

SHORT REPORT

Aberrant gene methylation implicated in the progression of monoclonal gammopathy of undetermined significance to multiple myeloma

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Malignant transformation is a multistep process that may involve dysregulation of oncogenes and tumour suppressor genes, and monoclonal gammopathy of undetermined significance (MGUS) is believed to be a precursor of multiple myeloma. To investigate whether aberrant promoter methylation might be involved in the evolution of MGUS to multiple myeloma, we examined the *p16*, *protein tyrosine phosphatase, non-receptor type 6 (SHP1)*, *death-associated protein (DAP) kinase*, *E-cadherin* and *oestrogen receptor* genes, most being tumour suppressor genes, by methylation-specific polymerase chain reaction. In 32 cases of multiple myeloma and 19 cases of MGUS, significantly more frequent methylation of *p16* ($p=0.001$), *SHP1* ($p\leq 0.001$) and *E-cadherin* ($p\leq 0.001$) genes was found in multiple myeloma than in MGUS. Methylation of *DAP kinase* and *oestrogen receptor* genes was comparable in multiple myeloma and MGUS. In conclusion, methylation of *p16*, *SHP1* and *E-cadherin* genes might be involved in the progression of MGUS to multiple myeloma.

Oncogenesis is a multistep process involving dysregulation of oncogenes and tumour suppressor genes. Multiple myeloma is characterised by neoplastic proliferation of monoclonal plasma cells. Multiple myeloma cells arise from postgerminal centre B cells, which migrate to the bone marrow, adhere to the stroma, proliferate, and trigger subsequent bone resorption and a paracrine cytokine loop.^{1,2} The disease may progress from monoclonal gammopathy of undetermined significance (MGUS) to multiple myeloma. MGUS occurs in approximately 3% of people >70 years old.² Multiple myeloma develops in about one quarter of MGUS patients after a median of 10 years.³ Therefore, MGUS is regarded as a precursor of multiple myeloma, with the rate of transformation to multiple myeloma estimated to be 1% per year.³

DNA methylation involves the addition of a methyl group to the carbon 5 position of the cytosine ring in CpG dinucleotide. At gene promoters, DNA methylation leads to recruitment of a transcriptional repressor complex, resulting in suppression of transcription.⁴ In cancers, the CpG islands of selected genes are aberrantly methylated, resulting in silencing of these genes. Therefore, methylation is an alternative mechanism of gene inactivation, in addition to deletion and mutation.

Frequent methylation of the *oestrogen receptor*, *E-cadherin*, *protein tyrosine phosphatase, non-receptor type 6 (SHP1)*, *death-associated protein (DAP) kinase* and *p16* genes has been shown in multiple myeloma.^{5–7} During the evolution of MGUS to multiple myeloma, multiple genetic aberrations are sequentially acquired. In this study, we tested the hypothesis that aberrant promoter methylation might be one of the steps involved in the progression of MGUS to multiple myeloma.

MATERIALS AND METHODS

Patients, diagnosis and treatment

Diagnosis of MGUS was based on an asymptomatic monoclonal gammopathy (immunoglobulin G (IgG) ≤ 3.5 g/dl, IgA ≤ 2 g/dl, and urinary Bence Jones protein ≤ 1 g/24 h), <10% plasma cells in the bone marrow and absence of lytic bone lesions. Diagnosis of multiple myeloma was based on standard criteria.³ Monoclonal paraproteins were identified by serum electrophoresis, immunoelectrophoresis or immunofixation. The study was approved by the review board of Queen Mary Hospital, Hong Kong.

Methylation-specific polymerase chain reaction

High-molecular-weight DNA was isolated by standard protocols from bone marrow aspirates at diagnosis, and from eight marrow aspirates of healthy donors. Methylation-specific polymerase chain reaction (MSP) was performed as described.⁶ Briefly, treatment of DNA with bisulphite for conversion of unmethylated, but not methylated, cytosine to uracil was performed with the CpGenome DNA modification kit (Intergen, New York, New York, USA). Primers for the methylated MSP and unmethylated MSP gene promoter regions were as described previously (fig 1). In all experiments, DNA from normal donors was used as negative control, and methylated control DNA (CpGenome Universal Methylated DNA, Intergen) as positive control. MSP was performed in a thermal cycler (9700, Perkin-Elmer Biosystems, Foster City, California, USA). The polymerase chain reaction mixture contained 50 ng of bisulphite-treated DNA, 0.2 mM deoxynucleoside triphosphates, 2 mM MgCl₂, 10 pmol of each primer, 1× PCR buffer II and 2.5 U AmpliTaq Gold (Perkin-Elmer Biosystems) in a final volume of 25 μ l. The identity of the MSP products was confirmed by DNA sequencing.

Statistical analysis

Frequencies of gene methylation in multiple myeloma and MGUS were compared by χ^2 or Fisher's exact test (SPSS V.12). *p* Values are two sided.

RESULTS

Methylation-specific polymerase chain reaction

The positive control, but none of the eight normal marrow DNA samples, showed complete methylation of *DAP kinase*, *p16*, *SHP1*, *E-cadherin* and *oestrogen receptor* genes (fig 1). In samples showing negative methylated-MSP amplification, integrity of DNA was confirmed by positive unmethylated-MSP amplification.

Abbreviations: DAP, death-associated protein; MGUS, monoclonal gammopathy of undetermined significance; MSP, methylation-specific polymerase chain reaction; *SHP1*, *protein tyrosine phosphatase, non-receptor type 6*.

MSP of multiple myeloma and MGUS

There were 32 patients with multiple myeloma and 19 patients with MGUS (table 1). Frequencies of methylation of *DAP kinase* and *oestrogen receptor* genes were comparable in multiple myeloma and MGUS. *E-cadherin* gene methylation was found only in multiple myeloma ($p < 0.001$). *p16* and *SHP1* methylation was significantly more frequent in multiple myeloma than in MGUS (*p16* 53.1% v 5.3%, $p < 0.001$; *SHP1* 84.4% v 31.6%, $p < 0.001$; fig 1, table 2).

DISCUSSION

Our study is based on the hypothesis that if methylation of these genes were involved in the progression of MGUS to multiple myeloma, the frequency of gene hypermethylation would be significantly higher than that of MGUS. On the other hand, if hypermethylation of these genes were part of the initiating event that leads to immortalisation of the plasma cell, the frequency of hypermethylation of these genes in multiple myeloma would be expected to be comparable to that of MGUS. We showed that *p16*, *SHP1* and *E-cadherin* gene methylation was significantly more frequent in multiple myeloma than in MGUS, suggesting that methylation of these genes might be involved in clonal evolution of MGUS to multiple myeloma.

p16 is a cyclin-dependent kinase inhibitor targeting cyclin-dependent kinases 4 and 6. We showed preferential methylation of *p16* in multiple myeloma. Previous studies have also

shown more frequent *p16* methylation in multiple myeloma than in MGUS.^{8,9} Furthermore, *p16* methylation was also more frequent in plasma-cell leukaemia.¹⁰ However, in CD138-purified plasma cells, *p16* methylation has been found to be equally frequent in MGUS and multiple myeloma.¹⁰

E-cadherin, a homotypic cell adhesion molecule, is considered a suppressor of metastasis. Our finding of preferential *E-cadherin* gene methylation in multiple myeloma instead of MGUS was consistent with previous reported results.¹⁰ As the progression of MGUS involves metastasis of the myeloma plasma cells to other parts of the bone marrow, *E-cadherin* gene methylation might be expected to play a role in the evolution of MGUS to multiple myeloma.

SHP1, a cytoplasmic protein tyrosine phosphatase, is a putative tumour suppressor negatively regulating the interleukin 6/Jak/STAT signalling in plasma cells.⁶ In myeloma cells with complete *SHP1* methylation, STAT3 was constitutively activated. Demethylation with re-expression of *SHP1* led to down regulation of phosphorylated STAT3, showing that *SHP1* methylation is biologically relevant in myeloma pathogenesis.⁶

Taken together, *p16* and *SHP1* gene methylation might confer a proliferative and survival advantage on neoplastic plasma cells, and *E-cadherin* gene methylation might enhance the metastatic potential and thus progression of MGUS.

Similar to another study,¹¹ we showed a comparable frequency of *DAP kinase* methylation in patients with MGUS

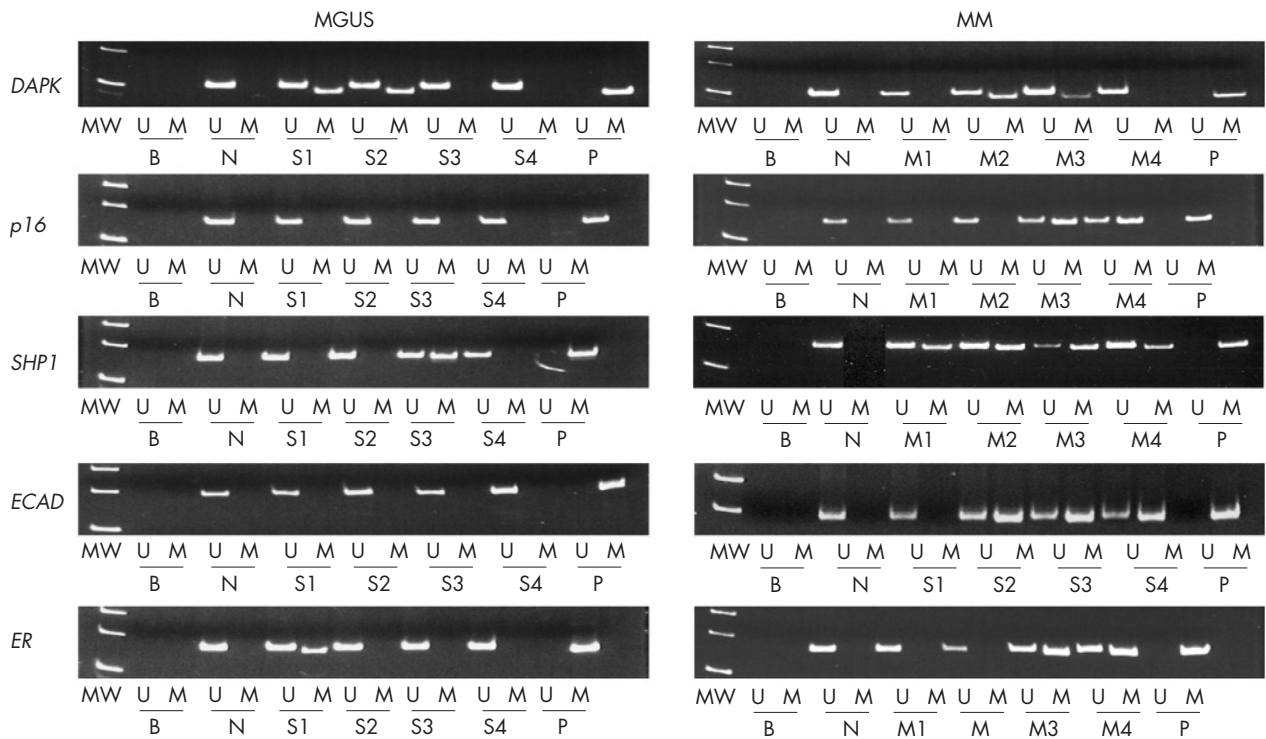


Figure 1 Methylation-specific polymerase chain reaction of *death-associated protein kinase (DAPK)*, *p16*, *suppressor of high-copy PP1 (SHP1)*, *E-cadherin (ECAD)* and *oestrogen receptor (ER)* genes in monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM). Samples tested were marrow aspirates obtained at diagnosis. The sequences for primers were *DAPK* (methylated, forward: 5'-GGA TAG TCG GAT CGA GTT AAC GTC-3', reverse: 5'-CCC TCC CAA ACG CCG A-3'; unmethylated, forward: 5'-GGA GGA TAG TTG GAT TGA GTT AAT GTT-3', reverse: 5'-CAA ATC CCT CCC AAA CAC CAA-3'), *p16* (methylated, forward: 5'-TTA TTA TTA GAG GGT GGG GCG GAT CGC-3', reverse: 5'-GAC CCC GAA CCG CGA CCG TAA-3'; unmethylated, forward: 5'-TTA TTA GAG GGT GGG GTG GAT TGT-3', reverse: 5'-TTA TTA GAG GGT GGG GTG GAT TGT-3'), *SHP1* (methylated, forward: 5'-GAA CGT TAT TAT AGT ATA GCG TTC-3', reverse: 5'-TCA CGC ATA CGA ACC CAA ACG-3'; unmethylated, forward: 5'-GTG AAT GTT ATT ATA GTA TAG TGT TTG G-3', reverse: 5'-TTC ACA CAT ACA AAC CCA AAC AAT-3'), *E-cadherin* (methylated, forward: 5'-GTG GGC GGG TCG TTA GTT TC-3', reverse: 5'-CTC ACA AAT ACT TTA CAA TTC CGA CG-3'; unmethylated, forward: 5'-GGT GGG TGG GTT GTT AGT TTT GT-3', reverse: 5'-AAC TCA CAA ATC TTT ACA ATT CCA ACA-3') and *oestrogen receptor* (methylated, forward: 5'-CGA GIT GGA GTT TTT GAA TCG TTC-3', reverse: 5'-CTA CGC GTT AAC GAC GAC CG-3'; unmethylated, forward: 5'-ATG AGT TGG AGT TTT TGA ATT GTT T-3', reverse: 5'-ATA AAC CTA CAC ATT AAC AAC AAC CA-3'). MW, molecular weight markers; U, unmethylated MSP; M, methylated MSP; B, reagent blank; N, normal negative control; P, methylated positive control; S1-S4, four MGUS marrow samples; M1-M4, four primary myeloma marrow samples.

Table 1 Demographic data of patients with monoclonal gammopathy of undetermined significance and multiple myeloma

	MGUS	MM
n	19	32
Sex		
Male	10 (52.6%)	17 (53.1%)
Female	9 (47.4%)	15 (46.9%)
Age (years)		
Median	76	63
Range	55–85	25–87
Paraprotein subtype		
IgG	12 (85.7%)	18 (56.3%)
IgA	2 (14.3%)	8 (25.0%)
IgD	–	1 (3.1%)
Light chain	–	5 (15.6%)
Bone marrow plasma cells	3–7%	38–93%
Paraprotein level		
Median	–	37.5 g/l
Range	–	12–78 g/l
Durie–Salmon stage		
I	–	4 (12.5%)
II	–	9 (28.1%)
III	–	19 (59.4%)

–, not applicable.

MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma.

Take-home messages

- Tumour suppressor genes are implicated in the pathogenesis of multiple myeloma.
- Hypermethylation of *ECAD*, *SHP1* and *p16* is implicated in the progression from MGUS to multiple myeloma.

and multiple myeloma, implying that *DAP kinase* is probably not involved in the progression of MGUS. However, there was a relatively high frequency of *DAP kinase* methylation (35.7%) in MGUS, suggesting that this might be involved in the development of MGUS.

Ideally, methylation of *p16*, *E-cadherin* and *SHP1* genes should be investigated serially in MGUS and multiple myeloma samples in the same patient. However, as the annual rate of progression of MGUS to multiple myeloma is only 1%, this would require a long period of observation.

In conclusion, there was preferential methylation of some tumour suppressor genes in MGUS. Therefore, inactivation of tumour suppressor genes by gene methylation might contribute to disease progression from MGUS to multiple myeloma.

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Table 2 Frequencies of aberrant methylation of *death-associated protein kinase*, *p16*, *SHP1*, *E-cadherin* and *oestrogen receptor* genes

	MGUS	MM (%)	p Value
n	19	32	
<i>DAP kinase</i>			
M	6 (31.6)	18 (56.3)	0.146
U	13 (68.4)	14 (43.7)	
<i>p16</i>			
M	1 (5.3)	17 (53.1)	0.001
U	18 (94.7)	15 (46.9)	
<i>SHP1</i>			
M	6 (31.6)	27 (84.4)	<0.001
U	13 (68.4)	5 (15.6)	
<i>ECAD</i>			
M	0 (0)	18 (56.3)	<0.001
U	19 (100)	14 (43.7)	
<i>ER</i>			
M	3 (15.8)	13 (40.6)	0.117
U	16 (84.2)	19 (59.3)	

DAP, death-associated protein gene; *ECAD*, E-cadherin gene; *ER*, oestrogen receptor gene; M, methylated gene status; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; *SHP1*, suppressor of high-copy *PP1* gene; U, unmethylated gene status. M and U values are n (%).

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