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Methylation and mRNA expression levels of P_{15}, death-associated protein kinase, and suppressor of cytokine signaling-1 genes in multiple myeloma

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ABSTRACT

Objective(s): The aim of this study was to investigate the methylation status and mRNA expression levels of P_{15}, death-associated protein kinase (DAPK), and suppressor of cytokine signaling-1 ($SOCS_1$) genes in multiple myeloma (MM).

Materials and Methods: The bone marrow samples of 54 MM patients were collected and the methylation status of the P_{15}, DAPK, and $SOCS_1$ gene promoter regions was determined by methylation-specific polymerase chain reaction. Automated sequencing technology was used to sequence the amplified products in order to analyze the base methylation sites. mRNA expression levels were determined using real-time fluorescent quantitative polymerase chain reaction .

Results: Among the 54 MM patients, the positive methylation rates of the P_{15}, DAPK, and $SOCS_1$ genes were 27.78%, 18.52%, and 16.67%, respectively. The methylation results were confirmed by sequencing. The positive methylation rates of the P_{15}, DAPK, and $SOCS_1$ genes showed no correlation with patient gender, age, typing, staging, and grouping (P>0.05). There was no significant difference in the mRNA expression levels of the P_{15}, DAPK, and $SOCS_1$ genes between the MM patient group and the control group (P>0.05).

Conclusions: Aberrant methylation of the P_{15}, DAPK, and $SOCS_1$ genes exists in MM, and these genes may play certain roles in pathogenesis of MM. There was no significant difference in mRNA expression levels between the methylated group and the non-methylated group, suggesting that these genes are regulated by other mechanisms during their transcription.

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Introduction

Multiple myeloma (MM) is a cancer formed by malignant plasma cells and its complex multistep pathogenesis has not yet been elucidated. Previous studies have shown that genetic mutations, such as point mutations, translocations or deletions, are important aspects of the pathogenesis of MM (1, 2). Abnormal chromosomal numbers and structures are detected in approximately 50% of MM patients and these anomalies are closely associated with disease prognosis (3, 4). Increasing attention is being paid to the important roles of epigenetic changes such as DNA methylation and histone modifications, or abnormal miRNA expression in the pathogenesis of MM (5). DNA methylation occurs mainly on CpG islands. The methylation of a CpG island, for example in a tumor suppressor gene promoter region, would inhibit transcription of this gene, resulting in gene silencing. In tumor tissue, this phenomenon might be much more common than mutations (6).

It had been found that the inactivation of a series of tumor suppressor genes involved in cell cycle, cell signaling regulation, and apoptosis, was closely related to the occurrence and development of MM. The methylation of tumor suppressor genes involved in the cyclin/cyclin dependent kinase (cdk)/-retinoblastoma (Rb) pathway, the death-associated protein kinase (DAPK)/p14RAF/p53 pathway, and the interleukin-6 (IL_6) and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling system is reported to play an important role in the pathogenesis of MM (7). P_{15} gene is a classic tumor suppressor gene and an important member of the cyclin-dependent kinase inhibitor (CDK_I) family. Expression of the P_{15} gene is considered to be closely associated with not only the occurrence of MM, but also with the malignant transformation and disease progression of the plasma cells (8). DAPK is a calcium/calmodulin-dependent serine threonine protein kinase, which

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can activate p53 in a p19ARF-dependent manner, thus inducing apoptosis. Therefore, it is considered to be an independent poor prognostic indicator of diffuse large B-cell lymphoma (9). The suppressor of cytokine signaling (SOCS)-1 gene is an important member of the SOCS family; it regulates cytokines, but it is also related to a variety of immune responses and tumorigenesis *in vivo* (10). This study investigated the methylation and mRNA expression levels of P_{15}, DAPK, and $SOCS_1$ genes in MM. The aim was to explore the relationship between the aberrant methylation of tumor suppressor genes and the occurrence, development, and prognosis of MM.

Materials and Methods

Clinical data

The bone marrow samples of 54 MM patients were collected from the department of Hematology, Second Affiliated Hospital and First Affiliated Hospital of Kunming Medical University, including 27 newly diagnosed patients and 27 relapsed patients. Totally, 35 male and 19 female patients (male:female ratio, 1.84:1) were enrolled. Patients were aged between 38 and 79 years old (median age, 64 years old; mean age, 63.44 ± 8.94 years). For all cases, staging and grouping were performed according to the standard Durie-Salmon classification system: 26 cases of IgG myeloma, 14 cases of IgA myeloma, 9 cases of non-secretory myeloma, and 5 cases of light chain myeloma. Group A consisted of 2 patients at stage I, 16 patients at stage II, and 19 patients stage III; group B consisted of 16 patients at stage II and 13 patients at stage III. The control group consisted of a total of 40 non-tumor patients (mainly malnutritional anemia and immune thrombocytopenia patients), including 15 males and 25 females. The median age of the control group was 52 years (range 19-68 years). This study was conducted in accordance with the Declaration of Helsinki. This study was conducted with approval from the Ethics Committee of our Medical University. Written informed consent was obtained from all participants.

DNA extraction and bisulfite modification

Three milliliters of bone marrow samples were extracted from the MM and the control group patients and anticoagulated with ethylene diamine tetraacetic acid (EDTA). Ficoll fluid was then used to isolate single karyoplasts for DNA extraction with the whole blood genomic DNA Miniprep kit (Axygen Biosciences, Hangzhou, China). The EZ DNA Methylation-Gold™ Kit (ZYMO Research Co, CA, USA) was used for the bisulfite conversion of genomic DNA, according to the manufacturer's instructions. CpG methyltransferase (M. SssI, New England Biolabs, Inc., MA, USA) was used to prepare the methylated products and the non-methylated positive control.

Methylation specific polymerase chain reaction

According to previous studies (11-13), Meth-Primer program (http://www.urogene.org/-methprimer/) was applied to design the primers for CpG islands in the upstream 2000 bp from transcription initiation site of target genes. The span of CpG islands was at least 200 bp, with GC content>50% and CpG frequency>0.6. The methylation specific PCR (MSP) primers were synthesized by Invitrogen Biotechnology Co., Ltd. (Shanghai, China). The P_{15} gene MSP primers were as follows: 5'-GCGTTCGTATTTTGCGGTT-3' (upstream), 5'-GCGTT-CGTATTTT-GCGGTT-3' (downstream); non-methylation primers: 5'-TGTGATGTGTTTGTATTTTGTGGTT-3' (upstream), 5'-CCATACAATAACCAAACAACCAA-3' (downstream). The DAPK gene MSP primers were as follows: 5'-GGATAGTCGGATCGAGTTAACGTC-3' (upstream), 5'-CCCTCCCAAACGCCGA-3' (downstream); non-methylation primers: 5'-GGAGGATAGTTGGATTGAG-TTAATGTT-3' (upstream), 5'-CAAATCCCTCCCAAA-CACCAA-3'(down stream). The $SOCS_1$ gene methylation primers were as follows: 5'-TTCGCGTGTATTTTTAGGTCGGTC-3' (upstream), 5'-CGACACAACTCCTACAACGACCG-3' (downstream); non-methylation primers: 5'-TTATGAGTATTTGTGTGTATTTTTAGGTTGGTT-3' (up-stream), 5'-CACTAACAACACAACTCCTA CAACAA CCA-3' (downstream). The MSP was performed according to the reported procedure (14). The PCR amplification products underwent agarose gel electrophoresis. The image was developed and photographed under ultraviolet light with an automatic gel image analyzer, according to the presence or absence of specific bands. The results were recorded as positive and negative.

MSP product sequencing

MSP amplification products (50 μl) were stored in an ice box and sent to Shanghai Invitrogen Co. for automated sequencing. The sequencing results were concatenated with DNASTAR software and comparatively analyzed with BiQ Analyzer software.

Reverse transcription quantitative polymerase chain reaction

The total RNA was extracted using TRIzol reagent (Invitrogen Biotechnology Co, Ltd, Shanghai, China). The cDNA was synthesized using TransScript First-Strand cDNA Synthesis SuperMix cDNA Kit (Shanghai Sangon Biological Engineering Co, Ltd, Shanghai, China). According to the mRNA complete sequences of P_{15}, DAPK, and $SOCS_1$ genes in Gene Bank, Primer3.0 software (Primer Inc, TX, USA) was used to design the q-PCR primers. The uniqueness of primers was confirmed in NCBI primer blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers were synthesized by Invitrogen Biotechnology Co, Ltd (Shanghai, China). In the same method, the primer of GAPDH was designed and

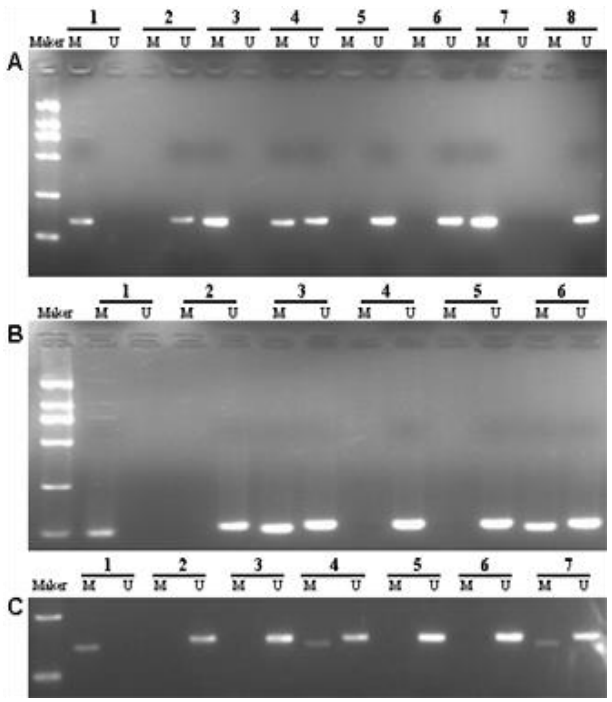

Figure 1. Electrophoresis of methylation in P_{15} gene promoter region of partial test samples (A). Marker: DL-2000; M: methylation; U: non-methylation. Lane 1: positive methylation control, lane 2: non-methylation control. Electrophoresis of methylation in DAPK gene promoter region of partial test samples (B). Comparison of MSP amplified products in the 3' end of $SOCS_1$ promoter region and non-methylation products (C)

synthesized. The sequences of primers were as follows: P_{15} gene: upstream primer, 5'-ATGCGCG-AGGAGAACAAG-3'; downstream primer, 5'-CTCCC-GAAACGG TTGACTC-3'; amplified fragment length, 143bp. DAPK gene: upstream primer, 5'-AGAGTTT-GTCGCTCCTGAGATAGT-3'; downstream primer, 5'-TGCTAACGTTTCTTGCTTAGTGTC-3'; amplified frag-ment length, 139bp. $SOCS_1$ gene: upstream primer, 5'-TATTACTTGCCTGGAACCATGTG-3'; downstream primer, 5'-TCAAGAGGTGAGAAGGGGTCT-3'; ampli-fied fragment length, 127bp. The GAPDH gene was used as the internal standard: upstream primer, 5'-AGTCAACGGATTTGGTCGTATT-3'; downstream primer, 5'-AACCATGTAGTTGAGGTCAATGAAG-3. The reverse transcription quantitative polymerase chain reaction (RT-qPCR) was conducted on CFX96 Touch PCR detection system (Bio-Rad Laboratories, CA, USA), with using Platinum SYBR Green qPCR SuperMix-UDG Kit (Bio-Rad Laboratories, CA, USA). GAPDH was used as the internal reference. The PCR steps were as follows: pre-denaturation, 2 min at 50 ˚C; denaturation, 2 min at 95 ˚C; annealing, 15 sec at 95 ˚C; elongation, 30 sec at 60 ˚C. Above steps were performed for 40 cycles. The expression amount was presented by cycle-threshold. The relative mRNA expression values were expressed as fold increase or decrease relative to GAPDH.

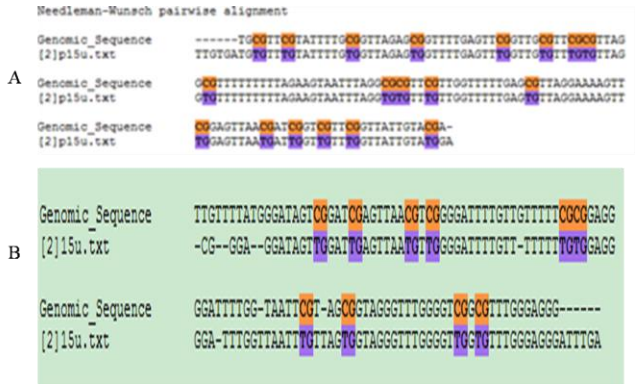

Figure 2. Sequencing comparison of methylation products and non-methylation products in the 5' end of P15 gene promoter region (A). Sequencing comparison of methylation products (orange marker) and non-methylation products (purple marker) in DAPK gene of 1 MM patient (B)

Statistical methods

The SPSS11.5 statistical package was used for statistical analysis. Methylation analysis used the fourfold table χ^2 test; gene expression analysis was described as median±interquartile; the intergroup comparison used the random sample rank sum test for statistical analysis. $P \leq 0.05$ was considered significant.

Results

Methylation status

The positive methylation rate of the control group was 0.00% (0/40). The positive methylation rates in the MM patients were as follows: 27.778% (15/54) in CpG islands of the P_{15} gene promoter region (the corrected χ^2 test showed that the χ^2 value was 13.22, $P<0.05$); 18.52% (10/54) in the DAPK gene promoter region, ($\chi^2=7.50$, $P<0.05$); and 16.67% (9/54) in the 3 'end of $SOCS_1$ gene promoter region, ($\chi^2=7.37$, $P<0.05$). There was a significant difference in the methylation statuse of the MM group promoter regions and the control group promoter regions ($P<0.05$). Figure 1 shows the electrophoresis of the MSP products.

Sequencing results

The positive MSP amplification samples were sequenced. The sequencing results were concatenat-ed using DNASTAR software and then analyzed with BIQ analyzer software. The presence of methylation in the gene promoter region was determined by a C → T conversion in the sequencing results. The specific methylation sites were then analyzed. The results are shown in Figure 2: 19 nucleotides were methylated at the 5' end of the P_{15} gene promoter region, and 10 nucleotides were methylated at the 5' end of the DAPK gene promoter region. Theoretically, at the 3' and 5' ends of the $SOCS_1$ gene promoter region, 19 and 22 bases, respectively, could undergo C → T conversion.

Table 1. Relationships of methylation of p15 gene promoter region with some clinical characteristics of MM patients

	Methylation of p15 gene promoter region		Positive rate(%)	χ2	*P*
	Positive	Negative			
Gender				3.00	0.08
M	7	28	20.00		
F	8	11	42.11		
Age				0.02	0.89
≥65	7	19	26.92		
<65	8	20	28.57		
Staging				0.01	0.95
Stage I+II	6	16	27.27		
Stage III	9	23	28.12		
Grouping				0.64	0.42
Group A	12	25	32.43		
Group B	3	14	17.65		
Firstly diagnosed or not				0.83	0.36
Yes	9	22	33.33		
Recurrent	6	21	22.22		

However, there were a number of differences in specific base methylation results across different samples, and the methylation frequency was lower than in the positive samples; therefore, a full assessment could not be carried out.

Correlations analysis

There was no statistically significant association between the positive methylation rates of the P_{15}, DAPK, and $SCOS_1$ gene promoter regions and patient gender or age (≥ 65 years or <65 years); stage I + II and stage III; group A and group B; and newly diagnosed patients and recurrent patients (P>0.05) (Table 1-3).

Reverse transcription-qPCR efficiency

After PCR, the baseline and threshold were set, and the amplification curve and dissolution curve were automatically drawn. At the same time, the standard S1, S0.1, S0.01, S0.001, and blank control in the reaction plate hole were set, and the standard curve was automatically generated. The PCR efficiency was obtained. Results showed that, the χ^2 values of P_{15}, DAPK, $SOCS_1$, and GAPDH genes were more than 0.99. The PCR efficiency was 90%-110%, and melting curve had a single peak. This indicated that, the condition of RT-qPCR was well controlled.

Expression of P_{15}, DAPK, and $SOCS_1$ mRNAs

Expression of P_{15} gene mRNA was as follows: MM group, 28.64±28.77 fold; control group, 25.79±78.16 fold; methylated group, 28.64±19.13 fold; non-methylated group, 29.86±29.24 fold; initial diagnosis group, 23.92±26.46 fold; and recurrent group, 36.13±27.65 fold. There was no statistically significant difference between the MM group and the control group (P>0.05), between the methylated group and the non-methylated group (P>0.05), or between the initial diagnosis group and the recurrent group (P>0.05). The expression level of DAPK mRNA was lower in the MM group than in the control group (1.09±1.35 fold vs. 1.26±1.38 fold), although the difference was not statistically significant (P>0.05). DAPK mRNA levels were lower in the methylated group than in the non-methylated group (0.76±1.09 fold vs. 1.18±1.28 fold); the differences between the methylated, non methylated, and control groups were not statistically significant (P>0.0167). DAPK mRNA levels were lower in the initial diagnosis group than in the recurrent group

Table 2. Relationships of methylation of DAPK gene promoter region with some clinical characteristics of MM patients

	Methylation of DAPK gene promoter region		Positive rate (%)	χ2	*P*
	Positive	Negative			
Gender				0.518	0.472
M	5	30	14.29		
F	5	14	26.32		
Age				0.231	0.631
≥65	6	20	23.08		
<65	4	24	14.29		
Staging				2.992	0.84
Stage I+II	7	15	31.82		
Stage III	3	29	9.38		
Grouping				0.089	0.765
Group A	8	29	21.62		
Group B	2	13	13.33		
Firstly diagnosed or not				0.000	1.000
Yes	5	22	18.52		
Recurrent	5	22	18.52		

Table 3. Relationships of methylation of SOCS-1 gene promoter region with some clinical characteristics of MM patients

	Methylation of SOCS-1 gene promoter region		Positive rate (%)	χ^2	*P*
	Positive	Negative			
Gender				0.07	0.80
M	5	30	14.29		
F	4	15	21.05		
Age				0.231	0.631
≥65	4	22	15.39		
<65	5	23	17.86		
Staging				0.02	0.90
Stage I+II	3	19	13.64		
Stage III	6	26	18.75		
Grouping				0.00	1.00
Group A	6	31	16.22		
Group B	3	14	17.65		
Firstly diagnosed or not				0.53	0.47
Yes	6	21	22.22		
Recurrent	3	24	11.11		

(0.98±1.19 fold vs. 1.17±1.72 fold), and the differences between the initial diagnosis, recurrent, and control groups were not statistically significant (P>0.0167). The expression levels of $SOCS_1$ gene mRNA were as follows: levels in the MM group were close to those in the control group (12.39±18.32 fold vs. 13.51±26.38 fold); levels in the methylated group were close to those in non-methylated group (17.00±16.52 fold vs. 16.45±17.97 fold); and levels in the initial diagnosis group (11.47±23.15 fold) were significantly lower than those in the recurrent group (18.93±17.59 fold). However, the intergroup comparison showed no statistically significant difference (P>0.05).

Discussion

More and more studies have shown (11,12)that tumor occurrence and development includes two main mechanisms: 1) nucleotide sequence changes that induce genetic mutations and deletions, namely genetic mechanisms, and 2) base modification changes that result in gene expression level changes, namely epigenetics. The epigenetic changes observed in tumors include DNA methylation, histone modifications, and chromatin remodeling, etc. Epigenetic changes occur in a variety of human tumors, and are associated with tumor occurrence and development; DNA methylation is the most intensively studied epigenetic mechanism. Modern cancer theory considers that, besides genetic structural variations such as mutations and deletions, there exists a third tumor suppressor gene inactivation mechanism, namely methylation. In some cases, it is the only mechanism involved in the inactivation of a tumor suppressor gene. Studies have shown (13,14) that changes in DNA methylation patterns are closely related to tumorigenesis. Reports (1, 2, 4) regarding abnormal methylation in tumor suppressor gene promoter regions of MM have been mainly focused on genes involved in cell cycle regulation, apoptosis, and cytokine signal transduction, such as P_{16}, P_{15}, P_{14}, E-Cadherin, SHP_1, DAPK, O6-methylguanine-DNA methyltransferase, and retinoic acid receptor β. But the methylation rate largely fluctuates among different reports, and its association with clinical features and prognosis were often contradictory. Therefore, further research is necessary.

The P_{15} gene (also known as cyclin-dependent kinase inhibitor 2B, $CDKN_{2B}$, inhibits CDK_4) is located on human chromosome 9 (9p21). It is an important member of the CDK_I family. P_{15} can inhibit the activity of cyclin-dependent kinases (CDK) 4/6, prevent the phosphorylation of Rb, and inhibit the dissociation of transcription factor E2F, thus blocking the cell cycle during the G → S conversion; cells would then be malignantly transformed. Studies have shown that inactivation of the P_{15} gene through methylation of its promoter region in a variety of hematological malignancies such as lymphoma, acute leukemia, and chronic myeloid leukemia(15,16, 17). Chim *et al* (18) reported the hypermethylation of the P_{15} gene as a common and important event in MM; methylation of the P_{15} gene promoter region was found in 75% of newly diagnosed patients and 80% of relapsed patients. However, Galm *et al* (19) concluded the opposite; this study reported that existence of methylation in the P_{15} gene promoter region of only 1 out of 53 patients, and proposed that the role of P_{15} gene methylation was overvalued in MM, and it might only be meaningful in acute leukemia.

In this study, the positive methylation rate of the P_{15} gene promoter region across 54 specimens was 27.78%, while that of the control group was 0. Sequencing of the MSP products revealed that methylation was present at 19 base sites at the 5' end of the P_{15} promoter region, confirming the existence of aberrant methylation in the P_{15} promoter region. Our research showed no significant difference in the methylation rates of P_{15} gene CpG islands among the patients in stage I, II, and III, or in group A and group B, indicating that methylation of the P_{15} gene might be an early event in MM

development. There was no significant difference with gender or age, consistent with most other findings (18).

DAPK is a calcium-calmodulin-dependent serine-threonine protein kinase that participates in apoptotic processes mediated by interferon (IFN)-γ, tumor necrosis factor (TNF), and Fas. In recent years, much attention has been paid to the association between DAPK gene promoter region methylation and cancer (20). Hypermethylation of the DAPK promoter region induces DAPK deficiency and this has been confirmed as a common phenomenon in B-cell malignancies, such as follicular lymphoma, diffuse large B-cell lymphoma, and Burkitt's lymphoma (21). Foreign studies (22, 23) found that the positive methylation rate of the DAPK gene promoter region in MM was between 5.9% and 67%; its rate was 18.52% in our study, close to those reported. The positive methylation rate of the control group was 0, indicating that methylation of the DAPK gene promoter region does exist in MM.

Esteban B *et al* (23) found that, although the positive methylation rate of the DAPK promoter region in MM was not high (4/68), it was significantly correlated with poor prognosis indicators such as high creatinine, calcium, and stage III, and that overall survival was significantly lower than in methylation-negative patients. But this study found that the positive methylation rate of stage I-II tumors was 31.82% (7/22), and that the rate of stage III tumors was 9.38% (3/32); there was no statistically significant difference between these two groups, suggesting that methylation of the DAPK promoter region might be an early event in the pathogenesis of MM. Hatzimichael E *et al* have compared the positive methylation rates of the DAPK promoter region between patients with monoclonal gammopathy of undetermined significance(MUGS) and patients with MM; no significant difference was observed, which also indicates that it may be an early event in the pathogenesis of MM (24). The positive methylation rate of the DAPK promoter region had no association with gender and age, and there was no statistically significant difference between newly diagnosed patients and relapsed patients. This could indicate that methylation of the DAPK promoter region has nothing to do with treatment. It may also be possible that the recurrence itself was associated with the abnormal methylation of tumor suppressor genes.

The SOCS family is a family of proteins that was discovered in recent decades. They negatively regulate the JAK/STAT pathway and exert its biological effects. The human $SOCS_1$ gene is located on chromosome 16p13.13. It consists of 1 exon and its full-length is 1776 bp. It encodes 211 amino acids, and is the most studied member of the SOCS family. Numerous studies have demonstrated that IL_6 is the critical growth factor for MM cells, and that $SOCS_1$ is the key that could regulate the expression of IL_6 in a negative feedback loop manner (25).

The positive methylation rates of the $SOCS_1$ promoter region in MM have been reported as 0-74.5% (26). Among the 54 MM patients in this study, the positive methylation rate of the $SOCS_1$ gene promoter region was 16.67% (9/54), consistent with the literature. Some authors believe that $SOCS_1$ methylation is an early event in the pathogenesis of MM, and there has been no evidence to date proving its correlation with the progression of MM (27, 28). Our study also showed that the positive methylation rate of the $SOCS_1$ promoter region had no significant correlation with clinical characteristics such as age, typing, staging, and grouping, or with new or recurrent diagnoses.

In this study, RT-qPCR was performed to study the mRNA expression levels of P_{15}, DAPK, and $SOCS_1$. The results showed that, the expression levels of P_{15}, DAPK, $SOCS_1$ genes mRNA in the bone marrow fluid of MM patients were lower than those in the control group, although the difference was not significant ($P>0.05$). This indicated that the promoter region methylation of above genes might have some regulatory effect on the expression of the gene, but it was not the main regulatory factor. Toyooka *et al* (29) found that methylation of tumor suppressor gene promoter regions and histone deacetylation may downregulate the expression levels of mRNA, but that it was not the only mechanism. There exists a variety of methods to regulate gene expression. Besides promoter hypermethylation, the main mechanism of tumor suppressor gene inactivation in human tumors, there may also exist other mechanisms that can lead to the silencing of gene expression, such as gene heterozygosity deletion, or gene homozygosity deletion (30). There is not just one method of epigenetic regulation. DNA methylation regulation also includes histone acetylation, histone methylation, and histone phosphorylation (31). DNA methylation is not the predominant method of gene silencing (6). Jung *et al* (32) detected 193 myeloma specimens and found that, only the methylation of a few genes, such as interleukin-like growth factor 1 receptor (IGF1R), deleted in liver cancer-1 (DLC_1), P_{16}, and interleukin-17 receptor B ($IL_{17}RB$), was associated with gene transcription, while the methylation of other genes had no association with gene transcription. The reason for this lack of correlation may be that, 5-methylcytosine is necessary but not sufficient for regulating gene expression, and the nature of chromatin formed on a methylated template is what renders it transcriptionally active or inactive. In addition, the potential for bias that may have resulted from inadequate specimen size cannot be

ruled out. Therefore, an increased sample number will be needed for further studies.

Conclusion

There existed methylation in the promoter regions of P_{15}, DAPK, and $SOCS_1$ genes in the MM patients, and showed statistically significant difference than the control group (P<0.05), indicating that the methylation in the above genes' promoter regions plays certain roles in the pathogenesis of MM. The positive rates of the methylation in the above genes' promoter regions were not related with gender, age, staging, grouping, and treatment-or-not. In this study, the mRNA expression of P_{15}, DAPK, and $SOCS_1$ genes in the methylation group was lower than the non-methylation group and the control group; however, the differences showed no statistical significance. This result indicated that the DNA methylation in the gene promoter region might not be the main regulatory factor influencing the mRNA expression of this gene.

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Conflict of interest

All authors have no conflict of interest regarding this paper.

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