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Loss of *hMLH1* expression correlates with improved survival in stage III–IV ovarian cancer patients

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Abstract

Pre-clinical data suggest a relationship between DNA MisMatch Repair (MMR) system failure, particularly the inactivation of genes *hMLH1* and *hMSH2*, and resistance to drugs like cisplatin and carboplatin. We studied the correlation between loss of *hMLH1* expression in tumour cells and clinical outcome in 38 patients with ovarian cancer, who underwent cisplatin-based chemotherapy. 19 patients (56%) showed loss of *hMLH1* expression (Group A) while 15 patients (44%) showed normal *hMLH1* expression (Group B). 4 patients were not evaluable for *hMLH1* expression. The 2 groups of patients were similar for clinical characteristics, response to chemotherapy and time to progression. Group A patients showed a median survival of 55 months whereas Group B patients had a median survival of 12 months ($P = 0.014$). Loss of *hMLH1* expression was the only independent predictor of survival in the multivariate analysis. Our observations suggest a relationship between loss of hMLH1 and improved survival in advanced ovarian cancer.

Keywords: hMLH1; Advanced ovarian cancer; Chemotherapy; Platinum resistance

1. Introduction

Ovarian cancer ranks fifth in incidence among women and first in overall mortality among gynaecological cancers. Although it accounts for only 4% of all cancer diagnoses, ovarian cancer is the fourth leading cause of cancer death in females, showing a mortality rate of approximately 50%. Early stage at diagnosis is correlated with a 5-year survival rate of 93%. However, 75% of patients present with stage III and IV disease: these women have a 5 year survival rate as poor as 20% [1–3].

Platinum-based combination chemotherapy represents the main treatment option in advanced ovarian cancer, along with optimal (or sub-optimal) debulking surgery. The response rate to chemotherapy is high, ranging between 50 and 70%, with 15–20% complete responses. Patients treated with platinum-containing regimens achieve a median survival time of 25 to 30 months with a 5-year overall survival rate of 45%. Although substantial progress has been made in the treatment of ovarian cancer, 50–75% of advanced disease patients will ultimately relapse [3]. In these patients, the development of platinum-resistance still represents an unfavourable, but common, event which makes the tumour refractory to most of the subsequent salvage therapies. Several mechanisms have been implicated in the development of platinum resistance, such as increased expression of DNA repair genes like *ERCC-1* and *XPAC* and augmented intracellular platinum detoxification [4]. More recently, the inactivation of the postreplicative DNA MisMatch Repair (MMR) system has been linked to drug resistance [5,6]. The products of six genes, namely *hMLH1*, *hMSH2*, *hPMS1*, *hPMS2*, *hMSH6* and *hMSH3* participate in DNA MMR and they were originally identified for their involvement in the Hereditary Non Polyposis Colorectal Cancer

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(HNPCC) syndrome. The MMR system, particularly *hMLH1* and *hMSH2,* has been shown to recognise several types of drug-induced DNA adducts, for example, those caused by treatment with cisplatin, carboplatin, doxorubicin or thioguanine [7]. Recognition, and perhaps the attempt of the MMR system to process such adducts, triggers the activation of apoptosis, a mechanism which may enhance the cytotoxicity of chemotherapy. Failure of the MMR system would make the neoplastic cell less able to detect drug-induced DNA damage and less able to initiate apoptosis. Such inability to initiate the apoptotic response may represent one mechanism of resistance to treatment with platinum-based drugs [8]. Loss of hMLH1 protein expression has been found in certain cell lines selected for resistance to cisplatin and doxorubicin and restoration of MMR activity in these cells was sufficient to re-establish their susceptibility to chemotherapy [5,9]. However, it has also been suggested that the combined inactivation of p53 and of the MMR system is required for the development of cisplatin resistance and that in this mechanism the loss of p53 plays a more significant role than the MMR system failure alone [10]. In addition, clinical studies to date have generated inconclusive results: no correlation has been found between the patterns of MMR gene expression, response to treatment and overall survival in breast and ovarian cancer patients [11–13]. On the contrary, inactivation of MMR functions was associated with a better survival in colorectal cancer patients [14].

In this study, we investigated the impact of hMLH1 protein expression on overall survival, the response to chemotherapy and time to progression in 38 patients with the International Federation of Gynecology and Obstetrics (FIGO) stage III and IV ovarian cancers, treated with a cisplatin-containing chemotherapy regimen.

2. Patients and methods

2.1. Patients

38 patients with stage III or IV epithelial ovarian cancers who were referred to our Institution were included in the study. Patients were treated with chemotherapy according to the PEC regimen (cisplatin 60 mg/m^2, epirubicin 60 mg/m^2 and cyclophosphamide 750 mg/m^2, day 1, cycles repeated every 3 weeks), as part of their first-line treatment. Response was assessed after 3 cycles of treatment, according to the World Health Organisation (WHO) criteria for evaluation of response in cancer patients. Second- and third-line chemotherapy was administered when clinically indicated. All the relevant data regarding patient characteristics, response to treatment, overall survival and time to progression were available for analysis.

2.2. Immunohistochemical analysis

Immunohistochemical investigation of hMLH1 protein expression was performed on paraffin-embedded tissue sections [15]. Only the primary ovarian tumour was sampled in each case and analysed for immunohistochemical expression of hMLH1. The expression of hMLH1 was not investigated in the metastases. Five micrometre sections containing tumour tissue and normal ovarian tissue as internal controls, were dewaxed and rehydrated using xylene and alcohol. Endogenous peroxidase was blocked by dipping the sections in 3% aqueous H_2O_2 for 10 min and antigen retrieval was performed with a 10 min microwave treatment in 10 mM citrate buffer, pH 6.00. Following antigen retrieval, sections were incubated overnight at 4 °C with a mouse monoclonal antibody to the hMLH1 protein (clone G168-15, 1:100 dilution; PharMingen, San Diego, CA) and lightly counterstained with haematoxylin [16]. Immunostaining was performed using the avidin-biotin peroxidase complex technique, using diaminobenzidine as a chromogen. The normal staining pattern of hMLH1 was assessed and the percentage of malignant cells positive for hMLH1 staining (from 0 to 100% in 10% increments) was estimated by a pathologist who was unaware of the patient's clinical outcome. The immunohistochemical expression of hMLH1 was nuclear and was found in both epithelial and stromal cells. Tumour cells were defined as negative for hMLH1 expression when there was no detectable nuclear staining in the presence of internal positive controls represented by normal epithelial cells, stromal cells or lymphocytes [11,12]. Fig. 1 shows an example of a tumour negative for hMLH1 immunostaining and an example of a tumour with a strong immunoreactivity to the anti-hMLH1 antibody. Patients were arbitrarily divided in 2 groups according to the percentage of tumour cells expressing hMLH1. Group A included patients showing loss of hMLH1 expression in $\geqslant 50\%$ of the tumour cells analysed. Group B included patients in which a normal hMLH1 expression was detected in $>50\%$ of the tumour cells.

2.3. Statistical analysis

The SAS® System (version 8.2, SAS Institute, Cary, NC, USA) was used for all statistical analyses. Observed differences were considered significant if $P<0.05$. Group A and Group B were examined to test whether a statistically significant difference existed in tumour residuum $\leqslant 2$ cm or tumour residuum >2 cm (Fisher's Exact Test), stage III or IV (Fisher's Exact Test) and tumour grading (Fisher's Exact Test). In addition, Group A and Group B were tested for any correlations between hMLH1 expression and the response to treatment (Fisher's Exact Test), time to

progression and overall survival (comparison of Kaplan–Meier curves using log-rank statistics). Chi square test and logistic regression analysis were performed to study variables associated with the response to treatment in the whole group. The impact on survival of stage at diagnosis, response to treatment, post-surgical residuum and hMLH1 status was assessed in the whole group of patients by comparison of Kaplan–Meier curves using log-rank statistics for univariate analysis and the proportional hazards model of Cox for multivariate analysis.

3. Results

Tumour samples were available from 38 patients. However, in 4 samples hMLH1 immunohistochemical analysis could not be performed due to tissue degradation. The clinical characteristics and treatment outcome of the 34 evaluable patients are summarised in Table 1. Median age at diagnosis was 60 years (range 32–77 years), 25 patients (74%) had FIGO stage III disease whereas 9 patients (26%) had FIGO stage IV disease. Post-surgical residuum was $\leqslant 2$ cm in 11 patients (32%) and >2 cm in 16 patients (47%); in the remaining 7 patients (21%) an assessment of post-surgical disease residuum was not available, however in all 7 cases post-surgical residuum was described as macroscopic. 2 patients had mucinous tumours and a further 2 patients clear cell tumours; 19 patients (56%) showed a high grade histology (Grade 3). Five patients (15%) achieved a complete response (CR) and 10 patients (29%) a partial response (PR) resulting in an overall response rate of 44%. 13 patients (38%) showed stable disease and 3 patients (9%) progressive disease. Response to treatment could not be assessed in 3 patients (9%), one of which died after the first cycle of chemotherapy because of therapy-related toxicity. Median survival time of the whole group of patients was 28 months and median time to progression was 9 months. At univariate analysis, patients who achieved a response to chemotherapy, either complete or partial response, had a median survival of 55 months whereas non-responders demonstrated a median survival of 12 months ($P<0.025$). Patients with FIGO stage III tumours showed a median survival of 51 months whereas those with FIGO stage IV had a median survival of 12 months ($P<0.05$). Median survival was 50 months in patients with post-surgical disease residuum $\leqslant 2$ cm. Patients with a disease residuum >2 cm had a median survival of 14 months. None of these variables resulted in differences in overall survival in the multivariate analysis.

Next, we assessed the impact of hMLH1 expression on survival, response to treatment and time to progression. Patients were divided in 2 groups according to the percentage of tumour cells expressing hMLH1: 19 patients (56%) showed loss of hMLH1 expression in $\geqslant 50\%$ of neoplastic cells (Group A), 15 patients (44%) showed a normal hMLH1 expression in $>50\%$ of neoplastic cells (Group B). The median survival time was 55 months for the patients in Group A and 12 months for the patients in Group B (Fig. 2); this survival difference was statistically significant ($P=0.014$). The 2 groups of patients were comparable for the clinical characteristics and the major prognostic factors: median age at diagnosis was 60 years in Group A (range 32–77 years) and 60 years in Group B (range 42–66 years). 14 patients (74%) in Group A and 11 patients (73%) in Group B had FIGO stage III disease at diagnosis, whereas 5 patients (26%) in Group A and 4 patients (27%) in Group B showed FIGO stage IV disease ($P=$ non-significant (N.S.)). Six patients (32%) in Group A and 5 patients (33%) in Group B showed a post-surgical residuum $\leqslant 2$ cm, while 10 patients (53%) in Group A and

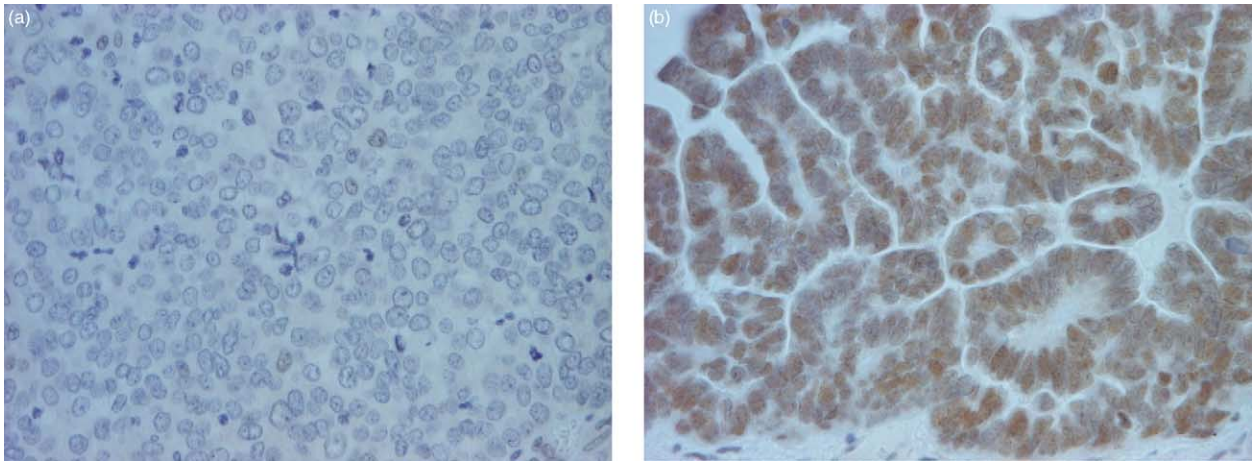

Fig. 1. Immunohistochemical staining for hMLH1. (a) Serous cystadenocarcinoma of the ovary in which the staining of hMLH1 is negative (original magnification $\times 250$). (b) Serous cystadenocarcinoma of the ovary demonstrating positive staining of tumour cells for hMLH1 (original magnification $\times 250$).

6 patients (40%) in Group B had a post-surgical residuum >2 cm (P = N.S.). 6 patients (32%) from Group A and none of the patients from Group B showed a tumour grading of 1 and 2, whereas 10 patients (53%) from Group A and 9 patients (60%) from Group B had a poorly differentiated, G3, tumour (P = N.S.). 3 patients in Group A (16%) and 2 patients in Group B (13%) obtained a CR to chemotherapy, PRs were 5 (26%) in Group A and 5 in Group B (33%). Overall response rate was 42% in Group A and 47% in Group B (P = N.S.). Time to progression was 12 months in Group A and 10 months in Group B (P = N.S.). At multivariate analysis, the only variable associated with objective responses was a post-surgical residuum ⩽2 cm (P = 0.0107), whereas loss of expression of hMLH1 was the only independent prognostic marker linked to overall survival (P = 0.0065).

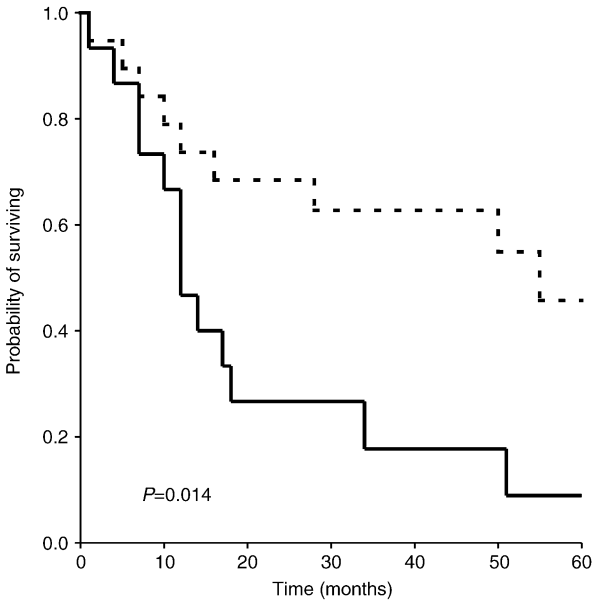

Fig. 2. Kaplan–Meier survival curves for patients showing loss of hMLH1 expression in ⩾50% of tumour cells (Group A , - - - - -), and for patients showing normal hMLH1 expression in >50% of tumour cells (Group B , —).

4. Discussion

We studied the correlation between expression abnormalities of hMLH1 and response to chemotherapy,

Table 1
Patients' characteristics and hMLH1 staining results

	Group A	Group B	All patients
No. of patients	19	15	34
Median age (range) (years)	60 (range 320-77)	60 (range 42–66)	60 (range 32–77)
FIGO stage			
III	14 (74%)	11 (73%)	25 (74%)
IV	5 (26%)	4 (27%)	9 (26%)
Surgical residuum:			
⩽2 cm	6 (32%)	5 (33%)	11 (32%)
>2 cm	10 (53%)	6 (40%)	16 (47%)
NA	3 (16%)	4 (27%)	7 (21%)
Histology			
Serous	9 (47%)	6 (40%)	15 (44%)
Endometrioid	6 (32%)	1 (7%)	7 (21%)
Clear cell	2 (11%)	0 (0%)	2 (6%)
Mucinous	1 (5%)	1 (7%)	2 (6%)
Not otherwise specified	1 (5%)	7 (47%)	8 (24%)
Grading			
G1, G2	6 (32%)	0 (0%)	6 (18%)
G3	10 (53%)	9 (60%)	19 (56%)
NA	3 (16%)	6 (40%)	9 (26%)
Response rate			
CR	3 (16%)	2 (13%)	5 (15%)
PR	5 (26%)	5 (33%)	10 (29%)
Overall (CR + PR)	8 (42%)	7 (47%)	15 (44%)
NA	1 (5%)	2 (13%)	3 (9%)
Median survival (months)	55*	12*	28
Time to progression (months)	12	10	9

Group A: patients showing loss of hMLH1 immunostaining in ⩾50% of tumour cells. Group B: patients showing normal hMLH1 immunostaining in >50% of tumour cells. *P = 0.014. FIGO, International Federation of Gynecology and Obstetrics, NA, not available; G, grade, CR, complete response; PR, partial response.

time to progression, and overall survival in a group of 38 patients with stage III and IV ovarian cancer, who were treated with the platinum based 'PEC' regimen. Of the 34 evaluable patients, 19 (56%) showed loss of hMLH1 staining in $\geqslant 50\%$ of tumour cells (Group A), whereas the remaining 15 patients (44%) showed a normal hMLH1 expression in $>50\%$ of the neoplastic cells analysed (Group B). The 2 groups of patients demonstrated similar characteristics, as there was no relationship between the expression of hMLH1 and age, stage at diagnosis, post-surgical residuum, histology and grading (Table 1). We didn't find any significant differences in the response to chemotherapy and time to progression between the patients in Group A and Group B. Preclinical studies showed that the *in vitro* sensitivity of ovarian cancer cells to cisplatin and to other drugs was linked to the expression of hMLH1 protein [5,9]. However, our data, consistent with observations made by other authors, suggest that the level of hMLH1 expression in the tumour does not predict the response or the resistance of ovarian cancer to platinum-based chemotherapy [11]. This discrepancy between pre-clinical and clinical studies could have several explanations involving other cellular functions, such as the p53 stress response or the efficiency of drug detoxification, which also determines the sensitivity to the treatment [4,10]. It is also important to consider that immunostaining of hMLH1 is an indirect method to assess MMR activity, which may not represent the actual efficiency of DNA repair functions in the cell [11].

In our study, patients in the 2 groups differed in their median survival rates (55 months compared with 12 months for Groups A and B, respectively). These findings were confirmed by multivariate analysis in which loss of expression of hMLH1 was the only independent prognostic marker linked to overall survival ($P=0.0065$). A similar survival advantage, and a reduced likelihood of developing metastases, has been described in colorectal cancer patients showing microsatellite instability or loss of hMLH1 expression [14,17]. By contrast, other studies have failed to find a relationship between MMR function and prognosis in ovarian and breast cancer patients [11,12]. Although the sample size of our study could have affected the results of statistical analysis, the observation that the loss of hMLH1 expression in tumour cells may confer a survival advantage in ovarian cancer patients is novel and deserves further study. So far, the biological mechanisms which may be implicated in such a survival advantage have not been identified. It has been hypothesised that the widespread accumulation of replication errors in the tumour DNA could affect cell cycle progression and decrease the rate of dividing cells [18]. Studies carried out in colon cancer have shown that tumours with inactivation of the MMR system preferentially harbour mutations of the TGF β receptor type II and of the β-catenin genes. These data have suggested that loss of MMR functions could result in the mutation of a specific panel of genes which may give the tumour unique pathological features and a less aggressive phenotype [14].

The management of advanced ovarian cancer requires a multidisciplinary approach which combines surgical and medical procedures. In this setting, the choice of the appropriate treatment strategy is critical and the availability of prognostic markers could facilitate the decision-making process. Our data indicate that loss of hMLH1 immunostaining could represent a useful prognostic marker in patients diagnosed with stage III–IV ovarian cancer. However, more research is warranted to confirm our observations and to identify novel prognostic indicators to be employed in the clinic.

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