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hMLH1 and MGMT inactivation as a mechanism of tumorigenesis in monoclonal gammopathies

Paloma Martin[1], Almudena Santón[2], Mónica García-Cosio[2] and Carmen Bellas[1]

[1]*Laboratory of Molecular Pathology, Department of Pathology, Hospital Universitario Puerta de Hierro, Madrid, Spain and* [2]*Laboratory of Molecular Pathology, Hospital Ramón y Cajal, Madrid, Spain*

Monoclonal gammopathies are a group of disorders characterized by clonal proliferation and accumulation of immunoglobulin-producing plasma cells. Multiple myeloma and monoclonal gammopathy of undetermined significance are the most common monoclonal gammopathies; the two comprise a spectrum of disorders, ranging from a relatively benign disease, monoclonal gammopathy of undetermined significance, to a malignant disease, multiple myeloma. Aberrant promoter methylation represents a primary mechanism of gene silencing during tumorigenesis. DNA repair systems act to maintain genome integrity in the presence of replication errors, environmental insults, and the cumulative effects of aging. The methylation patterns of two genes implicated in DNA repair, O6 methylguanine DNA methyl-transferase (*MGMT*) and human mutL homologue1 (*hMLH1*) have been detected in various solid tumours. With the purpose of studying the gene silencing of *MGMT* and *hMLH1* in plasma cell disorders, we investigated the methylation status and expression of both genes in: 29 cases of multiple myeloma; one case of plasma cell leukaemia; 13 cases of monoclonal gammopathy of undetermined significance; and two cases of polyclonal plasmacytosis, using methylation-specific polymerase-chain reaction and immunohistochemical techniques. Methylation frequencies for *MGMT* were 23% in multiple myeloma and 8% in monoclonal gammopathy of undetermined significance. It was 10% for *hMLH1* in multiple myeloma. None of the patients diagnosed with monoclonal gammopathy of undetermined significance had *hMLH1* hypermethylated. In addition, 50% of myeloma cases had a loss of hMLH1 expression, whereas silencing of *MGMT* was observed in 43% of myeloma and 36% of samples with monoclonal gammopathy of undetermined significance. This study indicates that repair pathway defects play a role in the pathogenesis and evolution of monoclonal gammopathies, and suggests that inactivation of *hMLH1* could be implicated in multiple myeloma tumorigenesis.
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Multiple myeloma is a plasma-cell neoplasm characterised by multiorgan dysfunction as a result of bone marrow infiltration by malignant cells and the systemic damage of monoclonal circulating protein.[1] Multiple myeloma ranks as the second most frequently occurring haematological malignancy after non-Hodgkins lymphoma (NHL).[2] Despite advances in systemic and supportive therapies, multiple myeloma has remained an incurable disease. Myeloma is usually preceded by an age-dependent premalignant disease called monoclonal gammopathy of undetermined significance. This premalignant disease is characterised by low concentrations of monoclonal immunoglobulin and may progress to malignant myeloma at a rate of 1% per year. Currently, there are no reliable predictors for the progression of monoclonal gammopathy of undetermined significance to multiple myeloma or related disorders.[3] Development of multiple myeloma is a multistep process[4,5] associated with an increasing frequency of chromosomal abnormalities and complex translocations which induce mutations in several proto-oncogenes and tumour suppressor genes.

Recently, epigenetic silencing of gene expression by hypermethylation has been described as an important regulator of gene transcription, especially when involving CpG-rich areas, known as CpG islands, located in the promoter regions of many genes.[6] Extensive methylation of CpG islands has

Correspondence: Dr C Bellas, MD, PhD, Laboratory of Molecular Pathology, Department of Pathology, Hospital Universitario Puerta de Hierro, C/San Martín de Porres 4, Madrid 28035, Spain. E-mail: cbellas.hpth@salud.madrid.org
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been associated with transcriptional inactivation of selected genes[7] and represents the epigenetic equivalent of mutations and deletions in carcinogenesis.

Tumour-specific methylation changes in different genes have been identified,[8–11] and the methylation profile helps us distinguish tumour types and, perhaps, their response to chemotherapeutic agents.[12] Methylation changes are often detected in premalignant or precursor lesions, (before malignant changes) and, thus, may be of use in early detection of cancer.[13]

During replication, the primary function of the eukaryotic DNA mismatch repair (MMR) system is to recognise and correct mismatched base pairs within the DNA helix. The DNA MMR system is expressed in all tissues at various levels and plays an important role in the maintenance of genomic integrity.[14] Deficiencies in MMR have been reported previously in cases of hereditary nonpolyposis colorectal cancer and sporadic tumours occurring in a variety of tissues.[15] Human mutL homologue1 (*hMLH1*, 3p21.3) is a MMR gene responsible for correcting insertion/deletion loops and single base–base mismatched pairs that arise during normal DNA replication,[5,16] especially in the repeated sequence motifs, such as microsatellites.

Inactivation of genes encoding proteins involved in this system results in a mutator phenotype that is associated with a predisposition for tumour development.[5,17] A consequence of the mutator phenotype is the production of multiple replication errors in simple repetitive DNA sequences, resulting in microsatellite instability (MSI).[18] Although *hMLH1* mutation is the most frequent cause of inactivation of *hMLH1* in hereditary colorectal cancer, in sporadic tumours there is a lack of mutation in the MMR system, and the main cause of MSI is the gene silencing by promoter methylation.[19]

O6 methylguanine DNA methyl-transferase (*MGMT*, 10q26) encodes a DNA repair protein that removes mutagenics and cytotoxic adducts at O^6 of guanine and, therefore plays an important role in maintaining normal cell physiology and genomic stability.[20] Methylated regions of the *MGMT* promoter are in a 'closed' nucleosome structure, that is associated with alterations in heterochromatin and with promoter methylation of other cancer-associated genes. *MGMT* promoter methylation is only found in tumours. Loss of MGMT expression is rarely, if ever, due to deletion, rearrangement, or mutation of the *MGMT* gene.[21,22] On the other hand, inactivation of the *MGMT* gene through promoter hypermethylation is a common event in the carcinogenic process.[23,24]

To determine whether the status of genes implicated in the DNA repair process might be implicated in the development or progression of monoclonal gammopathy of undetermined significance and multiple myeloma, in this study we evaluated methylation patterns comparing the loss of protein expression in each gene.

Materials and methods

Human Tissue Samples

Bone marrow aspirates ($n = 45$) and bone marrow biopsies ($n = 29$) from the posterior iliac crest were collected from 45 consenting patients during routine clinical assessment. Patients were classified according to the current WHO classification. Of the 45 cases, 29 had been diagnosed with multiple myeloma, 13 with monoclonal gammopathy of undetermined significance, one with plasma cell leukaemia and two with polyclonal plasmacytosis. Analyses of age, gender, haemoglobin levels, presence of lytic bone lesions, creatinine, serum calcium levels, LDH, $\beta 2$ microglobulin, paraprotein isotype, percentage of plasma cells and stage of disease were obtained from all patients.

From bone marrow aspirates, mononuclear cell suspensions were prepared by Ficoll-Paque PLUS gradient centrifugation (Amersham Pharmacia AB, Uppsala, Sweden). Plasma cell isolation from the mononuclear cell suspension was performed by immunomagnetic bead selection with CD138 using the Automacs system (Miltenyi, CA, USA). Enriched fractions were assessed for purity by CD138-fluorescein isothiocyanate (FITC) monoclonal antibody labelling (BB4, Cytognos). CD138, also known as Syndecan-1, is expressed on plasma cells, but not on circulating B cells, T cells or monocytes.[25] The purity of plasma cells obtained by this method was more than 90%, as confirmed by flow cytometry (FAC sort: Becton Dickinson, San José, CA, USA) using the Paint a Gate program. Plasma cell leukaemia was not purified.

DNA was extracted from CD138-positive cells using Tri-Reagent (from Becton Dickinson, San José, USA) following manufacturer's instructions.

Methylation Analysis

DNA methylation patterns in the CpG islands of *MGMT* and *hMLH1* were determined by chemical modification of only the unmethylated cytosines to uracil and subsequent polymerase chain reactions (PCRs) using primers specifically designed to distinguish methylated from unmethylated sequences in bisulphite-modified DNA. Bisulphite modification was performed as previously described.[26] DNA (1 μg) was denatured by NaOH. The denatured DNA was treated with sodium bisulphite to chemically modify the unmethylated cytosines to uracil. Modified DNA was purified using the Wizard DNA Clean-up system (Promega, Madison, WI, USA), then denatured with NaOH, precipitated with ethanol and resuspended in 25 μl of water. *MGMT* primer sequences for the unmethylated (U) reaction were: 5′-TTT GTG TTT TGA TGT TTG TAG GTT TTT GT-3′ forward and 5′-AAC TCC ACA CTC TTC CAA AAA CAA AAC A-3′ reverse. *MGMT* primer sequences for the methylated (M) reaction were: 5′-

TTT CGA CGT TCG TAG GTT TTC GC-3′ forward and 5′-GCA CTC TTC CGA AAA CGA AAC G-3′ reverse. Samples were amplified under the following conditions: 94°C for 8 min, followed by 40 cycles at 94°C for 1 min, 62°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. *hMLH1* primer sequences for the unmethylated (U) reaction were: 5′-TTA ATA GGA AGA GTG GAT AGT G-3′ forward and 5′-TCT ATA AAT TAC TAA ATC TCT TCA-3′ reverse. *hMLH1* primer sequences for the methylated (M) reaction were: 5′-TTA ATA GGA AGA GCG GAT AGC-3′ forward and 5′-CTA TAA ATT ACT AAA TCT CTT CG-3′ reverse. The annealing temperature was 55°C for *hMLH1* (unmethylated and methylated reactions). DNA from peripheral blood lymphocytes from healthy donors was used as the unmethylated control, whereas universal methylated DNA (CpGenome™ Universal Methylated Control DNA, Chemicon) was used as the positive control. Also, control experiments without DNA were performed for each set of PCRs.

Each PCR product was loaded directly onto 3% agarose gels and electrophoresed; the gel was stained with ethidium bromide and visualized under UV illumination.

Immunohistochemical Staining

Paraffin-embedded sections from bone marrow biopsies were stained with hMLH1 and MGMT. Antigen retrieval was achieved by heat treatment in a pressure cooker for 4 min in 10 mM citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked in 0.3% H_2O_2 in methanol for 20 min. The sections were incubated for 1 h at 37°C with the primary antibodies. We used a monoclonal mouse anti-human hMLH1 (clone G-168-15, PharMingen International, CA, USA) at a dilution of 1:50, using the LSAB Visualization System (Dako, Glostgrup, Denmark). MGMT (clone MT3.1, Dako) was used at a dilution of 1:100 and immunodetection was performed with the Dako EnVision + ™ DAB system. Counterstaining of the nuclei was carried out with haematoxylin. Only nuclear staining was regarded as positive staining. The results were interpreted as positive when there were more than 30% of positive cells in each antibody.[27,28] Normal laryngeal epithelium and reactive lymphadenitis were used as positive controls for hMLH1 and MGMT, respectively.

Stained slides were evaluated for the presence of expression of MGMT or hMLH1 in the tumour by two independent observers (PMA and MGC).

Results

General characteristics of the patients are summarized in Table 1. The median age of the patients was 70 years (range 39–85). Of the 43 monoclonal gammopathies studied 20 cases were IgG, 16 cases were IgA, one IgM, one nonsecretory, and five cases excreted light chain only. Nineteen patients had stage III disease, five patients had stage II disease, and five patients had stage I disease. In the multiple myeloma group, 16 patients were analysed at diagnosis, and 14 patients were analysed at variable times after treatment.

Table 1 Clinical and laboratory characteristics of 43 patients with monoclonal gammopathies

Characteristics	
Median patient age (range), years	70 (39–85)
Gender (n)	
Male	20
Female	23
Paraprotein isotype (n)	
IgA	16
IgG	20
IgM	1
Light chain only	5
Nonsecretory	1
Stage	
MGUS	13
I	5
II	5
III	19
Plasma cell leukaemia (*n*)	1
Lytic bone lesions (n)	
Yes	16
No	27
At diagnosis (*n*)	29
During follow-up (*n*)	14

Methylation of *hMLH1* and *MGMT* genes was performed in 45 samples: 29 multiple myeloma, 13 monoclonal gammopathies of undetermined significance, one plasma cell leukaemia and two polyclonal plasmacytosis. In addition, hMLH1 and MGMT protein expression was analysed by immunohistochemistry in 29 samples: 15 multiple myeloma, one plasma cell leukaemia, 11 monoclonal gammopathies of undetermined significance, and two polyclonal plasmacytosis. Therefore, correlation between methylation status and protein expression was assessed in 27 plasma cell gammopathies. An overview of the methylation profile and immunohistochemistry is given in Figure 1.

hMLH1 and *MGMT* Promoter Hypermethylation

The frequencies of aberrant methylation among multiple myeloma and plasma cell leukaemia samples were 10% (3/30) for *hMLH1*, and 23% (7/30) for *MGMT*. For the monoclonal gammopathy of undetermined significance samples, we found no methylation for the *hMLH1* gene and 8% (1/13) for *MGMT* (see Table 2). Concurrent hypermethylation of the two genes occurred in one multiple myeloma sample. No methylation of the two genes was found

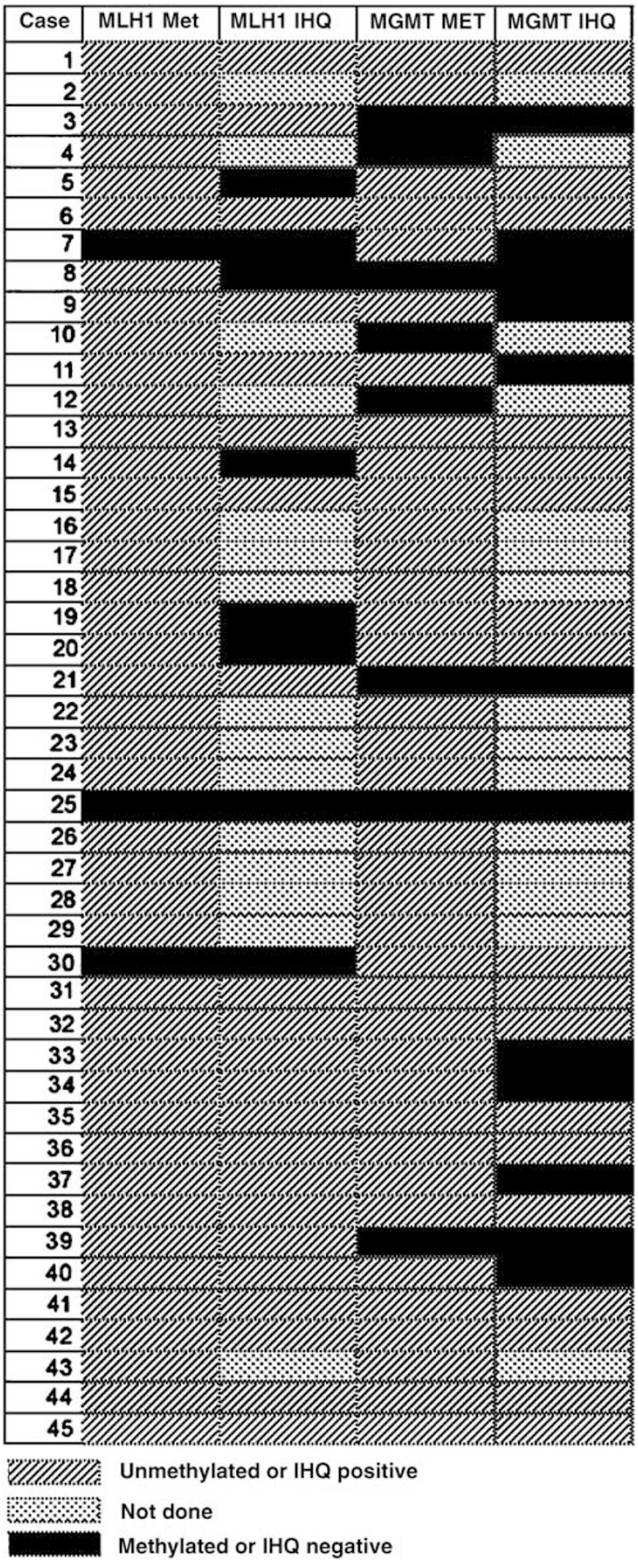

Figure 1 Summary of methylation and loss of protein expression in cases with multiple myeloma (cases 1–30), monoclonal gammopathy or undetermined significance (cases 31–43), and polyclonal plasmacytosis (cases 44 and 45). Black boxes represent methylated samples or loss of protein expression, and grey boxes represent unmethylated cases or cases with protein expression positive.

in polyclonal samples. Positive and negative controls worked appropriately in each round of PCR reaction.

Representative results of the methylation-specific PCR of *hMLH1* and *MGMT* are shown in Figure 2. The presence of a PCR product in lanes marked M indicates a methylated *hMLH1* or *MGMT* promoter, while a product in lanes marked U indicates an unmethylated *hMLH1* or *MGMT* promoter.

hMLH1 and MGMT Immunohistochemistry

Loss of hMLH1 expression revealed by immunohistochemistry was found in 50% (8/16) multiple myeloma samples (including plasma cell leukaemia); by contrast, none of the monoclonal gammopathy of undetermined significance cases had lost protein expression (see Table 2) (Figure 3a and b).

MGMT protein expression was absent in 44% (7/16) multiple myeloma samples, and in 36% (4/11) of monoclonal gammopathies of undetermined significance (Figure 4a and b). Polyclonal samples showed nuclear staining of both proteins. We found losses of both protein expressions in three multiple myeloma samples (Figure 4c).

We then explored possible correlation between the methylation data and clinical parameters of the 43 patients. We did not find any association between methylation/inactivation of any of the investigated genes and the clinical parameters; however, we found in the survival analysis that there was a trend between aberrant methylation of *hMLH1* and poorer survival, but due to sample size, it was not possible to do the statistical analysis.

Discussion

Alterations in the expression of hMLH1 and MGMT have been detected in various solid tumours[18,29,30] and in different haematological malignancies.[31,32] However, the involvement of the repair system in the pathogenesis of plasma cell dyscrasias is not yet fully elucidated. One molecular mechanism of gene inactivation of these DNA repair enzymes is promoter methylation of CpG islands.[33,34] In our analysis, we detected a loss of hMLH1 expression revealed by immunohistochemistry in 50% of multiple myeloma cases, whereas none of the monoclonal gammopathies of undetermined significance cases had a loss of expression of this repair enzyme. With the methylation-specific PCR technique, we also found that 10% of myeloma cases had *hMLH1* promoter methylation. None of the multiple myeloma cases with *hMLH1* hypermethylation had any detectable hMLH1 protein expression in neoplastic cells. The finding that no monoclonal gammopathy of undetermined significance cases had a loss of expression of hMLH1, whereas half of multiple myeloma cases lacked it, leads us to suggest that the loss of hMLH1 plays a role in the

Table 2 Results of methylation specific PCR and immunohistochemistry of hMLH1 and MGMT in monoclonal gammopathies

MSPCR (n = *45*)	*hMLH1 methylated*	*hMLH1 unmethylated*	*MGMT methylated*	*MGMT unmethylated*
MM *(n = 30)*	10% (3/30)	90% (27/30)	23% (7/30)	77 % (23/30)
MGUS *(n = 13)*	0%	100% (13/13)	8% (1/13)	92% (12/13)
Polyclonal *(n = 2)*	0%	100%	0%	100%
IHQ (n = 29)	*hMLH1 IHQ negative*	*hMLH1 IHQ positive*	*MGMT IHQ negative*	*MGMT IHQ positive*
MM *(n = 16)*	50% (8/16)	50% (8/16)	44% (7/16)	56% (9/16)
MGUS *(n = 11)*	0%	100% (11/11)	36% (4/11)	64% (7/11)
Polyclonal *(n = 2)*	0%	100%	0%	100%

MSPCR: methylation-specific PCR; MM: multiple myeloma; MGUS: monoclonal gammopathy of undetermined significance.

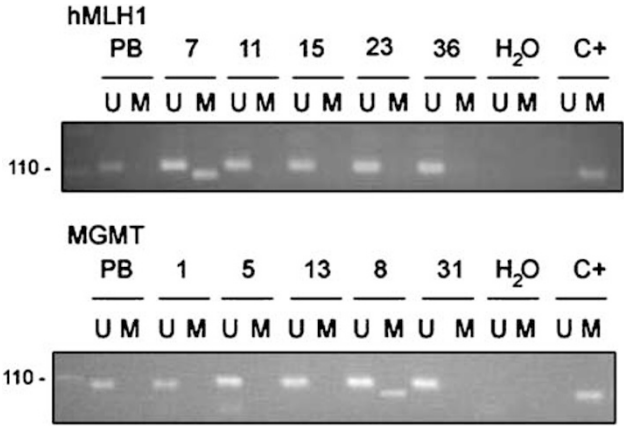

Figure 2 Representative methylation-specific PCR assays for *hMLH1* and *MGMT* in five cases. M: reactions with methylation-specific primer sets; U: reactions with unmethylation-specific primer. Multiple myeloma: cases 1–30, monoclonal gammopathy or undetermined significance: cases 31–43, C+: positive control for methylated alleles. Peripheral blood (PB) mononuclear cells were used as control for unmethylated alleles.

progression of the multistep process. We know that the MMR system is involved in signalling the presence of DNA damage to the apoptotic machinery conferring resistance to chemotherapy;[16] consequently, the silencing of *hMLH1* may be one of the causes of drug resistance, relapse, and refractory disease characteristic in multiple myeloma. We have observed that patients with *hMLH1* hypermethylation show a tendency to shortened survival, suggesting that lesions in this repair gene may be related to biological behaviour of the disease and, therefore, could have prognostic relevance.

To our knowledge, there is only one study that analyses *hMLH1* methylation in plasma cell dyscrasias[35] and did not find methylation of this gene in their cases. The difference in our results, compared with this study, may be accounted for the use of non-enriched plasma cells for the methylation-specific PCR technique; hence, Galm *et al*[35] probably would have underestimated the true frequency of this epigenetic event. Nevertheless, our findings support the work of Kotoula *et al*,[36] who described low expression or absence of hMLH1 in multiple myeloma samples with extensive bone marrow infiltration using a multiplex RT-PCR technique; they suggested that a deficiency of hMLH1 confers an unfavourable prognosis for multiple myeloma.

We also investigated the promoter status and expression of MGMT. Hypermethylation of *MGMT* was observed in seven cases of multiple myeloma (including one case of plasma cell leukaemia) while only one monoclonal gammopathy of undetermined significance sample showed methylation. The frequency of *MGMT* methylation was higher in our series, compared to other studies: Galm *et al*[35] (1.8% in multiple myeloma); Seidl *et al*[37] (7% in myeloma and 4% in monoclonal gammopathy of undetermined significance); Rossi *et al*[38] (12.5% in multiple myeloma); and Chim *et al*[39] (0% in multiple myeloma). Once more, this discrepancy may be attributed to the use in those studies of not-purified plasma cells. Loss of MGMT expression is rarely due to deletion, rearrangement or mutation of the *MGMT* gene, and approximately 20% of human tumour cell lines lack MGMT activity, suggesting that the gene is under epigenetic control.[21,22] Silencing of the *MGMT* gene has been reported in diffuse large B-cell lymphoma (36%)[40] and in immunodeficiency-related NHL with a similar frequency.[41] In addition, the pathogenetic role of *MGMT* inactivation is supported by the fact that *MGMT* knockout mice develop lymphoma with high frequency,[42] this inactivation being an important mechanism in lymphomagenesis. We propose that *MGMT* inactivation could have relevance in myelomagenesis, producing spontaneous G to A mutations and, consequently, genetic instability. In our study, all *MGMT*-methylated cases had lost protein expression, similar to that reported for immunodeficiency-related LNH.[41] Contrary to Rossi *et al*, who found that all lymphoma samples carrying unmethylated *MGMT* expressed the protein, we found in our series that seven unmethylated samples failed to express the MGMT protein. This fact argues for an additional inactivating mechanism being involved in the lack of MGMT expression in plasma cell dyscrasias.

Recently, Peng *et al*[43] reported that the silencing of DNA repair proteins may be one of the causes for the development of multiple myeloma, preceding

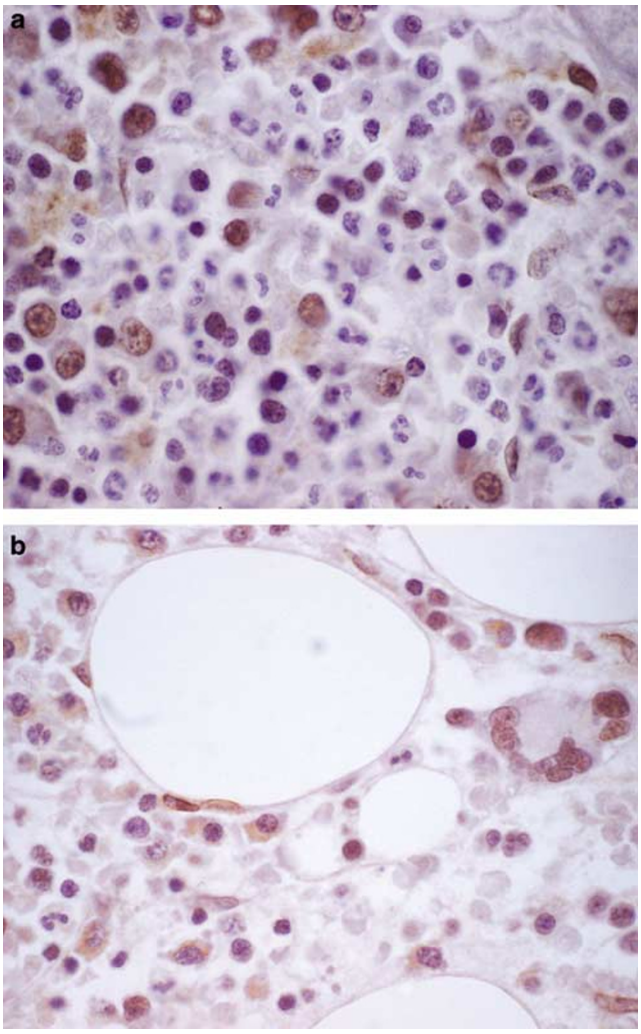

Figure 3 Immunohistochemical staining of hMLH1. Original magnification ×100. (**a**) Case 11, this myeloma (unmethylated) sample shows nuclear staining for hMLH1. (**b**) Case 36, monoclonal gammopathy or undetermined significance hMLH1 positive.

chromosomal aberrations. We only found simultaneous loss of expression of both genes in multiple myeloma samples (three cases: two IgG kappa and one myeloma Bence Jones lambda) and the silencing of MGMT expression was found with a higher frequency in myeloma (43%) than in monoclonal gammopathy of undetermined significance (36%). Multiple myeloma accumulates multiple genetic alterations, in contrast to monoclonal gammopathy of undetermined significance, and its genetic instability is increased when the DNA repair genes are silenced, confirming the finding reported by Peng.

Ten percent of colorectal tumours have simultaneous silencing of both genes, and this phenomenon confers[44] resistance to cell death, producing a high incidence of mutation. Although tumours with methylated *MGMT* are more sensitive to the apoptotic effects of alkylating agents, it has been reported that *Mgmt*$^{-/-}$ mice with a loss of MLH1 expres-

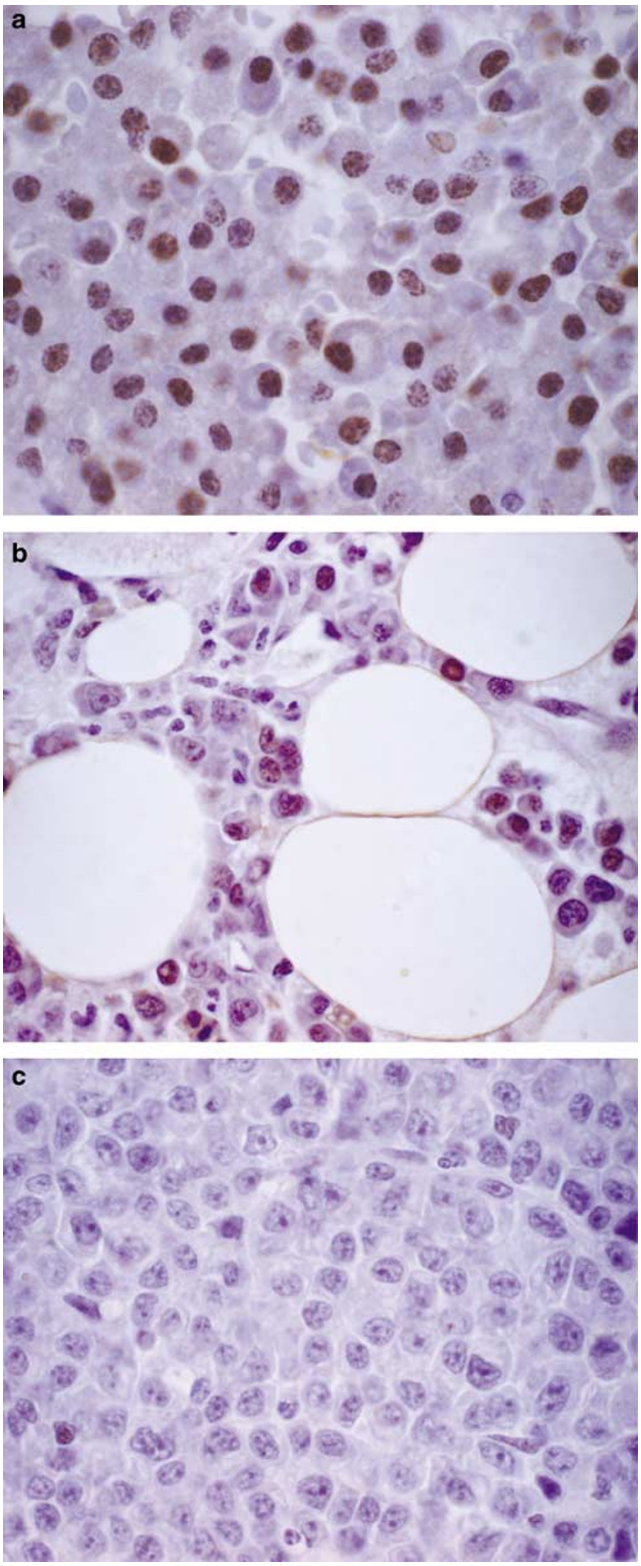

Figure 4 Immunohistochemical staining of MGMT. (**a**) Plasma cell leukaemia (case 30) MGMT positive (this case was unmethylated). (**b**) Multiple myeloma with low infiltration MGMT positive (case 19, unmethylated). (**c**) Loss of MGMT expression in multiple myeloma (case 8, methylated).

sion[45,46] are as resistant to alkylating drugs as are wild-type mice. Additionally, MMR-deficient cell lines display a mutator phenotype and resistance to several cytotoxic agents, including compounds widely used in cancer chemotherapy.[47] Recent studies[48] indicate that the lack of hMLH1 and MGMT expression could play a role in carcinogenic pathways in the colon; therefore, higher rates of loss of hMLH1 (33%) and MGMT (37%) were detected in mixed colon polyps with carcinomatous areas as compared to hyperplastic polyps and adenomas. Something similar could happen in the neoplastic progression of monoclonal gammopathies, where the lack of hMLH1 has been detected only in multiple myeloma cases and not in MGUS samples, and where the silencing of *MGMT* has been found with greater frequency in multiple myeloma than in monoclonal gammopathy of undetermined significance.

On the basis of our data, we believe that: (a) inactivation of the repair pathway plays an important role in genetic instability in plasma cell dyscrasias, which may reflect an underlying defect in the ability of malignant plasma cells to detect and eliminate mistakes, resulting in a mutator phenotype that would contribute to chemotherapy resistance; and (b) methylation is one of the silencing mechanisms of repair genes and therefore, the research and development of new demethylation drugs may improve multiple myeloma patient survival, although more studies with larger numbers of cases and follow-ups are needed to confirm these findings.

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