression level of the *BMI1* gene. BMI1 expression and its potential usefulness as a molecular factor of outcomes were analyzed in brain tumors but not in

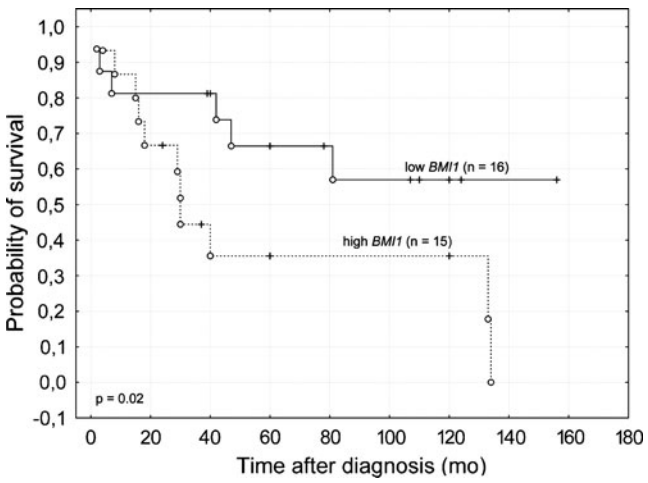

Fig. 2 Survival analysis of patients stratified by expression of *BMI1*. Kaplan–Meier analysis of survival in 31 children with medulloblastoma. Patients were stratified into two groups based on their median expression level. Follow-up is expressed as time from diagnosis, in months

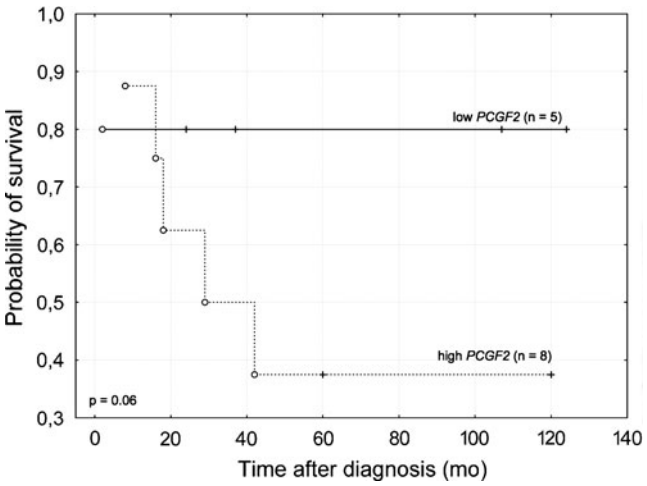

Fig. 4 Survival analysis of female patients stratified by expression of *PCGF2*. Kaplan–Meier analysis of survival in 13 females with medulloblastoma. Patients were stratified into two groups based on median expression level. Follow-up is expressed as time from diagnosis, in months

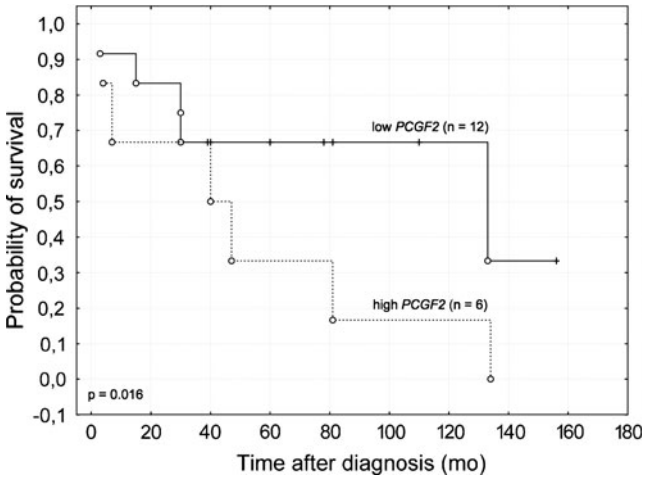

Fig. 5 Survival analysis of male patients stratified by expression of *PCGF2*. Kaplan–Meier analysis of survival in 18 males with medulloblastoma. Patients were stratified into two groups based on median expression level. Follow-up is expressed as time from diagnosis, in months

medulloblastoma. Tirabosco et al. and Häyry et al. found no correlation between BMI1 expression and outcome in astrocytomas, but such a link was observed in oligodendroglial tumors [18, 32]. Häyry et al., after reviewing analysis of *BMI1* loss in glial tumors, pointed out the association of gene deletion with poor outcomes in grade II–IV astrocytomas and considered that plausible *BMI1* gene function (oncogenic or suppressing) could vary according to the type of tumor, kind of molecular alteration, or transcriptional events [17].

A similar phenomenon was noted for the *PCGF2* gene, which encodes a functional homologue of BMI1 and

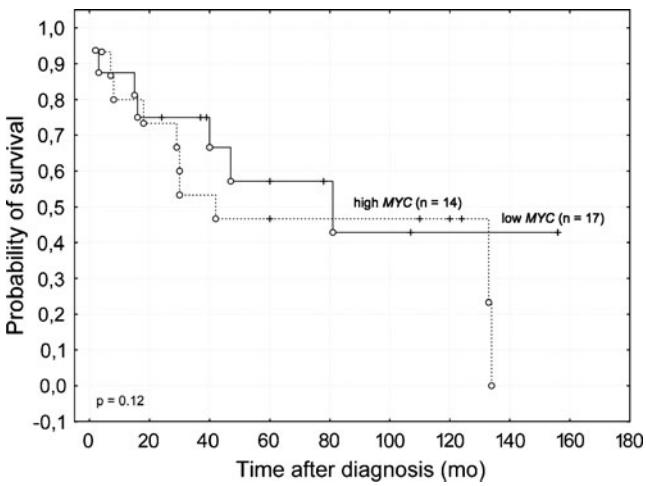

Fig. 6 Survival analysis of patients stratified by expression of *MYC*. Kaplan–Meier analysis of survival in 31 children with medulloblastoma. Patients were stratified into two groups based on median expression level. Follow-up is expressed as time from diagnosis, in months

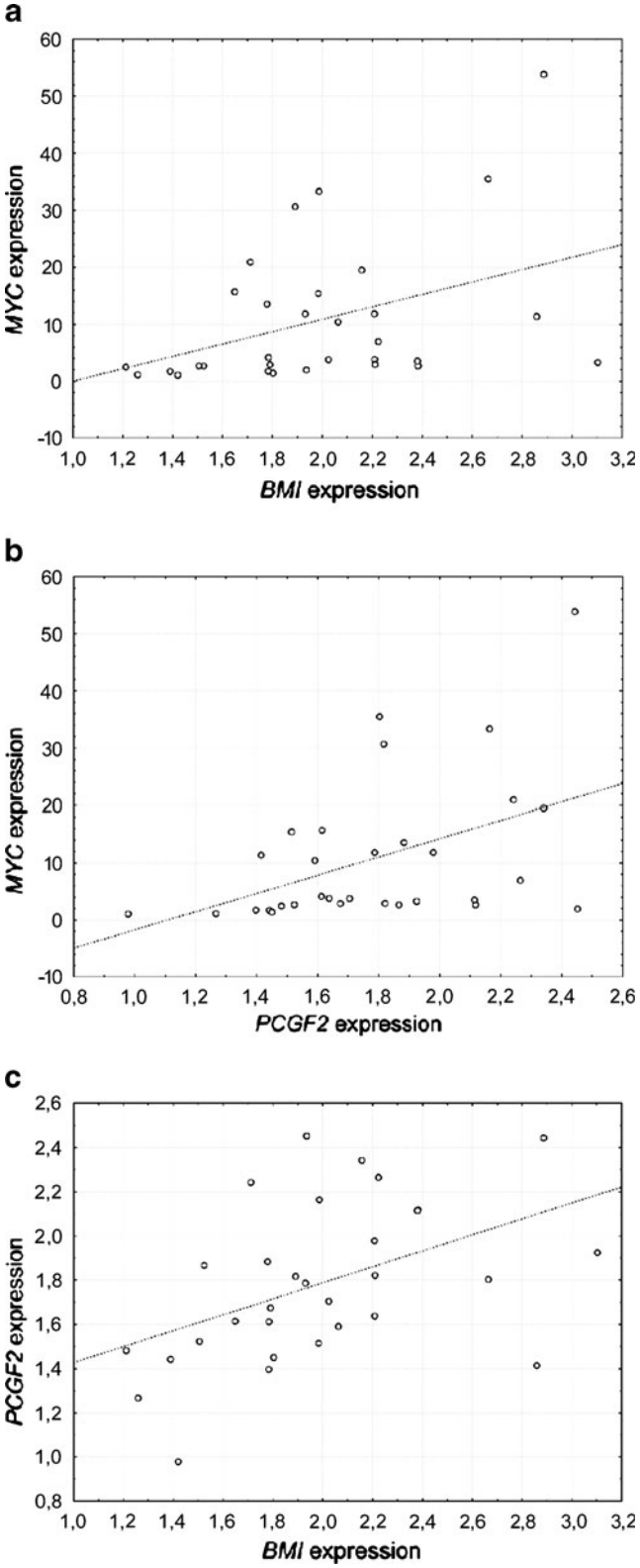

Fig. 7 The relationship between genes obtained using Spearman's correlation test. **a** *MYC* vs. *BMI1*, r^2=0.19, p=0.02; **b** *MYC* vs. *PCGF2*, r^2=0.24, p=0.005; **c** *PCGF2* vs. *BMI1*, r^2=0.26, p=0.003

regulates self-renewal processes in hematopoietic cells, as does *BMI1* [12, 21, 22, 36]. While in prostate and breast tissue *PCGF2* functioned as a tumor suppressor gene, there were evidences of its oncogenic function in medulloblastoma [15, 16, 34, 36]. Here, we demonstrated a link between a higher expression level of *PCGF2* and decreased patient survival especially among females. Our results indicated the oncogenic function of *PCGF2* in human medulloblastoma and confirmed the observation of Wiederschan et al. obtained in experimental study of medulloblastoma cell lines [36]. The data presented here showed also sex-dependent gene expression of the *PCGF2*. The higher gene expression in female patients may be interpreted as the consequence of the mechanism of chromosome X inactivation, induced via *Xist* RNA (X-inactive-specific transcript), in which PcG genes are involved during development [12, 19].

In our analysis, we also included the *MYC* gene, which was associated with *BMI1* in oncogenic processes and in breast cancer was repressed by *PCGF2* [15, 16]. *MYC* oncogene overexpression was described in approximately 50% of medulloblastomas, but its connection with prognosis predictions is unclear [9, 14, 20]. Rutkowski et al. and Herms et al. revealed the relevance of *MYC* expression for predicting outcomes, however Das et al. showed no correlation between the MYC labeling index and progress-free survival, similarly as De Bortoli et al., which presented no such link either for expression analysis [5, 6, 20, 30]. These discrepancies may be caused by the different criteria used in data interpretation, or the specificity of groups analyzed, and still needs elucidation, especially as regards the possibility of using the *MYC* as the therapeutic target inducing apoptosis in medulloblastoma [7, 33]. Here, we observed correlation between *MYC* expression level and risk stratification and no such relationship for PcG genes. In our opinion, lack of relation with clinical risk of *BMI1* and *PCGF2* genes could be connected with imperfect clinical risk classification of this tumor. Development of well-chosen molecular markers used in multivariate risk estimation together with clinical data must be concerned in case of medulloblastoma.

In summary, our analysis suggest that *BMI1* and *PCGF2* genes, acting as the epigenetic silencers engaged in the self-renewal processes of neoplastic stem cells, could be engaged in medulloblastoma oncogenesis. Higher *BMI1* and *PCGF2* gene's expression levels should be considered as the predictors of poor clinical outcome for children with medulloblastoma.

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