Annals of Hematology & Oncology

Open Access

Austin Publishing Group

Research Article

Different Gene Methylation Status of the CDKN2B and/or PDLIM4 as the Result of Comparative Analysis to the Global DNA Methylation in Unsorted Cell Population of Multiple Myeloma Patients

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Received: June 22, 2019; **Accepted:** July 04, 2019; **Published:** July 11, 2019

Abstract

Background: Multiple Myeloma (MM) is a hemato-oncological disease characterized by clonal expansion of malignant plasma cells in the Bone Marrow (BM). Apart from genetic changes, such as point mutations, deletions or translocations, it is well known, that in pathogenesis of MM are also involved epigenetic changes such as DNA methylation. Methylation of both *CDKN2B* gene, representing an inhibitor of cyclin dependent kinases, and *PDLIM4* gene, one of potential tumor suppressor genes engaged in MM evolution, were evaluated in newly diagnosed multiple myeloma patients.

Methods: The quantification of the global DNA methylation at 5´-CCGG-3´sequence using LU minometric Methylation Assay (LUMA) and the colorimetric quantification of the global DNA methylation were performed. Bisulfite-treated DNA in 13 CpGs of a promoter, and 16 CpGs of the first exon of the *CDKN2B* gene, 9 CpGs of the *PDLIM4* gene promoter were analyzed by pyrosequencing.

Results: Studied *CDKN2B* gene regions revealed CpGs methylation in the range 2.8 - 6%, whereas *PDLIM4* gene promoter showed increased level of methylated CpGs in the range 13.1 - 27%. We found a strong positive correlation between the global DNA hypomethylation (LUMA) and *CDKN2B* expression ($r = 0.766$, $P < 0.01$), and strong negative correlation between global DNA hypermethylation (LUMA) and *PDLIM4* promoter methylation level ($r = -0.994$, $P < 0.01$). Our data indicate functional unmethylated *CDKN2B* gene, in contrast to methylated tumor-suppressor *PDLIM4* gene in newly diagnosed multiple myeloma patients.

Conclusion: In unsorted bone marrow cells of newly diagnosed multiple myeloma patients, the CpG methylation pattern of the studied *CDKN2B* and *PDLIM4* genes varies depending on overall DNA methylation level. Their different methylation status determined in both global DNA hypomethylated and hypermethylatied groups of patients could be related to a followed progression of the multiple myeloma disease. On the base of statistical analysis, the *PDLIM4* gene show significantly increased methylation state with negative correlation to the detected DNA methylation level. These methylation changes of the *PDLIM4* gene can contribute to pathogenesis of myeloma and its methylation status acts as a prognostic factor.

Keywords: Methylation; Neoplastic cells; DNA

Abbreviations

AML: Acute Myelogenous Leukemia; BM: Bone Marrow; CDKN2A and CDKN2B: Cyclin Dependent Kinase Of Inhibitors 2A and 2B; CML: Chronic Myelogenous Leukemia; CML: Chronic Myelogenousleukemia; LUMA: Luminometricmethylaion Assay; MGUS: Monoclonagammopathy of Undetermined Significance; MDS: Myelodysplastic Syndrome; MM: Multiple Myeloma; MtL: Methylation Level; NF-κβ: Nuclear Factor - κβ; SMM: Smoldering Multiple Myeloma; *PDLIM4*: PDZ and LIM Domain Protein 4; TGF-β: Transforming Growth Factor - β.

Background

Monoclonal gammopathies are clonal proliferations of plasma cells, also referred as plasma cell dyscrasias. The most important condition among plasma cell dyscrasias is Multiple Myeloma (MM). Multiple myeloma is characterized by malignant proliferation of clonal Plasma Cells (PC). The overt/active form of the disease is accompanied by various extent of organ impairment. The organ and tissue alteration is usually described with the acronyme "CRAB" - hypercalcaemia, renal insufficiency, anemia and lytic bone lesions. Smoldering Multiple Myeloma (SMM) is an intermediate

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Citation: Luzna P, Flodrova P, Janovska L, Zapletalova J, Minarik J, Kolar Z, et al. Different Gene Methylation Status of the CDKN2B and/or PDLIM4 as the Result of Comparative Analysis to the Global DNA Methylation in Unsorted Cell Population of Multiple Myeloma Patients. Ann Hematol Oncol. 2019; 6(7): 1257.

Table 1: Primer sequences corresponding to bisulfite-treated DNA used for pyrosequencing.

Gene	PCR Primer Forward	PCR Primer Reverse	Pyrosequencing Primer	CpGs
RILpromot (216 bp)	5´GGGTTTATG- AGGAGGTATT-TGAGTTG-3´	5´-biotin-ACACC-CCCACTCAACT-CTC-3´	5´-TGTAGATA9 GTTGGGTTTG-G-3´	
P15promot (223bp)	5´-AGGAGTTG- AGGGTAGTGG-T-3´	5´-biotin-TCCCC-ACCCCCTTAAA-CT-3´	5´-GGATATTT13 AGAGAGTAG-TGTAGTTA-3´	
P15exon (138bp)	5´-AGGAGGGG- TAGTGAGGAT-3´	5´-biotin-ACTTT-TCCTAACCCTC-AAAAACCAAC-3´	5´-GGGGTAG- TGAGGATT-3´	16

asymptomatic malignant plasma cell disorder, with a risk of progression to symptomatic MM of ~10 % per year, for the first 5 years [1,2]. Multiple myeloma is a multistep transformation process evolving from its premalignant state - Monoclonal Gammopathy of Undetermined Significance (MGUS), with transformation rate of ~1% per year. Molecular biology of MM is complex and involves important and not yet fully understood relationship between neoplastic cells and microenvironment of the bone marrow, which is known to assert one of the most important processes in the disease´s evolution [3]. Very important role in MM pathogenesis belongs to different cytogenetic abnormalities - hyperdiploid or hypodiploid genome, translocations with very high incidence of IgH gene involvement, deletions and amplifications. Some of these changes are associated with poor prognosis in MM patients. Apart from genetic abnormalities, epigenetic modifications are known to participate in pathogenesis of monoclonal gammopathies.

DNA methylation belongs to a group of epigenetic changes together with histone posttranslational modifications and changes in microRNA. Epigenetic modifications deal with affecting of gene expression in other levels than in changes in DNA sequence. The key role of DNA methylation during the physiological processes as embryogenesis, ontogenesis and aging is well known. Tumor development and progression caused by DNA methylation changes have been already described as well [4-6]. Aberrant DNA methylation seems to be an important event in multiple myeloma pathogenesis. DNA methylation patterns have been found to change as multiple myeloma progresses [7], and differential methylation at certain gene loci has an association with adverse outcomes [8,9].

In this study, we used two methodological procedures – colorimetric and Luminometric Methylation (LUMA) assays to detect the global DNA methylation with the followed CpG methylation pattern analysis of *CDKN2B* and *PDLIM4* genes detected by bisulfite pyrosequencing. In unsorted bone marrow cells of newly diagnosed multiple myeloma patients, the CpG methylation pattern of the studied *CDKN2B* and *PDLIM4* genes varies depending on overall DNA methylation level. On the base of statistical analysis, the *PDLIM4* gene show significantly increased methylation state with negative correlation to the detected DNA methylation level. These methylation changes of the *PDLIM4* gene can contribute to pathogenesis of myeloma or could act as a prognostic factor.

Methods

Patient samples

Unsorted cell population in the bone marrow aspirate of patients with symptomatic multiple myeloma were studied. The diagnosis of MM followed International Myeloma Working Group criteria. The study was approved by the Ethics Committee of University Hospital Olomouc, and samples were collected after informed consent.

ELISA analysis of global DNA methylation

Genomic DNA was obtained from unsorted cell population in the bone-marrow aspirates. The extraction was performed using QIAamp® DNA Blood Kit (Qiagen, Hilden, Germany) according to manufacturer´s protocol and the 260/280 nm absorbance ratio was used to assess the purity of DNA. Isolated DNA (100 ng) was used for a colorimetric detection of the global methylation status (% 5mC) by MethylFlash™ Methylated DNA Quantification Kit (Epigentek, Farmingdale, NY, USA).

Luminometric Methylation Assay (LUMA)

Quantification of global DNA methylation was examined using Luminometric Methylation Assay [10,11]. Briefly, genomic DNA was isolated by the Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA).Extracted genomic DNA (~400 ng) was treated with sodium bisulfite using EpiTect® Bisulfite kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). DNA after bisulfite modification was cleaved using two restriction enzymes, *HpaII* (methylation sensitive) and *MspI* (methylation insensitive) in two separate reactions in the presence of *EcoRI* to standardize for DNA amounts. The LUMA assay is a global rather than a gene-specific assay and from this reason, it is performed without a reference genome [10]. Digested DNA were then used as templates for pyrosequencing with the Pyromark Q96 ID instrument and Pyromark Gold Q96 reagents (Qiagen, Hilden, Germany). The luminometric signals produced by either the sequential incorporation of C and G nucleotides (reflecting the number of 5´-CCGG- 3´ sequence digested by HpaII or MspI) or the sequential incorporation of A and T nucleotides (reflecting the number of 5´-AATT- 3´ sequence digested by EcoRI), were then quantified using Pyromark Q96 ID software 1.0. The global methylation percentage per sample was then calculated as follows [12]: (Average signal obtained with *HpaII* after *EcoRI* normalization / Average signal obtained with *MspI* after *EcoRI* normalization) • 100.

Bisulfite methylation analysis by pyrosequencing

DNA methylation of *PDLIM4* promoter and the *CDKN2B* promoter and first exon gene regions was determined by pyrosequencing method [13-15]. Bisulfite treatment of extracted genomic DNA was done as describe above. Primers for PCR and following pyrosequencing reaction were designed using PyroMark Assay Design SW 2.0 (Qiagen, Hilden, Germany) (Table 1). For PCR reaction 1 µl of bisulfite treated DNA was added in a 25-µl PCR reaction mixture containing 1 x PyroMark PCR Master Mix (Qiagen, Hilden, Germany), 1 x CoralLoad Concentrate (Qiagen, Hilden, Germany), 1.5 mM $MgCl_2$, 0.2 µM forward primer and 0.2

Table 2: The set of 42 MM samples was divided on the basis of the global DNA methylation parameter determined by both LUMA and ELISA assays, and the level of both CDKN2B and PDLIM4 gene expressions. *Values were not included in the correlation analysis calculation.

Hypomethylated group		Global methylation
<μ-σ	0.57157	ELISA LUMA (%)
1/M56	0.405	62.73
2/M57	0.293	29.55
3/M58	0.08	75.71
4/M59	0.379	68.49
5/M64	0.273	75.44
7/M50	0.539	74.96
6/M73	0.172	44.26
8/M86	0.546	65.65

Moderately methylated group	Global methylation	
	ELISA (%)	LUMA (%)
1/M61	0.589	56.04
2/M62	1.301	67.05
3/M66	0.725	71.15
4/M68	0.745	73.41
5/M69	1.127	70.46
6/M77	1.185	72.15
7/M78	1.14	72.9
8/M79	0.785	75.54
9/M80	1.189	72.47
10/M81	1.118	66.24
11/M83	1.22	73.92
12/M84	0.993	76.07
13/M85	1.384	48.66
14/M25	0.643	77.57
15/M10	0.742	72.83
16/M08	0.977	74.66
17/M07	1.417	72.42
18/M14	1.268	79.21
19/M04	1.116	73.49
20/M03	1.34	55.81
21/M02	0.998	55.77
22/M06	1.39	62.95
23/M21	1.054	72.5
24/M26	0.99	72.99
25/M27	1.127	73.02
26/M28	0.8	73.65
27/M37	1.174	74.28
28/M39	1.072	73.11

Hypermethylated group		Global methylation	
>μσ	1.42781	ELISA (%)	LUMA (%)
1/M70		1.774	56.76
2/M20		1.688	80.77
3/M29		1.805	74.81
4/M30		1.463	71.09
5/M33		1.431	66.54
6/M38		1.530	75.82

μM biotinylated reverse primers. For Hot Start Taq Polymerase activation, the PCR reaction mixture was initially denaturated at 95°C for 15 min followed by 45 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, elongation at 72°C for 30 sec, and the final extension at 72°C for an additional 10 min after the last cycle. The final biotinylated PCR product was immobilized on Streptavidin Sepharose® HP (GE Healthcare, Pittsburg, PA), precipitated with 70% ethanol, passed through denaturation step and then washing step using PyroMark Q96 Vacuum Workstation (Qiagen, Hilden, Germany). The amplicons were transferred to each well of the PyroMark Q96 plate containing 40 μl of 0.4 μM sequencing primer diluted in annealing buffer (Qiagen, Hilden, Germany). Control unmethylated and methylated DNA (Qiagen, Hilden, Germany) after bilusulfite treatment were part of a set of analyzed MM patient samples. Pyrosequencing analysis was performed according to the PyroMark CpG Sofware 1.0.11 (Qiagen, Hilden, Germany). Methylation was quantified in terms of the methylation level (MtL) as the average percentage of cytosines methylated per CpG: MtL (%) = (Σ% methylated cytosines) / No. of CpGs analyzed).

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from unsorted population of bone marrow samples using TRI Reagemt® BD (Molecular Research Center, Inc., OH, USA). Input of 100 ng of total RNA was reverse transcribed using Transcriptor First Strand cDNA Synthesis kit (Roche, Basel, Switzerland). qRT-PCR with TaqMan probes and Xceed qPCR Probe Mix (Institute of Applied Biotechnologies, Prague, Czech Republic) was performed using Light cycler®480 System (Roche, Basel, Switzerland). Expression of *CDKN2B* (Hs00793225_m1) and *PDLIM4* (Hs00896441_m1) genes was normalized to expression of endogenous housekeeping control *GAPDH* (Hs01041237_g1) gene. All probes were provided by Thermo Fisher Scientific (Waltham, MA, USA) and commercially available human BM total RNA (Takara Bio USA, Inc., Mountain View, CA, USA) was used as the calibrator for $2^{-\Delta\Delta Ct}$ quantification approach.

Statistical analysis

Both methylation and gene expression data were quantile-normalized to make the distributions the same across patient samples. A Pearson correlation coefficient (r) was calculated for each global DNA methylation and gene expression probe pair, for global DNA methylation and gene methylation, and for gene methylation and gene expression. A negative correlation was defined when the directionality of changes for gene expression and DNA methylation were in the opposite direction (*e.g.* presence of methylation and low expression, or *viceversa*). A positive correlation occurred when the directionality of changes was the same between DNA methylation and gene expression (*e.g.* presence of methylation and positive expression, or *viceversa*).

Table 3: Correlation analysis between global DNA methylation determined by LUMA **(A) and/**or ELISA **(B**) and *CDKN2B* and *PDLIM4* gene expressions in the whole set of MM patients.

A		*CDKN2B* Gene Expression	*PDLIM4* Gene Expression
LUMA (%) Gl. DNA Methylation	Pearson (r) p-Value	0,786 < 0,01	0,445 < 0,01
Hypomethyl.gr. Gl. DNA Methylation	Pearson (r) p-Value	0,766 < 0,01	0,709 0,0326
Moderately m. Gl. DNA Methylation	Pearson (r) p-Value	0,430 0,0223	0,447 0,010
Hypermethyl.gr. Gl. DNA Methylation	Pearson (r) p-Value	0,592 0,4084	0,707 0,1818

B		CDKN2B Gene Expression	PDLIM4 Gene Expression
ELISA Gl. DNA Methylation	Pearson (r) p-Value	5	0,556 < 0,01
Hypomethyl.gr. Gl. DNA Methylation	Pearson (r) p-Value	0,601 0,1151	0,528 0,1783
Moderately m. Gl. DNA Methylation	Pearson (r) p-Value	0,472 0,0129	0,587 < 0,01
Hypermethyl.gr. Gl. DNA Methylation	Pearson (r) p-Value	0,776 0,224	0,643 0,1685

Table 4: Correlation analysis between global DNA methylation determined by LUMA **(A) and/**or ELISA **(B**) and *CDKN2B* and *PDLIM4* gene expressions in the whole set of MM patients (**Table 2**) and different methylated group of MM patients.

A		*CDKN2B* Gene Expression	*PDLIM4* Gene Expression
LUMA (%) Gl. DNA Methylation	Pearson (r) p-Value	0,786 < 0,01	0,445 < 0,01
Hypomethyl.gr. Gl. DNA Methylation	Pearson (r) p-Value	0,766 < 0,01	0,709 0,0326
Moderately m. Gl. DNA Methylation	Pearson (r) p-Value	0,430 0,0223	0,447 0,010
Hypermethyl.gr. Gl. DNA Methylation	Pearson (r) p-Value	0,592 0,4084	0,707 0,1818

B		CDKN2B Gene Expression	PDLIM4 Gene Expression
ELISA Gl. DNA Methylation	Pearson (r) p-Value	0,471 < 0,01	0,556 < 0,01
Hypomethyl.gr. Gl. DNA Methylation	Pearson (r) p-Value	0,601 0,1151	0,528 0,1783
Moderately m. Gl. DNA Methylation	Pearson (r) p-Value	0,472 0,0129	0,587 < 0,01
Hypermethyl.gr. Gl. DNA Methylation	Pearson (r) p-Value	0,776 0,224	0,643 0,1685

In the methylation parameter, for measure of central tendency, median as well as mean (MtL) were included in calculations. The set of 42 MM patient samples were divided on the basis of global methylation status determined: hypomethylated, moderately methylated or hypermethylated. This approach is referred as "the discretization approach" because samples are discretized into one of the three groups [16]. Thus, samples with methylation level < $\mu - \delta$ (mean plus one standard deviation) were classified into the hypomethylated group; samples with methylation level > $\mu + \delta$ were classified as hypermethylated; and all other remaining samples vere considered as moderately methylated samples. Followed, the global DNA methylation was determined in MM samples using both colorimetric detection (ELISA) and Luminometric Methylation Assay (LUMA) as well. Based on the global methylation status (% 5mC) detected by LUMA assay, the hypomethylated group (68,28 < $\mu - \sigma$), the moderately methylated and the hypermethylated group (75,724 > $\mu - \sigma$) of the same set of MM patient samples were formed (**Supplement I**). Furthermore, on the basis of values obtained by ELISA method, we classified the hypomethylated group (0,572 < $\mu - \sigma$), the moderately methylated group, and the hypermethylated group (1,428 > $\mu - \sigma$) of MM patient samples (**Supplement II**). Statistical significance of difference among tested groups of patients was analyzed using post- hoc test Mann-Whitney U test with Bonferroni´s correction of significance. All tests were made at a significance level of 0,05. Data were statistical analyzed with IBM SPSS Statistics, v.22.

Results

Global DNA Methylation *Versus* Gene Expression

Generally, at both monitored files detecting global DNA methylation LUMA and ELISA assays, a level of the *PDLIM4* expression was increased in comparison to the *CDKN2B* gene expression (**Table 2**). Furthermore, the highest level of *PDLIM4* expression was detected in MM patients with the lowest level of global DNA methylation - the hypomethylated group (**Figure 1, Table 2**). On the other hand, a high level of the global DNA methylation, in the hypermethylated group was accompanied by a high *CDKN2B* expression (mean value 3,85) (**Figure 2, Table 2**).

Following correlation analysis of the set of 42 MM samples (**Table 2**) was performed between the global DNA methylation parameter determined by both LUMA and ELISA assays, and the level of both *CDKN2B* and *PDLIM4* gene expressions. In the hypomethylated group of MM patients (LUMA), a strong positive correlation ($P<$ 0,01; r = 0,766) between the level of global DNA methylation and the *CDKN2B* gene expression was demonstrated (**Table 3A**). Positive correlation of the PDLIM4 expression (r = 0,587) with the moderately methylated group based on the ELISA assay was at significant P-value < 0,01 (**Table 3B**).

Global DNA Methylation *Versus* Gene Methylation

In search for causes of aberrant methylation of *CDKN2B* gene, a 223-bp promoter region with 13 CpGs, and a 138-bp region of the first exon of the gene with 16 CpGs were analyzed by pyrosequncing. For the assessment of the *PDLIM4* gene, the 216-bp region containing 9 CpGs localized in the promoter and 3´-UTR region was used. As described by [17], there are 2 alleles in the population, long and short, with a polymorphic area near a CGG repeat sequence adjacent to the transcription start site, where the long allele is created by an insertion of a 12-bp fragment (5´-CGGCGGCGGCTC- 3´) and substitution

Table 5: Correlation analysis between *CDKN2B* gene expression and *CDKN2B* promoter methylation (**A**), *PDLIM4* gene expression and *PDLIM4* promoter methylation (**B**) in the whole set of MM patients (**Table 2**) and different methylated group of MM patients divided on the basis of the global DNA mehtylation detected by LUMA assay).

A		***CDKN2B* Promoter Methylation (% MtL)**	***CDKN2B* Promoter Methylation (Median)**
LUMA (%) CDKN2B Gene expres.	Pearson (r) p-Value	0,276 0,3402	-0,345 0,227
Hypomethyl.gr. CDKN2B Gene expr.	Pearson (r) p-Value	0,451 0,5494	-0,398 0,6019
Moderately m. CDKN2B Gene expr.	Pearson (r) p-Value	0,229 0,621	-0,426 0,34
Hypermethyl.gr. CDKN2B Gene expr.	Pearson (r) p-Value	0,806 0,4032	-0,5416 0,6353

B		***PDLIM4* Promoter Methylation (% MtL)**	***PDLIM4* Promoter Methylation (Median)**
LUMA (%) PDLIM4 Gene expr.	Pearson (r) p-Value	-0,612 < 0,01	-0,659 < 0,01
Hypomethyl.gr. PDLIM4 Gene expr.	Pearson (r) p-Value	-0,605 0,395	-0,742 0,258
Moderately m. PDLIM4 Gene expr.	Pearson (r) p-Value	-0,654< 0,01	-0,686 < 0,01
Hypermethyl.gr. PDLIM4 Gene expr.	Pearson (r) p-Value	-0,561 0,3252	-0,5677 0,3189

Table 6: Correlation analysis between *CDKN2B* gene expression and *CDKN2B* promoter methylation (**A**), *PDLIM4* gene expression and *PDLIM4* promoter methylation (**B**) in the whole set of MM patients (**Table 2**) and different methylated group of MM patients divided on the basis of the global DNA mehtylation detected by ELISA assay.

A		**CDKN2B Promoter Methylation (% MtL)**	**CDKN2B Promoter Methylation (Median)**
ELISA CDKN2B Gene expr.	Pearson (r) p-Value	0,276 0,3621	-0,345 0,227
Hypomethyl.gr. CDKN2B Gene expr.	Pearson (r) p-Value	N.A.	N.A.
Moderately m. CDKN2B Gene expr.	Pearson (r) p-Value	0,154 0,692	-0,432 0,2456
Hypermethyl.gr. CDKN2B Gene expr.	Pearson (r) p-Value	0,89 0,3011	-0,3871 0,747

B		**PDLIM4 Promoter Methylation (% MtL)**	**PDLIM4 Promoter Methylation (Median)**
ELISA PDLIM4 Gene expr.	Pearson (r) p-Value	-0,612 < 0,01	-0,659 < 0,01
Hypomethyl.gr. PDLIM4 Gene expr.	Pearson (r) p-Value	-0,543 0,457	-0,523 0,477
Moderately m. PDLIM4 Gene expr.	Pearson (r) p-Value	-0,68 < 0,01	-0,714 < 0,01
Hypermethyl.gr. PDLIM4 Gene expr.	Pearson (r) p-Value	-0,538 0,2709	-0,5964 0,2118

of T to G 3 bases upstream of the insertion site in the short allele (**Figure 1**). Despite the awareness of suspicion, the short allele being methylated more than the long one, we analyzed the 216-bp region where both alleles are shared.

To determine the *CDKN2B* gene methylation level (MtL), i.e. the promoter and the first exon, we calculated the mean of all measured values, and the median calculated for each sample from all 13 CpG promoter and 16CpG first exon values, for each of the three groups of MM patients: hypomethylated, moderately and hypermethylated (**Supplement II**). We found significantly higher MtL mean (21,4) of *PDLIM4* promoter CpGs methylation in the hypermethylated group than in the hypomethylated group (MtL mean = 17,7; P = 0,026) as well as the moderately methylated group (MtL mean = 15,8; P = 0,023) (**Table 7, Table 8**, **Figure 4**).

For correlation analysis between the global DNA methylation and gene expression, the methylation level (mean and median) of both *CDKN2B* and *PDLIM4* gene promoters were calculated.

In the three selected groups of MM patients, we found the methylation level of the *CDKN2B* promoter in the range 2,8 - 6% (patient mean MtL) and/or 0 - 7% (median). The patient MtL of the *PDLIM4* promoter was in the range 13,1 - 27% and/or 12 - 26 % (median). (**Supplement I, Supplement II**). In the whole set of 42 samples (**Table 2**), data obtained from both methods for detection of the global DNA methylation status showed positive correlations with the *CDKN2B* gene promoter methylation and negative correlations with the *PDLIM4* gene promoter methylation. We have found very strong negative correlation in the hypermethylated group (P< 0,01; r = -0,994) between the global DNA methylation detected by LUMA assay and the *PDLIM4* promoter region methylation, in which median of 9 CpGs was used for calculation (**Table 4A**, **Supplement I**). In the hypermethylated group of MM samples examined by ELISA kit (**Table 4B**), strong negative correlations for average MtL (P = 0,0105; r = -0,915) and for median (P = 0,0162, r = -0,894) were found, these both were significant at P< 0,05. The negative correlations, were detected in the moderately methylated group (LUMA) and *PDLIM4* expression (r = -0,414 for average MtL; r = -0,88 for median) (**Table 4A, Table 4B**). Strong negative correlation between global DNA methylation (LUMA) and *PDLIM4* gene promoter methylation were found in the hypomethylated group of MM patients for average MtL (P = 0,035, r = -0,843) and for median of 9 CpGs of the *PDLIM4* promoter (P = 0,0157, r = -0,896).

Gene expression *Versus* gene methylation

To identify *CDKN2B* and *PDLIM4* gene promoter methylation to correlate to their gene expression, we performed Pearson correlation analysis in two normalized cohorts according to the method of global DNA methylation detection (**Suplement I**, **Supplement II**). We found that both ELISA and LUMA assays detecting global DNA methylation level in the moderately methylated group, show strong negative correlation between *PDLIM4* gene expression and *PDLIM4* promoter methylation at significant level < 0,01 (**Table 5**, **Table 6**). In both groups of MM patients, *i.e.* with low or high level of global DNA methylation (the hypomethylated group as well as

Table 7: Mean of all measured values were obtained from all CpGs of the *CDKN2B* gene tested regions - promoter (13 CpGs) and first exon (16 CpGs).

CDKN2B (CpGs): promoter + first exon	Hypermethylated group (mean MtL)	Moderately methylated group (mean MtL)	Hypomethylated group (mean MtL)
Mean	3,9	3,9	4,7
SD	3,3	4,3	3,3
Median	4,0	4,0	5,0
Minimum	0,0	0,0	0,0
Maximum	16	20	16

Table 8: Mean of all measured values were obtained from all CpGs of the *PDLIM4* gene promoter (9 CpGs).

PDLIM4 (CpGs): promoter	Hypermethylated group (mean MtL)	Moderately methylated group (mean MtL)	Hypomethylated group (mean MtL)
Mean	21,4	15,8	17,7
SD	10,1	7,4	8,9
Median	19,0	15,0	15,0
Minimum	8,0	7,0	6,0
Maximum	49	42	53

the hypermethylated group), the effect of methylation changes on *PDLIM4* expression was not significant. We found no correlation between the *CDKN2B* gene expression and the *CDKN2B* promoter methylation, suggesting there is a limited effect of DNA methylation on the *CDKN2B* expression or, that this gene expression is probably not affected by DNA methylation at all.

Discussion

In our study, DNA methylation and gene expression analysis from 42 newly diagnosed multiple myeloma patients were performed. The global DNA methylation status was determined by two methods - Luminomentric methylation assay and ELISA colorimetric assay, and for methylation analysis, *CDKN2B* and *PDLIM4* genes were selected for quantification of DNA methylation changes of unsorted cell population in bone marrow aspirates. We found that in newly diagnosed patients, the level of global DNA methylation reflects the *CDKN2B* gene expression, but not an expression of the *PDLIM4* gene, whose expression is negatively affected the *PDLIM4* promoter methylation (**Figure 3**).

CpG islands are genomic regions with a high frequency of CpG sites, which are often located near the transcripton start site of genes. DNA methylation changes, including hypermethylation, in gene promoter CpG islands are responsible for altering the expression of many different tumor suppressor genes and oncogenes in cancer diseases [18,19]. On the other hand, global DNA hypomethylation, which occurs predominantly outside CpG islands, leads to a genomic instability. Moreover, global hypomethylation supports the transition towards an increasingly aggressive phenotype: normal plasma cells / MGUS / asymptomatic myeloma / symptomatic myeloma [7,20], whereas distinct patterns of hypermethylation are presented within sub-groups of symptomatic myeloma [20].

Methylation patterns in cells relevant to the pathogenesis of myeloma plasma cells (Blymphoma cells, normal plasma cells, MGUS and MM cells) are capable of distinguishing nonmalignant and malignant cells [7]. The reason is due to the difference in hypomethylation of global DNA, which is accompanied by gene-specific hypermethylation [21]. For this, the *PDLIM4* gene, a potential tumor suppressor gene in MM progression, and the *CDKN2B* gene, representing an inhibitor of cyclin dependent kinases - $p15^{INK4B}$ and $p16^{INK4A,}$ were chosen for our analysis. The $p15^{INK4B}$ and $p16^{INK4A}$ located on chromosome 9p21 encoded by *CDKN2B* and *CDKN2A* genes, belong to the INK4 family proteins. Frequencies of *CDKN2B* and *CDKN2A* promoter methylation in multiple myeloma samples are significantly higher in comparison to normal samples [22] as well as frequent methylations of these genes were detected [23-25]. Different prognostic values of $p15^{INK4B}$ and $p16^{INK4A}$ methylation in multiple myeloma course suggest a distinct influence on the outcome of MM, and as described Guillerm et al. (2003), survival is impaired in patients with methylated$p16^{INK4A}$, but is not different between patients with methylated and unmethylated *CDKN2B* gene. Methylation of the *CDKN2A* gene is significantly associated with short survival [26], while expression of the *CDKN2B* gene is induced by transforming growth factor - β (TGF-β) [27]. The TGF-β acts as a potent tumor suppressor through regulation of variety of physiological processes, including adhesion of myeloma cells to stromal cells, growth inhibition and stimulation of apoptosis [28-30]. With this being related, the possible inactivation of *CDKN2B* by methylation could suggest a mechanism of escape from regulatory signals provided by the bone marrow microenvironment [31]. However, the role of $p15^{INK4B}$ methylation in the development and progression of the multiple myeloma disease remains unknown and other analysis are needed.

In MM patients, interactions between malignant plasma cells derived from multiple myeloma stem cells and bone marrow tumor microenvironment occur directly *via* cell receptors and adhesion molecules. Some of the adhesion molecules, such as cadherins, facilitate formation of multicellular structures in the bone marrow by anchoring to the actincytosleleton. Our selected RIL (product of *PDLIM4* gene) is an actin associated nuclear protein containing both PDZ and LIM domains, which belongs to a large family of LIM proteins [32]. PDZ-LIM proteins, including *PDLIM4* may act as adaptors between cytoskeleton and intracellular signaling components, thereby regulating diverse cell signaling pathways [33], In the process of adhesion of myeloma cells to stromal cells, an important role is also played by extracellular factor, such as cytokines

and growth factors, which have the ability to activate multiple signalling pathways (such as TGF-β, NF-κβ and Notch), and increase the expression of cell cycle regulatory proteins (D-type cyclins) and Bcl-2 family anti-apoptotic proteins in both stromal and myeloma cells [28-30].Transforming growth factor - β induced growth arrest is attenuated in association with aberrant activation of Nuclear Factor - κβ (NF-κβ) [34], which is in activated cells degraded by PDLIM2 [31]. Among PDZ-LIM proteins, PDLIM1, PDLIM3 and *PDLIM4* are most closely related to PDLIM2, since they all contain one PDZ domain and one LIM domain [35], but their role in this signaling pathway has not yet been described. Furthermore, [33] found that PDLIM1 is also highly expressed in dendritic cells, while *PDLIM4* is exclusively expressed in $CD4^{+}$T cells, but not in dendritic cells.

The LIM protein RIL, *PDLIM4* gene is located on 5q31.1, a region frequently deleted in the malignant cells of patients with Myelodysplastic Syndrome (MDS) and Acute Myelogenous Leukemia (AML) [36,37], and appears to be a candidate for tumor-suppressor gene resides in this area. Reversion Induced LIM (RIL) frequent in abnormal methylation of a Src suppressor gene *PDLIM4* was associated with shortened survival independently of Chronic Myelogenous Leukemia (CML) stage [38]. [39] show that RIL methylation is associated with loss of expression, and that RIL re-expression leads to suppression of cell growth and sensitizes cells to apoptosis. In addition, its methylation status could be a marker of adverse prognosis independent of chromosome 5 and 7 deletions [39,40]. Furthermore, [40] describe a polymorphism in the RIL promoter that creates an Sp1/Sp3 binding site on a 12-bp polymorphic sequence around its transcription start site that creates a long allele. Methylation analysis showed, the short allele had 2,1-3,1-fold higher methylation than the long allele, suggesting that the polymorphic Sp1 site protects against time-dependent silencing.

In our study, we found the significant positive correlation between the groups of both hypomethylated and moderately methylated multiple myeloma patients, and the *CDKN2B* gene expression. The CDKN2B expression corresponds to a level of the global DNA methylation and is the highest in hypermethylated patients (**Figure 1**). On the other hand, the level of *PDLIM4* expression is the highest in the hypomethylated group of patients (**Figure 2**). The revealed low *PDLIM4* expression in hypermethylated patients seems to be a consequence of the determined increased promoter methylation. This findings indicate a different gene methylation status between $p15^{INK4B}$ and *PDLIM4*, which was supported of the strong negative correlation between the *PDLIM4* promoter methylation and global DNA hypermethylation. Furthermore, patients with global DNA hypermethylation have got significantly higher MtL mean values of *PDLIM4* promoter CpGs than hypomethylated or moderately methylated groups (**Table 8**). Obviously, the PDLIM4 promoter methylation is present in MM patients and their methylation status may affect a reduction of the PDLIM4 expression. This is supported by the significant correlation between PDLIM4 expression and PDLIM4 promoter methylation.

Our findings show that newly diagnosed hypermethylated patients, as well as the hypomethylated group of patients, show expression variabilites of CDKN2B and *PDLIM4* genes in comparison to patients in moderately methylated global DNA methylation state. If we assume that the global DNA hypomethylation is a marker of cancer diseases [21], then the global DNA hypermethylation as the potential initial stage of the myeloma disease progression should be accompanied by slightly changed expression of genes of anti-tumor defense - including *CDKN2B* gene. Still not clear whether the *CDKN2B* gene promoter contains methylation changes leading to the gene silencing. There is a lack of systematic analysis of $p15^{INK4B}$ epigenetic modification such as promoter or body gene methylations. Although a number of reports have determined that the inactivation of *CDKN2B* gene is mainly induced by hypermethylation in MM, the reported rates of *CDKN2B* hypermethylation in MM are remarkably diverse. As described [42] in the set of 72 MM patients, in both tested regions of the *CDKN2B* gene, *i.e.* the promoter and the first exon, there were no significant differences between the methylation levels - mean MtL ranged from 3% to 6% in the *CDKN2B* gene promoter and from 3% to 12% in the first exon. Therefore, detailed pyrosequencing methylation analysis of promoter and first exon the *CDKN2B* gene regions were performed, but for our comparison analysis we used methylation data from the promoter region of both studied genes.

Although we present a pilot study and data, which should be further extended and complemented by comparison with clinical data of tested patients, in our analyzed the set of newly diagnosed multiple myeloma patients, a level of the global DNA methylation reflects the *CDKN2B* gene expression, while the *PDLIM4* gene expression is negatively affected by the *PDLIM4* promoter methylation. Our results indicate that increased methylation in *PDLIM4* promoter has an effect on the *PDLIM4* gene decreased expression as is generally described in cancer proliferations [18,19]. Moreover, we have found no correlations between both used methods - Luminomentric methylation assay and ELISA colorimetric assay detecting the global DNA hypermethylation, and the *CDKN2B* gene expression, suggesting a possibility that this gene expression is not affected by DNA methylation. The *CDKN2B* gene hypomethylated status corresponding to the *CDKN2B* gene low expression could be associated with impaired prognosis in newly diagnosed hypermethylated MM patients rather than in patients with hypomethylated and moderatetely methylated global DNA methylation states. Finally, our findings indicate that both global DNA hypermethylation and hypomethylation states of newly diagnosed patients could be related to a followed progression of the multiple myeloma disease.

Acknowledgement

We thank the Hemato-Oncology Department of the University Hospital Olomouc to providing samples of selected multiple myeloma patients. We thank Dr. Karin Jöhrer from Tyrolean Cancer Research Institute in Innsbruck for reading the manuscript.

Funding

This study was supported in part by NV18-03-0050 from the Ministry of Health of the Czech Republic and LF_2018_001 from Palacky University Olomouc.

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