

Clinicopathological significance and potential drug target of $p15^{INK4B}$ in multiple myeloma

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Abstract: Multiple myeloma (MM) is a clonal malignancy characterized by the proliferation of malignant plasma cells in the bone marrow and the production of monoclonal immunoglobulin. In addition to genetic changes, gene hypermethylation is an alternative mechanism of tumor suppressor gene inactivation in MM. The cyclin-dependent kinase inhibitor 1 (*CDKN2B* or $p15^{INK4B}$) gene lies adjacent to the tumor suppressor gene, cyclin-dependent kinase inhibitor 2 (*CDKN2A*), and is frequently mutated and deleted in a wide variety of tumors, including MM. However, there is a lack of systematic analysis of $p15$ epigenetic modification such as methylation in MM from different studies that can provide more powerful estimation of an effect. In this study, we have systematically reviewed the studies of $p15^{INK4B}$ promoter methylation in MM and quantified the association between $p15^{INK4B}$ promoter methylation and MM using meta-analysis methods. We observed that the frequency of $p15^{INK4B}$ methylation is significantly higher in MM patients than in normal healthy controls. The pooled odds ratio (OR) from ten studies including 394 MM and 99 normal individuals is 0.08, while confidence interval (CI) is 0.03–0.21 ($P < 0.00001$). This indicates that $p15^{INK4B}$ inactivation through methylation plays an important role in the pathogenesis of MM. In addition, the frequency of $p15^{INK4B}$ methylation was significantly higher in patients with MM than in those with asymptomatic monoclonal gammopathy of undetermined significance. The pooled OR from four studies is 0.40, 95% CI = 0.21–0.78 ($P = 0.007$). These results suggest that silencing of $p15^{INK4B}$ gene expression by epigenetic modification such as promoter hypermethylation plays a role not only in the initiation of MM but also in plasma cell malignant transformation, disease progression, and development.

Keywords: multiple myeloma, asymptomatic monoclonal gammopathy of undetermined significance (MGUS), $p15$, methylation, meta-analysis

Introduction

In tumor cells, the pattern of methylation is changed. There is a growing list of tumor suppressor genes (TSGs) with increased methylation frequency at promoter region¹ with detectable gene-body DNA hypomethylation.² Since aberrant hypermethylation of CpG dinucleotides in the promoter of TSG is a commonly observed epigenetic modification in human tumors,^{3–5} analysis of specific gene promoter methylation as a tool for diagnosis and prognosis of tumors has been widely applied in many different tumors, including multiple myeloma (MM).^{6,7}

MM is a clonal malignancy characterized by the proliferation of malignant plasma cells in the bone marrow and the production of monoclonal immunoglobulin.⁸ In the US, MM accounts for ~1% of all malignant diseases or ~15% of all hematological malignancies and is the second most common form of hematological malignancies.⁹ Clinically, MM starts with immortalization of a postgerminal center B-cell and presents as asymptomatic monoclonal gammopathy of undetermined significance (MGUS).

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MGUS is considered as the precursor of MM, since it is able to progress to symptomatic MM at a rate of 1% per year.¹⁰ MGUS plasma cells share many abnormal characteristics with MM plasma cells. Cytogenetic abnormalities, such as 14q32 translocations, deletions of 13q, and numeric gains of multiple chromosomes, have been discovered at the level of MGUS, indicating early onset of karyotypic instability in monoclonal gammopathies.¹¹ Genetically, MM develops and progresses due to the accumulation of multiple defined pathological genetic events. By genome-wide methylation microarrays or gene-specific hypermethylation analysis, aberrant DNA methylation in MM has been found to inactivate a number of protein-coding TSGs in the process of regulating cell cycle progression, cell growth, or apoptosis, which include cyclin-dependent kinase inhibitor 2A (*p16^{CDKN2A}*), cyclin-dependent kinase inhibitor 2B (*p15^{CDKN2B}* or *p15^{INK4B}*), death-associated protein kinase (*DAP*), secreted frizzled-related protein 2, suppressor of cytokine signaling 1 (*SOCS1*),^{12,13} and microribonucleic acid.¹⁴ However, there is a lack of a thorough summary of different studies on *p15^{INK4B}* epigenetic methylation in MM that can provide the reader with extensive information on whether an effect exists and what size the effect is. In the present study, we will carry out a meta-analysis of *p15^{INK4B}* methylation status in MM and in its precursor MGUS.

Methods

Search strategy

Medline, PubMed, Web of Science, Scopus, and Embase were searched in April 2014 using the search terms “*p15*”, “*p15^{INK4B}*”, “cyclin-dependent kinase inhibitor 2B”, “methylation”, “multiple myeloma”, and “clinical studies”. Investigations identified through the search approach as described were screened by titles first, then by abstracts of the publications. After exclusion of nonrelevant publications and identification of duplicates from the different databases, the remaining papers were evaluated in the full-text version for inclusion and exclusion criteria and for relevant articles in the reference lists. All clinical studies except case reports were chosen: eg, randomized controlled trials, cohort studies, case control studies, and case series. The language of publication was restricted to English and Chinese. All searched data were retrieved. Authors’ bibliographies and references of selected studies were also searched for other relevant studies. The most complete study was chosen to avoid duplication if the same patient populations were reported in several publications.

Selection criteria

We collected all eligible articles about the relationship between *p15^{INK4B}* methylation and/or expression and clinicopathological features and clinical outcomes in MM patients in this meta-analysis. Diagnosis and staging classification of MM must follow the standard criteria as described previously.^{15,16} In detail, MM requires evidence of bone marrow plasmacytosis, M-protein in serum or urine, and osteolytic bone lesions and the presence of myeloma-related organ or tissue impairment. MGUS is characterized^{15,16} by monoclonal protein <30 g/L and bone marrow clonal cells <10% with no evidence of MM, other B-cell proliferative disorders, or amyloidosis. Normal controls are referred to as the normal peripheral blood samples or bone marrow aspirates obtained from the healthy donors. Studies meeting the following inclusion criteria were included: 1) *p15^{INK4B}* methylation and/or expression evaluated in the circulation and/or bone marrow, 2) research that revealed the relationship between *p15^{INK4B}* methylation and/or expression and MM clinicopathological parameters and prognosis, 3) *p15^{INK4B}* methylation and/or expression examined by methylation-specific polymerase chain reaction (MSP) or Southern blot, 4) articles that were published as full papers in English or Chinese, and 5) articles that provided sufficient information to estimate hazard ratio (HR) about overall survival (OS) and 95% confidence interval (CI) and probabilities for progression-free survival (PFS) where applicable. The exclusion criteria included the following: 1) letters, reviews, case reports, conference abstracts, editorials, expert opinion, and non-English language papers; 2) articles with no information on OS or those that could not calculate the HR about OS from the given information; and 3) all publications regarding in vitro/ex vivo studies, cell lines, and human xenografts.

Data extraction

Two investigators independently extracted data from eligible studies. Disagreements were resolved by discussion and consensus. Two investigators reviewed all of the articles that fit inclusion and exclusion criteria. The following information was recorded for each study: the first author name, year of publication, sample source, number of cases, clinicopathological parameters, stage, *p15^{INK4B}* methylation and/or expression, and patient survival. Data for study characteristics and clinical response were summarized and turned into table format. Heterogeneity of investigation was evaluated to determine whether the data of the various studies could be analyzed in a meta-analysis.

Statistical analysis

Analysis was conducted using Stata 12.0 (Stata Corporation, TX, USA) and Review Manager 5.2 (Cochrane Collaboration, Oxford, UK). Comparisons of dichotomous measures were done by pooled estimates of odds ratios (ORs) as well as their 95% CIs. *P*-value of <0.05 was considered to be statistically significant. Heterogeneity was examined by a chi-square test with significance being set at *P*<0.10; the total variation among studies was estimated by *I* square. If there was heterogeneity among studies, we used a random effect model to pool the ORs; otherwise, a fixed effect model was selected.

The database search generated 25 articles from Medline, PubMed, the Web of Science, Scopus, and Embase. After initial screening of all titles, abstracts, and eligibility, 12 full-text studies were retracted for more detailed assessment. The search of the article references did not produce additional publications. Eventually, 12 publications met the inclusion criteria for qualitative study and for meta-analysis. The article search and study selection are depicted in Figure 1.

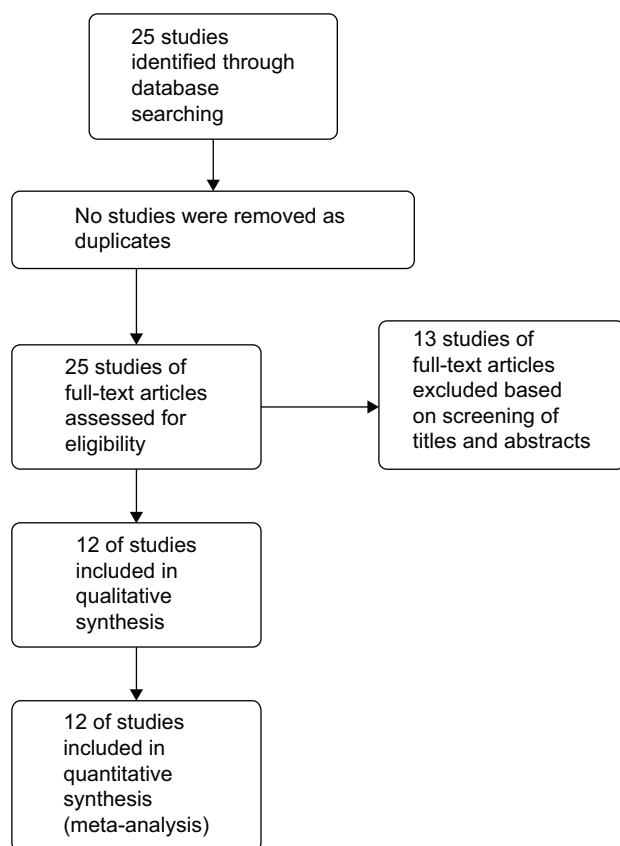


Figure 1 Flow chart of study selection.

Results

Identification of relevant studies

Twenty-five publications were identified by the search method as described. Thirteen of those were excluded due to laboratory studies, nonoriginal articles (review), or studies irrelevant to the current analysis. Eventually, there were 12 studies included in the final meta-analysis (Figure 1).^{17–28}

Study characteristics

Twelve studies published from 1997 to 2010 were eligible for meta-analysis. A total of 488 MM and 99 MGUS patients from the People's Republic of China, France, Germany, Austria, Spain, Brazil, and Argentina were enrolled. Their basic characteristics are summarized in Table 1.

p15 methylation and clinicopathological features

The inactivation of *p15^{INK4B}* through methylation in MGUS

We observed that *p15^{INK4B}* methylation is higher in MGUS than in normal individual controls, but it did not reach significant difference. The pooled ORs from three studies including 63 patients with MGUS and 19 healthy individuals are shown in Figure 2 (OR=0.30, 95% CI=0.05–1.72, *P*=0.18). These findings indicate that although MGUS is considered as a premalignant condition, *p15^{INK4B}* gene methylation is not the only one determinant factor for its potential malignancy. We excluded the other two studies that showed that *p15^{INK4B}* methylation in MGUS is 1.8%²¹ and 6.5%,²² because no healthy individual controls were available.

The inactivation of *p15^{INK4B}* through methylation in MM

We observed that *p15^{INK4B}* methylation is significantly higher in MM than in normal healthy controls. The pooled ORs from ten studies including 394 MM and 99 normal individuals are shown in Figure 3 (OR=0.08, 95% CI=0.03–0.21, *P*<0.00001), indicating that *p15* inactivation through methylation plays an important role in the pathogenesis of myeloma.

The role of *p15^{INK4B}* methylation in plasma cell malignant transformation

We analyzed 220 MM and 96 MGUS patients pooled in four studies to assess whether the aberrant *p15^{INK4B}* methylation/expression in serum/bone marrow DNA was associated with advanced stage. *P15^{INK4B}* methylation/expression estimated in

Table 1 Basic characteristics of the included studies

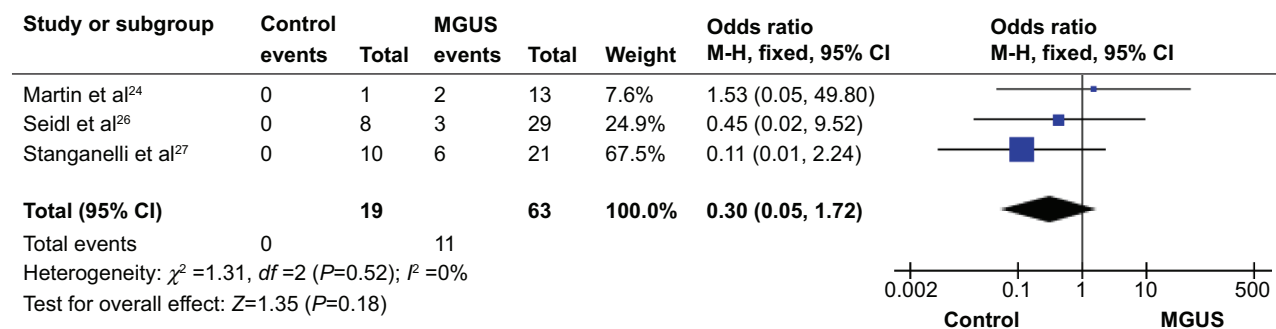
Study/country	Patients		Methods	Primary aim	Methylation site
	MGUS	MM			
Ng et al ²⁵ /People's Republic of China	12		MSP	Investigate whether <i>p16/p15</i> genes are inactivated by hypermethylation of the 5' CpG islands	Promoter, CpG islands
Fan et al ²⁰ /People's Republic of China	24		MSP	Study the action, characteristics, and expression of high methylation of <i>p15</i> and <i>p16</i> genes in MM	Promoter, CpG islands
Wu et al ²⁸ /People's Republic of China	12		MSP	Study the effect of operative region hypermethylation gene in human malignant hematopoietic tumors	Promoter, CpG islands
Guillerm et al ²³ /France	33	33	MSP	Determine how and when the alterations of <i>p15</i> and <i>p16</i> contribute to tumorigenesis	Promoter, CpG islands
Chen et al ¹⁸ /People's Republic of China	22		MSP	Investigate the frequency of <i>p16</i> and <i>p15</i> methylation and its relationship with bone marrow cell apoptosis and clinical outcome	Promoter, CpG islands
Guillerm et al ²² /France		61	MSP	Investigate different prognostic values of <i>p15</i> and <i>p16</i> gene methylation in MM	Promoter, CpG islands
Galm et al ²¹ /Germany	3	56	MSP	Gain insight into specific pathways that are relevant in the multiple step transformation process of normal plasma cells to MM	Promoter, CpG islands
Seidl et al ²⁶ /Austria	29	113	MSP	Investigate the methylation frequencies of ten genes in MGUS and compare with MM	Promoter, CpG islands
Chim et al ¹⁹ /People's Republic of China	13		MSP	Study the frequency and whether multiple genes are methylated in any one individual	Promoter, CpG islands
Martin et al ²⁴ /Spain	13	30	MSP	Explore the role of DNA methylation in plasma cell disorders	Promoter, CpG islands
Braggio et al ¹⁷ /Brazil		68	MSP	Gain insights on the prognostic implication resulting from epigenetic silencing	Promoter, CpG islands
Stanganelli et al ²⁷ /Argentina	21	44	MSP	Determine the role of aberrant promoter methylation as one of the steps involved in the progression of MGUS to MM	Promoter, CpG islands

Abbreviations: MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; MSP, methylation-specific polymerase chain reaction.

blood/bone marrow samples in early-stage MGUS and late MM stage was examined. The frequency of *p15^{INK4B}* inactivation was significantly higher in patients with MM than in those with MGUS (Figure 4), OR=0.40, 95% CI=0.21–0.78, $P=0.007$. These results suggest that silencing of *p15^{INK4B}* gene expression by promoter hypermethylation may contribute to plasma cell malignant transformation, disease progression, and development.

Prognostic values of *p15^{INK4B}* gene methylation in MM

Only three included studies^{21,22,26} estimated the relationship between OS/PFS and *p15^{INK4B}* methylation/expression. All of them showed the absence of prognostic impact of *p15^{INK4B}* gene methylation on MM patients. The data from Guillerm et al²² demonstrated that there is no significant difference in OS/PFS of 61 MM patients with (n=13; median survival

**Figure 2** *p15* methylation in MGUS.

Notes: Three of the included studies investigated *p15^{INK4B}* methylation status between 63 multiple myeloma patients and 19 normal individuals. The combined odds ratio was 0.30 (95% CI: 0.05–1.72; $Z=1.35$; $P=0.18$).

Abbreviations: MGUS, monoclonal gammopathy of undetermined significance; CI, confidence interval.

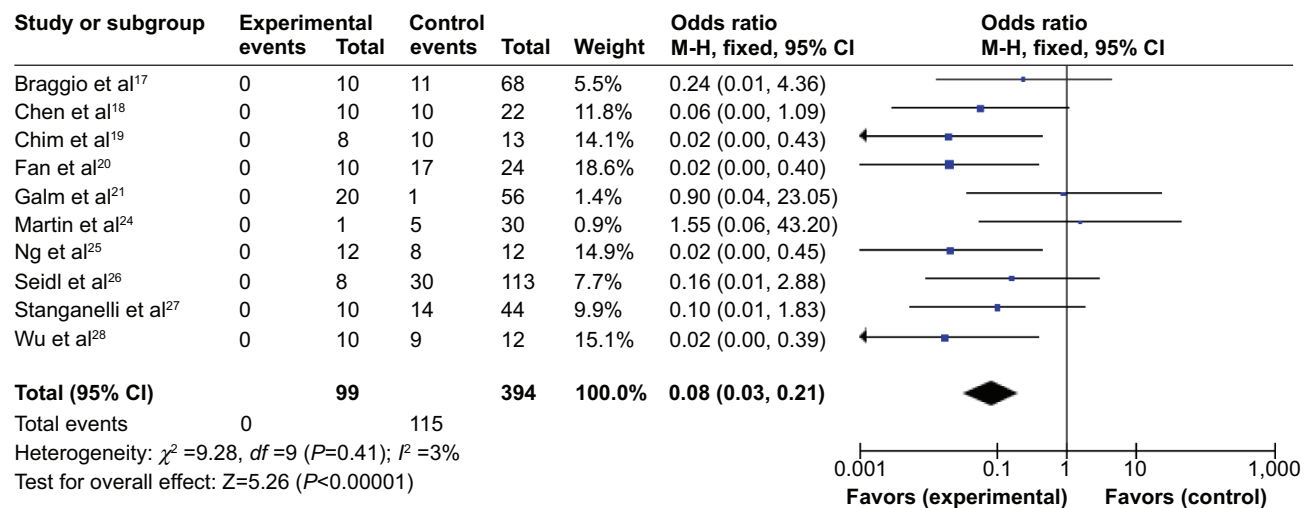


Figure 3 *p15* methylation in multiple myeloma.

Notes: Ten of included studies investigated *p15^{INK4B}* methylation status between 394 patients with multiple myeloma and 99 normal individuals. The combined odds ratio was 0.08 (95% confidence interval [CI]: 0.03–0.21; $Z = 5.26$; $P < 0.00001$).

8.6 years) or without ($n = 16$; median survival 8.6 years) *p15^{INK4B}* gene methylation ($P = 0.59/P = 0.28$, respectively). Two other studies^{21,26} contained just narrative description; therefore, we were unable to calculate the pooled HR for OS/PFS.

Sensitivity analyses and publication bias

A sensitivity analysis in which one study was removed at a time was conducted to assess the result stability. The pooled ORs were not significantly changed, indicating the stability of our analyses. The funnel plots were largely symmetric (Figure 5), suggesting there were no publication biases in the meta-analysis of *p15^{INK4B}* methylation/expression and clinicopathological features.

Discussion

MM is an incurable B-cell tumor. Pathogenesis involves upregulation of D-type cyclins, activation of oncogenes,

and inactivation of TSGs.¹² A better understanding of inactivation of TSGs by gene hypermethylation in MM pathogenesis will lead to more rational combinations of drugs targeting true epigenetic changes and cytotoxic actions. Since gene hypermethylation is a common mechanism of TSG inactivation, various approaches have been used to elucidate the role of gene hypermethylation in MM, including a candidate gene approach, microarray approach for genes upregulated by hypomethylating agents, and a cancer pathway approach, which enables a comprehensive picture of the involvement of multiple TSGs in MM.¹² The main TSGs inactivated by hypermethylation in MM are as follows: 1) the INK4 and CIP/KIP families of cyclin-dependent kinase inhibitors, CDKN2B (*p16^{INK4A}*) and CDKN2A (*p15^{INK4B}*); 2) SHP1 protein-tyrosine phosphatase and soluble Wnt inhibitors, which are associated with constitutive activation of JAK/STAT and Wnt signaling; and 3) the DAPK/P14/HDM2/P53/Apaf-1 pathway.¹²

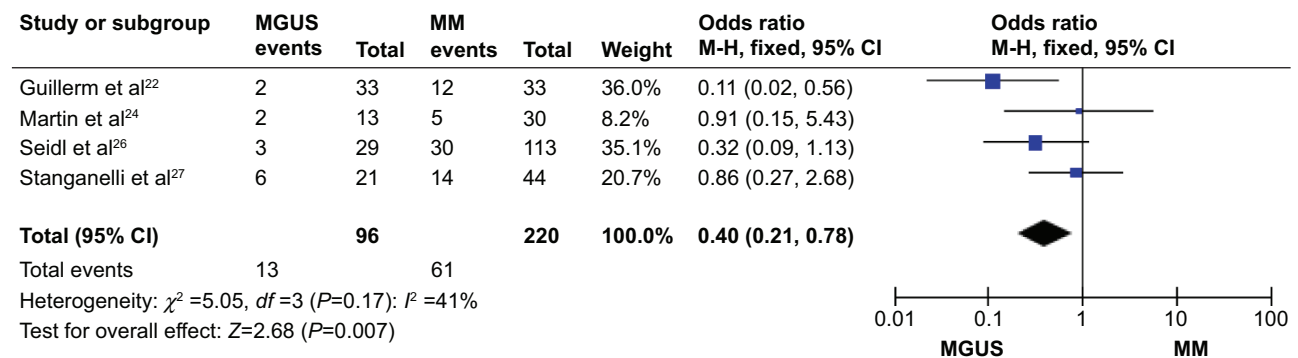


Figure 4 *p15* methylation in MM versus MGUS.

Notes: Four of the included studies investigated *p15^{INK4B}* methylation status between 220 patients with MM and 96 patients with MGUS. The combined odds ratio was 0.40 (95% CI: 0.21–0.78; $Z = 2.68$; $P = 0.007$).

Abbreviations: MM, multiple myeloma; MGUS, monoclonal gammopathy of undetermined significance; CI, confidence interval.

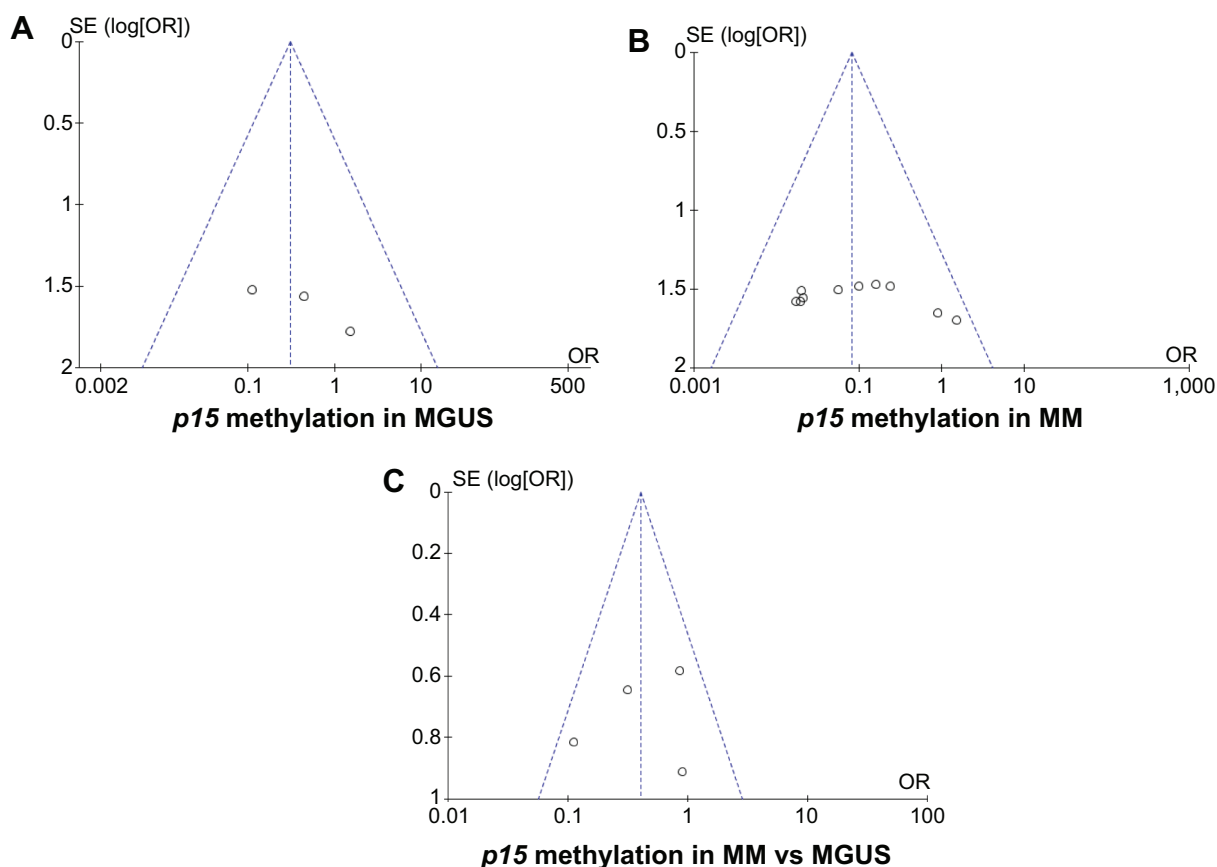


Figure 5 *p15* methylation in MM versus MGUS.

Notes: Funnel plot of publication bias in the meta-analysis of *p15^{INK4B}* expression and clinicopathological features. (A) *p15^{INK4B}* methylation in MGUS. (B) *p15^{INK4B}* methylation in MM. (C) *p15^{INK4B}* methylation in MM versus MGUS.

Abbreviations: MM, multiple myeloma; MGUS, monoclonal gammopathy of undetermined significance; SE, standard error; OR, odds ratio.

The present study focused on the involvement of epigenetic changes of *p15^{INK4B}* in MM pathogenesis and prognosis.

CD138 is a plasma biomarker. Several included studies in this meta-analysis sorted CD138⁺ plasma cells^{22,23} for MSP analysis, while others used unsorted mononuclear cells.²⁶ By reviewing various studies on methylation examination in B-cell malignancies for different TSGs such as *p16^{INK4B}* and *p15^{INK4B}*, we found that the reported methylation frequencies of these tested genes were comparable in both types of studies.^{22,29–34} Therefore, we concluded that CD138⁺ cells sorting is not mandatory for methylation evaluation by MSP technique. In other words, sorting CD138⁺ or not does not affect the included data analysis.

Epigenetic changes contribute to tumorigenesis and affect initial steps in malignant transformation by altering genome stability and regulating gene expression.³⁵ Analysis of the methylome of CD138⁺ cells by ligation-mediated polymerase chain reaction assay from different stages of myelomagenesis demonstrated qualitative epigenetic differences between premalignant and malignant stages.³⁵ Plasma cells from

MGUS, a premalignant and early stage of myeloma, were characterized by striking widespread hypomethylation, while gene-specific hypermethylation was found to occur in the advanced stages of myeloma. Furthermore, aberrant demethylation in MGUS occurred primarily in CpG islands, whereas differentially methylated loci in cases of myeloma occurred predominantly outside of CpG islands and affected distinct sets of gene pathways.³⁵ These results indicate widespread stage-specific epigenetic changes during myelomagenesis and that early demethylation can be a potential contributor to genome instability seen in myeloma.³⁵ In our included studies, there was a significant difference in hypermethylation status of *p15^{INK4B}* genes at promoter region between patients with MGUS and MM, which reflects the stage-specific epigenetic changes during myelomagenesis. However, all the studies fail to give information about the methylation situation outside promoter region.

Methylation patterns may be useful as prognostic indicators in MM. Despite possible variations between methods utilized, heterogeneity of patients, and other unknown

factors, p15^{INK4B} methylation analysis in MM provides interesting prognostic information. Patients with methylated p15^{INK4B} gene demonstrated significantly poorer OS ($P=0.01$, log-rank test 8.6 years)^{21,22} and PFS ($P=0.005$, 8.6 years).²² By univariate analysis, hypermethylation of *DAPK* ($P<0.001$) and *RARβ* ($P=0.01$) genes was also identified as an adverse prognostic factor. Median OS of MM patients with hypermethylation in *DAPK* (4 months) and *RARβ* (34 months) was significantly lower than in those without hypermethylation.¹⁷

Combined with the findings, our meta-analysis data suggest that p15^{INK4B} along with p16^{INK4A}, *DAPK*, and *RARβ* methylation might play a role in the initial transformation of plasma cells. However, p15^{INK4B} methylation might exert a lesser influence on subsequent tumor progression.²²

Recently, Wang et al³⁶ performed a meta-analysis to reveal the potential contribution of p15^{INK4B} and p16^{INK4A} methylation in the pathogenesis of MM. Their results indicated that the rates of p15^{INK4B} and p16^{INK4A} promoter methylation in MM samples were significantly higher compared with normal controls. Furthermore, they also showed that the aberrant methylation of p15^{INK4B} was significantly correlated with the risk of both Caucasian and Asian MM patients. Our current meta-analysis is consistent with their findings and further strengthens the conclusion that p15^{INK4B} promoter methylation may be an important contributor to the pathogenesis of MM.

Disclosure

The authors report no conflicts of interest in this work.

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