Clinicopathological significance of the p16 hypermethylation in multiple myeloma, a systematic review and meta-analysis

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ABSTRACT

It is well known that the loss of function of the p16INK4A gene is mainly caused by the hypermethylation of the p16 gene; however, whether or not the inactivation is associated with the clinical significance of multiple myeloma (MM) remains elusive. A meta-analysis was conducted to quantitatively determine the role of the p16 hypermethylation in the clinical significance of MM. We demonstrated that MM patients show much higher hypermethylation rates on the p16 gene in bone marrow compared to normal individuals, as well as monoclonal gammopathy of undetermined significance (MGUS). The difference of aberrant p16 hypermethylation between MM patients in advanced stage and MM patients in early stage is not statistically significant. Interestingly, the survival rate of MM patients with the p16 hypermethylation is much shorter compared to those without the p16 hypermethylation. Our results demonstrate that hypermethylation status of the p16 gene may play a role in the progression of MGUS to MM, as well as worse survival in MM. The p16 hypermethylation, which induces the loss of function of the p16 gene that plays a critical role in the early tumorigenesis of MM.

INTRODUCTION

Multiple myeloma (MM) is a clonal hematological cancer formed by malignant plasma cells and overproduction of monoclonal immunoglobulin [1]. Over the past decade, a number of approved drugs such as lenalidomide, bortezomib and thalidomide have demonstrated significant clinical benefits in heavily pretreated MM patients [2–5]. Unfortunately, most of MM patients still relapse; the investigation of potential drug target and biomarker is still essential to enhance the survival rate in the patients with recurrent MM or those refractory to chemotherapy. Based on previous reports, several prognostic biomarkers including β2-microglobulin, serum albumin, hemoglobin and cytogenetic aberrations in MM have been used in clinical settings [6–9]. Epigenetic aberrations, such as hypermethylation of CpG islands located within the promoter region of the gene could lead to transcriptional repression/inactivation of the gene and participate in a crucial role in the development and progression of MM [10, 11]. The loss of function due to aberrantly methylated (hypermethylation) of CpG islands in tumor suppressor genes was observed in many cancers including MM, and the effect of which is equivalent to gene deletion and mutation in tumorigenesis. The p16 gene is one of the most common cancer suppression genes, which is hypermethylated in many tumors, including MM [12, 13].

The p16/INK4A/CDKN2 gene belongs to a family of cell cycle-related genes located on chromosome 9p21. It encodes a protein that competitively interacts with cyclin-dependent kinase 4 protein (Cdk4), which compromises the connection of cyclin D1 and Cdk4 to facilitate transition from the G1 phase to the next stage of the cell cycle [14]. The inactivation of the p16 gene is caused by hypermethylation in MM, the published positive rates of the p16 hypermethylation in MM are remarkably diverse [15–17]. The heterogeneous reported results do need for further investigation and evaluation of the correlation between the hypermethylation status of the p16 gene.
and MM. Therefore, a meta-analysis was conducted to quantitatively examine whether or not the epigenetic changes are indicated by a higher level of methylation of the \( p16 \) affecting clinical significance with MM.

**RESULTS**

Forty two articles were selected by the search method. The articles were excluded if they are reviews, *in vitro* or *in vivo* investigations, or studies unrelated to the topics. Finally, twenty four of articles were stripped out and this meta-analysis included nineteen studies (Figure 1).

**Study characteristics**

Nineteen articles published from 1997 to 2013 and a number of 1348 patients from Argentina, South Korea, Brazil, China, Greece, Hong Kong, Germany, France, Spain, Austria, Poland, Japan and the United States were enrolled (Table 1).

**The \( p16 \) hypermethylation and clinicopathological characteristics**

The loss of the \( p16 \) expression through hypermethylation in MM and MGUS

MM patients showed higher proportion of the \( p16 \) hypermethylation compared to normal individuals. The pooled OR from 7 articles which include 736 MM and 73 normal bone marrow are presented in Figure 2A (odds ratios, \( OR=16.92, \) 95% confidence intervals, CI=5.86-48.87, \( p<0.00001 \)), which demonstrates that the loss of \( p16 \) expression by hypermethylation plays critical role in the tumorigenesis of MM. In addition, the \( p16 \) hypermethylation also is detected in monoclonal gammopathy of undetermined significance (MGUS) and is remarkably less than in MM (\( OR=2.53, \) 95% CI=1.54-4.17, \( p=0.0003 \)), as shown in Figure 2B. Due to limited studies of the \( p16 \) methylation on both normal individuals and MGUS patients [18, 19], we are unable to compare the difference of the \( p16 \) hypermethylation within these two groups of individuals.

**The \( p16 \) hypermethylation in the progression of MM**

We then analyzed 382 MM patients pooled in 8 investigations to evaluate if the role of inactivation of the \( p16 \) via hypermethylation on the progression of MM. In Figure 3A, aberrant \( p16 \) hypermethylation is not remarkably higher in advanced MM (III) than that in early staged MM (I &II), \( OR=1.07, \) 95% CI=0.65-1.74, \( p=0.80. \) We further analyzed 239 MM patients pooled in 4 studies and found there is no significant difference between the level of the \( p16 \) hypermethylation in stage III and stage I, \( OR=0.52, \) CI=0.24-1.16, \( p=0.11, \) as presented in Figure 3B. These results indicate that the inactivation of the \( p16 \) gene due to hypermethylation may not play a critical role in MM development from initial stage to advanced stage.

**Prognostic significance of the \( p16 \) hypermethylation in MM**

Five studies included investigated relationship between the \( p16 \) hypermethylation and overall survival
| Study                | Country          | Patients | Methods                     | Primary aim                                                                 | Methylation site                  | Detection of p16 protein |
|---------------------|------------------|----------|-----------------------------|----------------------------------------------------------------------------|-----------------------------------|-------------------------|
| Kim et al 2013 [16] | South Korea      | 103      | Methylation specific PCR (MSP) | Determine the methylation status of the p16 gene and the clinical significance | Promoter, CpG islands              | No                      |
| Park et al 2011 [17]| South Korea      | 99       | MSP                         | Determine the methylation status of the p16 and its association with common cytogenetic changes, clinicolaboratory findings | Promoter, CpG islands              | No                      |
| Braggio et al 2010 [49] | Brazil         | 68       | MSP                         | Determine the methylation status of nine tumor suppressor Genes including the p16 in MM | Promoter, CpG islands              | No                      |
| Stanganelli et al 2010 [41] | Argentina   | 44       | MSP                         | Determine the methylation status of 7 genes including the p16 in MM and MGUS | Promoter, CpG islands              | No                      |
| Hatzimichael et al 2009 [50] | Greece        | 45       | MSP                         | Aims to test CpG methylation of both p16 and TP73 occurs in MM              | Promoter, CpG islands              | No                      |
| Martin et al 2008 [51] | Spain           | 30       | MSP                         | Determine the methylation status of 6 genes including the p16 in MM and MGUS | Promoter, CpG islands              | No                      |
| Chim et al 2007 [42] | Hong Kong       | 32       | MSP                         | Determine aberrant p16 promoter methylation in the progression of MM         | Promoter, CpG islands              | No                      |
| Gonzalez-Paz et al 2007 [18] | The United States | 522 | MSP, RT-PCR              | Investigate the biological and clinical implications of the p16 gene methylation in MM | Promoter, CpG islands, First exon, | Yes                     |
| Liang et al 2006 [52] | China           | 28       | MSP, RT-PCR             | Determine the methylation status of p16 gene in MM                          | CpG islands                        | Yes                     |
| Seidl et al 2004 [25] | Austria         | 113      | MSP                         | Determine the methylation frequencies of 10 genes including the p16 in patients with monoclonal gammopathies. | Promoter, CpG islands              | No                      |
| Galm et al 2004 [23] | The United States | 56      | MSP                         | Determine the methylation status of 11 well characterized tumor suppressor genes including the p16 in MM. | Promoter, CpG islands              | No                      |
| Chim et al 2004 [53] | Hong Kong       | 8        | MSP                         | Determine the methylation status of 10 genes including the p16 in MM         | Promoter, CpG islands              | No                      |
| Kramer et al 2002 [54] | Germany         | 48       | MSP                         | Determine the frequency of Rb deletions, cyclin D1 alterations And hypermethylation of the p16 in MM | Promoter, CpG islands              | No                      |
| Chim et al 2003 [55] | Hong Kong       | 34       | MSP                         | Determine whether or not disruption of the INK4/cyclin D-CDK/RB pathway is a common mechanism in the pathogenesis of MM | Promoter, CpG islands              | No                      |

(Continued)
| Study               | Country     | Patients | Methods          | Primary aim                                                                 | Methylation site | Detection of p16 protein |
|---------------------|-------------|----------|------------------|-----------------------------------------------------------------------------|------------------|--------------------------|
| Guillerm et al 2001 [56] | France      | 33       | MSP              | Determine *p15* and *p16* methylation in the progression of MM              | Promoter, CpG islands | No                       |
| Ng et al 1997 [57]   | Hong Kong   | 12       | MSP              | To investigate whether *p15* and *p16* deactivated by deletions, mutations, and hypermethylation in MM | Promoter, CpG islands | No                       |
| Tasaka et al 1998 [58] | Japan       | 16       | MSP/RT-PCR       | Determine the *p16* methylation in the progression of MM                     | Promoter, CpG islands | Yes                      |
| Guillerm et al 2003 [24] | Poland     | 61       | MSP              | Determine the *p15*, *p16* methylation in the outcome of MM                 | Promoter, CpG islands | No                       |
| Fu et al 2002 [19]   | China       | 42       | MSP              | Determine the methylation status of the *p16* in MM and MGUS              | Promoter, CpG islands | No                       |

Note: MSP: Methylation specific PCR; Yes: p16 protein expression was detected; No: p16 protein expression was detected.

**Figure 2:** The pooled OR from 7 studies including 736 multiple myeloma (MM) and 73 normal bone marrow (OR=16.92, 95% CI=5.86-48.87, \( p < 0.00001 \)) (A). The pooled OR from 9 studies including 898 MM and 142 monoclonal gammopathy of undetermined significance (MGUS) (OR=2.53, 95% CI=1.54-4.17, \( p = 0.0003 \)) (B).
p16 gene is the most common methylated gene in MM [23–26]. The loss of the p16 function is significantly associated with the gene hypermethylated in a variety of cancers including MM [27–34]. Although there have been a number of studies are involved with the methylation level of the p16 in MM, the pathological significances of inactivation of the p16 in MM and clinical role are still elusive. The pooled data from this meta-analysis indicate that 1) remarkably higher the p16 hypermethylation was detected in MM than that from normal bone marrow; 2) the p16 hypermethylation was also detected in MGUS, but remarkably less than that from MM; 3) MM patients in advanced MM do not show high levels of the p16 hypermethylation compared with those at early stage; 4) MM patients with the p16 hypermethylation had a lower survival rate than those without the p16 hypermethylation. The analysis evidences revealed that the p16 hypermethylation proportion in MM was remarkably higher than that in the normal bone marrow, indicating that the p16 hypermethylation may play a role in the initiation of MM. Based on the

**DISCUSSION**

Aberrant DNA methylation, which has been well characterized in many tumors, is believed to cause tumor formation, progression and worse prognosis [20–22]. The p16 gene is the most common methylated gene in MM [23–26]. The loss of the p16 function is significantly associated with the gene hypermethylated in a variety of cancers including MM [27–34]. Although there have been a number of studies are involved with the methylation level of the p16 in MM, the pathological significances of inactivation of the p16 in MM and clinical role are still elusive. The pooled data from this meta-analysis indicate that 1) remarkably higher the p16 hypermethylation was detected in MM than that from normal bone marrow; 2) the p16 hypermethylation was also detected in MGUS, but remarkably less than that from MM; 3) MM patients in advanced MM do not show high levels of the p16 hypermethylation compared with those at early stage; 4) MM patients with the p16 hypermethylation had a lower survival rate than those without the p16 hypermethylation. The analysis evidences revealed that the p16 hypermethylation proportion in MM was remarkably higher than that in the normal bone marrow, indicating that the p16 hypermethylation may play a role in the initiation of MM. Based on the

**Publication bias and sensitivity analyses**

In the current study of the p16 hypermethylation and clinicopathological features using meta-analysis, the publication biases were evaluated by the funnel plots. The publication biases were ruled out by the symmetric funnel plot (Figure 5). The sensitivity analyses were conducted by removing one study at a time to determine the stability. The analysis results showed that the pooled ORs and HRs are not remarkably changed, suggesting the stability of these meta-analyses.

**Figure 3:** The pooled OR from 8 studies including 385 multiple myeloma (MM) patients. Aberrant p16 hypermethylation was not significantly higher in advanced MM (III) than that from early staged MM (I & II), OR=1.07, 95% CI=0.65-1.74, p=0.80 (A). The pooled OR from 4 studies including 239 MM patients. The p16 hypermethylation was also not significantly higher in stage III, than that from stage I, OR=0.52, CI=0.24-1.16, p=0.11 (B).
Figure 4: Five studies included investigated the relationship between overall survival (OS) and the \textit{p16} hypermethylation. The pooled HR for OS showed that the \textit{p16} hypermethylation was associated with worse survival in multiple myeloma (MM) (HR=2.77, 95\% CI=1.34-5.72, \textit{P}=0.006).

Figure 5: The funnel plots were largely symmetric suggesting there were no publication biases in the meta-analysis of the \textit{p16} hypermethylation and clinicopathological features. The funnel plot from 7 studies comparing multiple myeloma (MM) and normal bone marrow (A). The funnel plot from 9 studies comparing MM and monoclonal gammopathy of undetermined significance (MGUS) (B). The funnel plot from 8 studies comparing different staged MM patients (III VS. I &II) (C). The funnel plot from 4 studies comparing different staged MM (III VS. I) (D). The funnel plot from 6 studies in determining overall survival (OS) and the \textit{p16} hypermethylation in MM patient (E).
observation that the p16 hypermethylation status is reversible by the demethylation agents, currently several agents have been applied in clinics to slow down the process of carcinogenesis and progression, therefore improve prognosis. Histone deacetylase inhibitor, sodium phenylbutyrate, and 5-Aza-2'-deoxycytidine (5-Aza-CdR) were reported to induce p16 gene demethylation and tumor cell apoptosis in MM [35]. In another report, arsenic trioxide was also reported to induce p16 gene demethylation and tumor cell growth inhibition in MM [36]. This strategy may bring hope for tumor treatment through gene manipulation and gene-targeted therapy. Due to limited studies of the p16 methylation on both normal individuals and MGUS patients [18, 19], we are unable to compare the difference of the p16 hypermethylation within these two groups of individuals. Nevertheless, we can still conclude that the p16 hypermethylation status is associated with disease development and progression from benign MGUS to malignant MM and a stratification factor for patients with MM.

Recent studies show that MM is consistently preceded by a precursor state, MGUS [37–39]. Progression from MGUS to MM seems a result of the accumulation of genetic and epigenetic abnormalities, indicating a stepwise progression of alterations at genetic and epigenetic levels [40]. In fact, hypermethylation of a number of genes, such as p15INK4B, ARF, p27 KIP1, SOCS-1, RASSF1A, death-associated protein (DAP) kinase, non-receptor type 6 (SHP1), and TP73 genes were reported in MGUS [41, 42]. Among six studies, only one study detected the p16 gene hypermethylation in normal cohort (Figure 2A). In contrast, seven out of nine studies detected the p16 gene hypermethylation in MGUS (Figure 2B). Since only one study has relevant data, we were not able to determine whether or not the p16 gene hypermethylation in MGUS is significantly higher than that in normal cohort by meta analysis. Our results showed that significantly higher the p16 hypermethylation was detected in MGUS and significantly less than that from MM. Hypermethylation of the p16 gene could potentially be participants in the development of MGUS to MM.

The p16 hypermethylation in MM was not observed to associated with advanced stage. We also did not find that the p16 hypermethylation was remarkably higher in stage III, compared to that from stage I of MM. These evidences suggest that the p16 hypermethylation could be an early event. Only five studies examined the correlation between the overall survival and the p16 hypermethylation in MM, they showed homogenous results. The pooled HR (HR=2.77, 95 % CI=1.34-5.72, P=0.006) for OS showed that MM patients with high level of the p16 hypermethylation have significantly shorter survival (Figure 4). We observed that the pattern of the p16 methylation in different stages of MM were similar, however, several years after disease progression, MM patients with the p16-positive expression had remarkably improved survival rates compared to the p16-negative patients. There is no good explanation about that there is no significant difference of p16 hypermethylation in initial and advanced stage MM, however, there is significant difference in overall survival in MM. Maybe p16 hypermethylation status is more associated with stage- unrelated molecular changes and therapeutic response in MM patients. Galm et al [23] reported that hypermethylation of p16 was correlated with a poor prognostic impact in MM patients. Our analysis further supports that detection of p16 hypermethylation may provide an excellent prognostic marker for MM patients.

This study may have several potential limitations. The selection biases and unidentified confounders could not be completely excluded, since all of the included studies were experimental. Second, the identification of articles was based their publication only in English and Chinese, other articles with potentially meaningful data in other languages were not selected. Therefore, caution could be taken when our analysis are applied to the general populations.

In summary, this study using meta-analysis demonstrates that the p16 hypermethylation may play a role in the progression of MGUS to MM, as well as worse overall survival in MM. The p16 hypermethylation, which induces the loss of function of the p16 gene, plays an important pathologic role in the early carcinogenesis of MM.

**MATERIALS AND METHODS**

**Search method and selection criteria**

We searched Embase, Pubmed and ISI web of knowledge to select studies from June 1, 1996 to February, 2017 using the search terms: “multiple myeloma”, “plasma cell myeloma”, and “Kahler's disease”, “methylation”, and “p16”. We also checked the reference lists of the reviews and selected articles for related articles. Although our article or publication search did not have language limits initially, we only took into account studies published in English and Chinese for full-text reading and final evaluations. After the exclusion of the redundant and non-relevant publications from the different databases, the remaining papers were evaluated in the full text version for in- and exclusion criteria. Authors’ references of included studies were also checked for other related investigations. All searched data were retrieved. The most complete investigation was included if the same patient populations were published in different resources.

Criteria for identification that an eligible study has to meet were as follows: (1) the p16 methylation and/or expression evaluated in primary MM, (2) the p16 methylation determined by polymerase chain reaction (PCR), (3) researches revealed the relationship between the p16 methylation and/or expression of MM
clinicopathological parameters and prognosis, (4) studies which provided sufficient data to calculate Odds ratio (OR) and/or hazard ratio (HR) about overall survival (OS) and 95% confidence interval (CI). The exclusion criteria: (1) reviews, letters, case studies, editorials, conference abstracts, expert opinion, (2) articles that had no information of OS or 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 were also excluded. In addition, “aberrant” p16 methylation or p16 hypermethylation is defined by clear PCR product band detected by methylation specific PCR (MSP).

Data extraction and methodological assessment

Two authors (HY, LY) independently reviewed and analyzed data from eligible studies. Two authors (YF, MG) evaluated all of publications according to the inclusion and exclusion criteria. The following criteria were chosen for each study: the first author name, year of publication, number of cases, sample source, methylation detection method, clinicopathological parameters, methylation rate, and/or expression, and follow up. Data for study characteristics were summarized in a table format. Investigation heterogeneity was evaluated to determine whether or not the data of the various studies could be analyzed for a meta-analysis.

Three investigators (LY, YF and LT) read through each publication independently for the methodological evaluation of the studies, and assessed and scored them according to REMARK guidelines and ELCWP quality scale [43, 44]. Then they provided the quality scores, compared them, and then reached a consensus value for each item.

Statistical analysis

Analysis was performed using the STATA 12.0 (Stata Corporation, TX, USA) and Review Manager 5.2 (Cochrane Collaboration, Oxford, UK). The pooled frequency of the p16 hypermethylation and 95% confidence intervals (95% CI) were estimated. The frequency of the p16 hypermethylation was compared in different tumor characteristics. Heterogeneity among studies was examined with Cochran’s Q test [45] and the I² statistic [46, 47]. A fixed effect model was used to calculate parameters, when heterogeneity was not an issue (I² values <50%), while a random-effects model was used to pool data and attempt to identify potential sources of heterogeneity based on subgroup analyses, if there was substantial heterogeneity (I² values ≥50%). P values less than 0.05 were considered statistically significant.

Publication bias was determined by using a method described by Egger et al [48]. The analysis of meta-regression and publication bias was performed using STATA version 10.0. For statistical heterogeneity, we explored reasons for using meta-regression, subgroup analysis and sensitivity analysis.

Author contributions

HY, LT participated in the design of the study; HY, LY, YF and MG performed experiments; HY, LY analyzed data. LT wrote the manuscript. All authors reviewed the manuscript.

CONFLICTS OF INTEREST

Authors declare that they have no competing interests.

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