The Acquisition of *hMLH1* Methylation in Plasma DNA after Chemotherapy Predicts Poor Survival for Ovarian Cancer Patients

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ABSTRACT

Aberrant epigenetic regulation, such as CpG island methylation and associated transcriptional silencing of genes, has been implicated in a variety of human diseases, including cancer. Methylation of genes involved in apoptosis, including the DNA mismatch repair (MMR) gene *hMLH1*, can occur in tumor models of resistance to chemotherapeutic drugs. However, the relevance for acquired resistance to chemotherapy of patients' tumors remains unsubstantiated. Plasma DNA from cancer patients, including those with ovarian cancer, often contains identical DNA changes as the tumor and provides a means to monitor CpG island methylation changes. We have examined plasma DNA of patients with epithelial ovarian cancer enrolled in the SCOTROC1 Phase III clinical trial for methylation of the *hMLH1* CpG island before carboplatin/taxoid chemotherapy and at relapse. Methylation of *hMLH1* is increased at relapse, and 25% (34 of 138) of relapse samples have *hMLH1* methylation that is not detected in matched prechemotherapy plasma samples. Furthermore, *hMLH1* methylation is significantly associated with increased microsatellite instability in plasma DNA at relapse, providing an independent measure of function of the MMR pathway. Acquisition of *hMLH1* methylation in plasma DNA at relapse predicts poor overall survival of patients, independent from time to progression and age (hazard ratio, 1.99; 95% confidence interval, 1.20–3.30; $P = 0.007$). These data support the clinical relevance of acquired *hMLH1* methylation and concomitant loss of DNA MMR after chemotherapy of ovarian cancer patients. DNA methylation changes in plasma provide the potential to define patterns of methylation during therapy and identify those patient populations who would be suitable for novel epigenetic therapies.

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INTRODUCTION

Transcriptional silencing of proapoptotic genes, including the DNA mismatch repair (MMR) gene *hMLH1*, due to aberrant CpG island methylation has been implicated in acquired resistance to chemotherapeutic drugs *in vitro* (1, 2). A major limitation in confirming the clinical relevance of drug resistance mechanisms is the difficulty in obtaining tumor biopsies after initial treatment, at a time when resistant subpopulations may be more apparent. However, plasma DNA from cancer patients, including those with ovarian cancer, often contains the same genetic changes as the tumor (3, 4), raising the possibility of using plasma DNA to monitor genetic and epigenetic changes after treatment.

Although relatively chemosensitive, with response rates to primary chemotherapy of 60–80%, the majority of ovarian tumors will recur, leading to failure of treatment using conventional cytotoxic drugs and resulting in an overall 5-year survival for patients with advanced disease of <30% (5). Many reports have been published on potential drug resistance markers in ovarian cancer, derived mainly from the study of acquired resistance in experimental models (6). However, most clinical studies of drug resistance have focused on tumor characteristics at presentation, rather than at relapse. Whereas studies of tumors before chemotherapy are important for identifying prognostic markers and possible mechanisms of intrinsic resistance, they will provide limited information on mechanisms of acquired resistance. Thus, tumors at presentation will be heterogeneous, consisting of chemosensitive and chemoresistant subpopulations, making it difficult to identify the subpopulations that lead to treatment failure of an initially responsive tumor. Because chemotherapy positively selects for resistant subpopulations, analysis of tumors at relapse may allow these subpopulations of cells to become more apparent and will allow mechanisms of acquired rather than intrinsic drug resistance to be identified and analyzed for associations with patient survival.

Due to the difficulties in obtaining tumor samples routinely, especially from patients at relapse, and for ease of sample collection in the context of large, multicenter clinical trials, there has been increasing interest in the use of markers in plasma and serum for the prognostication and monitoring of cancer (3). DNA can be detected in plasma from cancer patients with the same characteristic changes, including CpG island methylation, found in the corresponding tumor (7). In the case of ovarian cancer, such changes have been detected with high specificity and have been suggested as a diagnostic tool (4, 8). DNA methylation is particularly suited for such analysis of

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plasma DNA because sensitive methylation-specific PCR (MSP)-based assays require only small amounts of DNA, and methylation of genes frequently aberrantly methylated in tumors is rarely observed in normal tissue, including peripheral blood mononuclear cell (PBMC) DNA that may be present with tumor DNA in plasma (9).

In vitro studies of ovarian cancer cell lines have implicated loss of DNA damage-dependent apoptotic pathway in acquired resistance to clinically important cytotoxic drugs (10). Methylation of *hMLH1* and loss of a MMR-dependent apoptotic response lead to increased resistance to cisplatin and carboplatin, drugs that are the cornerstone of treatment of ovarian cancer and a wide variety of tumors (2, 11). Restoration of *hMLH1* expression, either by gene transfer or by reversal of epigenetic silencing, leads to increased sensitivity to carboplatin and other cytotoxic agents (12, 13). However, the clinical relevance for acquired resistance to chemotherapy of epigenetic inactivation of proapoptotic genes such as *hMLH1* by DNA methylation remains uncertain. Indeed, methylation of antiapoptotic genes or genes involved in protecting cells from DNA damage can also occur in cancer and could lead to increased drug sensitivity (14). Therefore, identification of epigenetic changes that influence clinical outcome after chemotherapy, as well as allow target validation, will have potential for disease stratification and treatment individualization.

MATERIALS AND METHODS

Patients and Collection of Plasma. In the SCOTROC1 randomized trial, all patients had histologically confirmed epithelial ovarian carcinoma and FIGO (International Federation of Gynecologists and Obstetricians) stage Ic–IV disease with or without cytoreductive surgery at staging laprotomy. All patients gave written informed consent, and appropriate ethical review boards approved the study. Patients were randomized to receive six cycles of either paclitaxel (175 mg/m^2) as a 3-h infusion or docetaxel (75 mg/m^2) as a 1-h infusion in combination with carboplatin AUC5 as 1-h infusion. Ten ml of blood were collected before chemotherapy or at the time of disease progression (relapse) after primary chemotherapy into EDTA tubes, and the plasma was separated from PBMCs before being sent to the Cancer Research UK Beatson Laboratories (Glasgow, United Kingdom). All laboratory analyses were conducted blinded to clinical outcome. Progressive disease was defined as either clinical evidence of progressive disease based on the South Western Oncology Group Solid Tumor Response Criteria or elevated CA125 levels as defined previously (15) and clinical or radiographic findings indicative of progression. Five relapse blood samples were collected at the time of clinically suspected progression, but before progression was verified. More than 90% of the relapse samples were taken <4 weeks after the date of confirmed progression.

DNA Isolation. DNA was extracted from 200 μl of blood and 1 ml of plasma using the QIAmp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions, with plasma elute passed through the column an additional four times to increase yield and eluting in a final volume of 200 μl. The mean yield of DNA at presentation was 2.8 μg/ml plasma (SD, 1.7 μg/ml plasma), and at relapse, it was 3.6 μg/ml plasma (SD, 1.8 μg/ml plasma). However, it should be noted that this will represent DNA from lysed normal blood cells as well as tumor DNA.

MSP. MSP was done essentially as described previously (16). Fifty μl of DNA from each serum sample were bisulphite modified using the CpGenome DNA Modification Kit (Serologicals Corp.) according to the manufacturer's instructions. For each modification, a negative control of 100 and 500 ng of human Genomic DNA (Promega) was modified, and positive controls of serial dilutions (1:5, 1:10, 1:20, 1:50, 1:100, 1:200, and 1:500) of CpGenome Universally Methylated human DNA were diluted into human genomic DNA before modification. PCR for hMLH1 CpG island was performed essentially as described previously but with the primer sequences 5′-GGGTTAACGTTAGAAAGGTCG and 5′-CGCTTACGCGTTAAAAATCGC (17). The forward primer was fluorescently labeled with 5′-FAM dye (Applied Biosystems). PCR products were denatured at 95°C for 5 min and electrophoresed for 12 h at 2500 V on a 6% denaturing polyacrylamide gel (Flowgen) using a 373XL Stretch DNA Sequencer (Applied Biosystems). Samples were run with the internal lane size standard GS500XL ROX and analyzed using Genescan 3.1 Analysis Software (Applied Biosystems) to determine the size of the PCR products and the amount of fluorescent signal.

Microsatellite PCR. The choice of microsatellite loci used reflects those used in previous colorectal cancer and ovarian analysis (8, 18). PCR was carried out using primers (synthesized by Oswel DNA Service, Southampton, United Kingdom) specific for six polymorphic DNA microsatellite loci (*Mfd15CA, D2S123, P53, D5S346, D18S69*, and *D18S58*). The forward primer in each case was fluorescently labeled with 5′-FAM or HEX (Applied Biosystems). PCR products were denatured at 95°C for 5 min and electrophoresed for 12 h at 2500 V on a 6% denaturing polyacrylamide gel (Flowgen) using a 373XL Stretch DNA Sequencer (Applied Biosystems). Samples were run with the internal lane size standard GS500XL ROX and analyzed using Genescan 3.1 Analysis Software (Applied Biosystems) to determine the size of the PCR products and the amount of fluorescent signal. Allelic shifts were defined as a shift in the major PCR product(s) by >2 bases of plasma DNA compared with PBMC DNA. Samples with a major peak of PCR products of <30 fluorescence units were not included in the analysis.

Statistics. The change in methylation and microsatellite instability (MSI) status from prechemotherapy to progression was compared in the paired samples using the Wilcoxon signed rank-sum test. The examination of the association between MSI status and methylation status was conducted using Fisher's exact test. Kaplan-Meier methods were used to produce the survival curves in Fig. 4. All causes of death were considered as events. Cox regression techniques were used to analyze associations with survival; in the adjusted analysis, time to progression was included as an unordered categorical variable (<6 months, 6–12 months, >12 months), and age was included as a continuous variable.

RESULTS

***hMLH1* Methylation in Plasma DNA.** We have examined CpG island methylation changes of the *hMLH1* gene in

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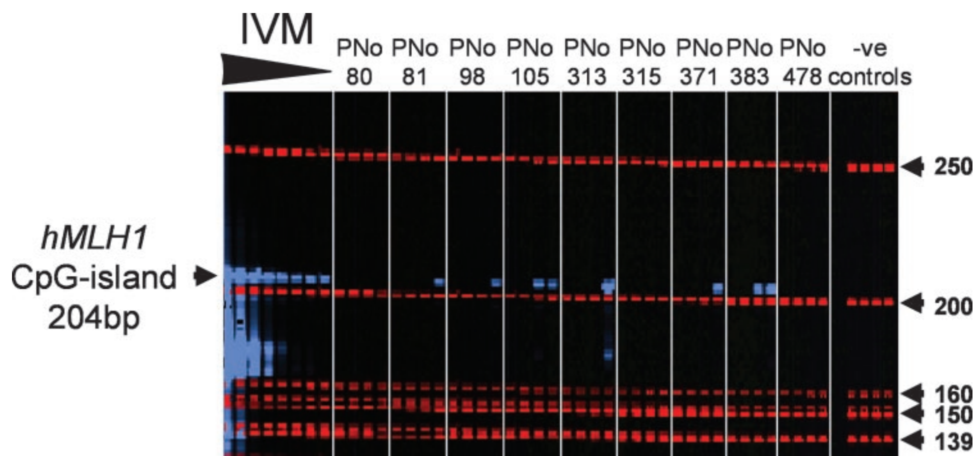

Fig. 1 Fluorescent methylation-specific PCR (MSP) of *hMLH1* CpG island. The *hMLH1* CpG island MSP product (204 bp) is shown in *blue*. The *first eight lanes* show dilutions of *in vitro* methylated (*IVM*) human DNA into peripheral blood mononuclear cell (PBMC) DNA (dilutions of 1:1, 1:5, 1:10, 1:20, 1:50, 1:100, 1:200, and 1:500). The last four lanes show negative controls of 100 and 500 ng of PBMC DNA and two water controls. The MSP products of SCOTROC1 patient number (*PNo*) samples are shown in groups of four and are, in order, DNA from PBMCs prechemotherapy, PBMCs at relapse, plasma prechemotherapy, and plasma at relapse. The sizes (in bp) of the markers (*red*) are shown.

plasma DNA from patients at presentation and at relapse from the SCOTROC1 Phase III clinical trial, a randomized, prospective comparison of paclitaxel-carboplatin *versus* docetaxel-carboplatin as first-line chemotherapy in stage Ic–IV epithelial ovarian cancer. The results of this clinical study have not yet been published, but preliminary analysis has been presented, and at that time, similar response rates, progression-free survival, and overall survival rates were observed for both treatment arms (19). Examining *hMLH1* methylation and MSI in plasma DNA were additional objectives of the trial. Of the first 480 patients enrolled on the SCOTROC1 trial, 351 had relapsed at the time of analysis, and matched lymphocyte and plasma samples before chemotherapy and at relapse, with informed patient consent, were received from 149 of these patients. Samples were removed from analysis if it became clear that the prechemotherapy and relapse samples did not match (*e.g.,* showed divergent microsatellite alleles in PBMC DNA) or that insufficient DNA was recovered; reliable results were available for 138 samples prechemotherapy and 144 samples at relapse (138 paired samples).

We have used MSP with fluorescently labeled primers to detect methylation of the *hMLH1* CpG island (Fig. 1). We have shown previously in ovarian tumor samples that methylation of this CpG island is associated with reduced expression of MLH1 (17) and that reversal of methylation of the CpG island in ovarian cell lines by DNA methyltransferase inhibitors leads to MLH1 reexpression (2). The fluorescent MSP assay is very sensitive, detecting down to a 1:500 dilution of *in vitro* methylated human DNA diluted into human lymphocyte DNA. As shown in Fig. 1, methylation of *hMLH1* is generally not detected in DNA from PBMCs from matched patients before chemotherapy or at relapse using the same DNA concentrations and PCR conditions as used for the plasma DNA. Examples of plasma DNAs positive for *hMLH1* CpG island methylation at prechemotherapy and at relapse are shown for patients 105 and 383, whereas patients 81, 98, 313, and 371 show evidence of *hMLH1* methylation only in the relapse plasma sample.

As shown in Table 1, the proportion of samples positive for *hMLH1* methylation increases from 12% (16 of 138) of plasma DNA samples before chemotherapy to 33% (45 of 138) at relapse, a significant ($P < 0.001$) increase in *hMLH1* methylation. Twenty five percent (34 of 138) showed *hMLH1* methylation in the relapse sample but not in the prechemotherapy sample and hence evidence of acquisition of *hMLH1* methylation after chemotherapy, consistent with selection for loss of DNA MMR and hence loss of MMR-dependent apoptotic responses. Although 11 of 16 plasma samples with methylated MLH1 prechemotherapy retain methylation at relapse, 5 show apparent loss of methylation. This is most likely to be due to a proportion of patients not having detectable tumor DNA in plasma at relapse and is consistent with the 73% sensitivity of detecting the same genetic change in plasma DNA as present in

Table 1 Plasma DNAs with *hMLH1* methylation and microsatellite instability phenotype

	hMLH1 methylation		Microsatellite phenotype		
	Negative	Positive	MS-S[a]	MSI-L	MSI-H
Pre-chemo	122(88%)	16(12%)	72(84%)	12(14%)	2(2%)
Relapse	93(67%)	45(33%)	48(56%)	27(31%)	11(13%)

[a] MS-S, microsatellite stable; MSI-L, low microsatellite instability (a shift in one locus of at least five loci examined); MSI-H, high microsatellite instability (a shift in more than one locus); Pre-chemo, before chemotherapy.

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the tumor reported previously for ovarian cancer at presentation (8). The observation that three of the relapse plasma samples that had apparently lost MLH1 methylation had very low DNA yields (<100 ng/ml plasma) further supports this interpretation.

Because surgical intervention at the time of the relapse is rare, we are unable to compare the changes we observe in plasma DNA with relapse tumor DNA. We have been able to obtain fixed tumor samples taken at presentation for 12 of 138 of these patients. Unfortunately, only two of these show *hMLH1* methylation in plasma DNA, but we do detect *hMLH1* methylation in DNA isolated from the fixed tumor material from these two patients (data not shown). As an alternative approach to validating the detection of methylated *hMLH1* in plasma, we have examined whether there is an association between *hMLH1* methylation and MSI in plasma DNA.

Association between MSI and *hMLH1* Methylation in Plasma DNA. Loss of MMR due to *hMLH1* methylation is associated with a mutator phenotype and acquisition of MSI (20). As a validation of loss of *hMLH1* expression and MMR function in these patient samples, we have examined MSI in plasma DNA from the same patients. DNA was isolated from 96 matched plasma samples from the same patients prechemotherapy or at relapse, as well as from PBMCs, and examined for allelic shifts at six microsatellite loci. Ten prechemotherapy and eight relapse samples were unsuitable for MSI analysis because <5 loci gave sufficient PCR product in one of the plasma DNA samples (86 paired samples were available for analysis). To reduce the probability of false positives, 30 PCR cycles were used for all samples, and products of <30 fluorescent units were excluded from the analysis. Allelic shifts were defined as the appearance of a new allele that is altered in size compared with the PBMC alleles by at least 2 bases (this cutoff was used to avoid confusion with so-called PCR stutter fragments produced during the PCR reaction). Examples of allelic shifts are shown in Fig. 2. MSI was defined as either a shift in one locus of at least five loci examined [low MSI (MSI-L)] or a shift in more than one locus [high MSI (MSI-H)]. For the purposes of the present analysis, if no allelic shift was detected in at least five loci, the sample was defined as microsatellite stable (MS-S).

Evidence of MSI, as defined by MSI-H, is observed in

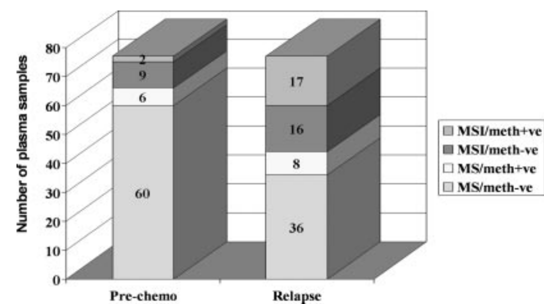

Fig. 3 *hMLH1* methylation and/or microsatellite instability (MSI) phenotype distribution of plasma DNA. Microsatellite-stable (MS-S) samples are those with no allelic shifts in at least five microsatellite loci examined. MSI includes MSI-L and MSI-H. *meth+ve,* samples positive for *hMLH1* methylation by fluorescent methylation-specific PCR; *meth-ve*, samples negative for *hMLH1* methylation by fluorescent methylation-specific PCR.

plasma DNA from 2% of patients at presentation, but this increases to 13% at relapse (Table 1). Similarly, MSI-L in plasma increases from 14% in patients at presentation to 31% at relapse. The increase in MSI observed in relapse samples compared with prechemotherapy samples is statistically significant ($P < 0.001$). The increase in MSI we observe after chemotherapy is consistent with previous observations made in small retrospective studies of ovarian cancer that there is increased MSI in residual tumor cells surviving chemotherapy (21).

For 77 of the patient samples, we were able to obtain both MSI and *hMLH1* methylation status in matched samples before chemotherapy and at progression. The proportion of samples with each phenotype is shown in Fig. 3. The percentage of relapse samples that have *hMLH1* methylation has a statistically significant association with MSI-positive status ($P = 0.001$). The proportion of plasma DNA that has methylation of *hMLH1* and is MSI positive (MSI-H and MSI-L) increases from 3% (2 of 77) prechemotherapy to 22% (17 of 77) at relapse. The proportion of MSI-positive DNAs that do not show evidence of *hMLH1* methylation also increases from 12% (9 of 77) prechemotherapy to 21% (16 of 77) at

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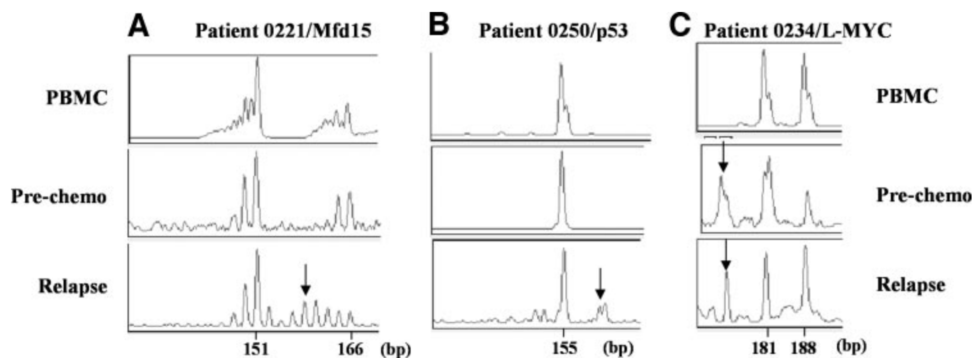
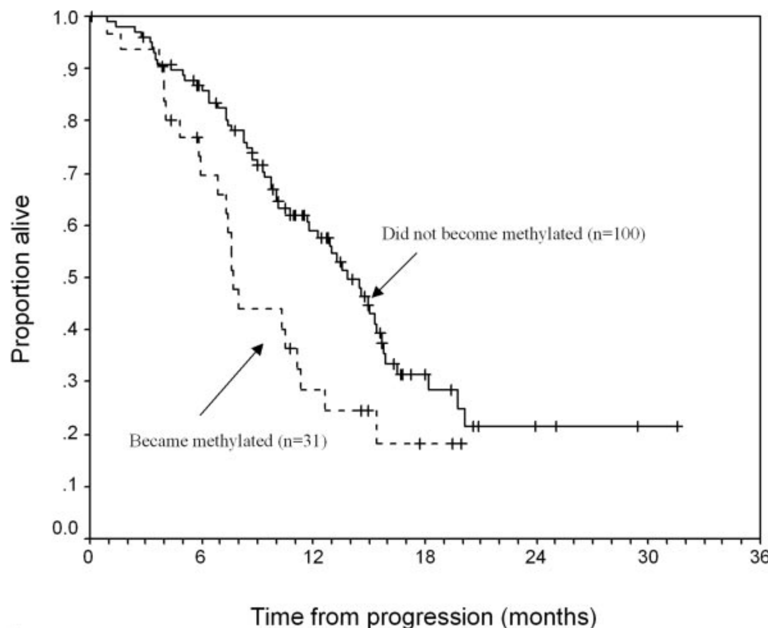


Fig. 2 Examples of allelic shift in plasma DNA. Allele sizes of PCR products at the loci shown were determined in DNA isolated from peripheral blood mononuclear cells (PBMCs), pretreatment plasma (*Pre-chemo*), and relapse plasma (*Relapse*). The sizes (in bp) of the major alleles detected in PBMCs are shown. *Arrows* indicate the presence of new alleles detected in plasma DNA that are not present in PBMC DNA. The microsatellite analyses shown are for (*A*) Mfd15, (*B*) p53, and (*C*) L-Myc.

relapse. Although *hMLH1* methylation is the main mechanism of loss of MMR in sporadic tumors, a proportion of tumors can acquire a MSI phenotype due to alterations in MMR genes other than *hMLH1* methylation (22). A number of plasma DNA samples show methylation of *hMLH1* but no evidence of MSI. It should be noted that only six loci were examined for allelic shifts, and this may be too few to identify instability. However, it is also possible that methylation of *hMLH1* does not cause detectable MSI if only one allele is methylated or inactivation of MMR does not cause a detectable MSI phenotype (for example, inactivation of MMR in cells will only give a detectable MSI phenotype if clonal growth occurs). We have observed ovarian tumors with methylated *hMLH1* that also do not show a detectable MSI phenotype (data not shown). It is possible that methylation of *hMLH1* could reduce MLH1 expression without abolishing MMR activity, and in this context, it is worth noting that reduced expression of MMR proteins can affect drug sensitivity without affecting MSI (23).

Acquired *hMLH1* Methylation and Patient Survival. We have shown that increased *hMLH1* methylation, which correlates with acquisition of a MSI phenotype, is observed in plasma samples at relapse. Next we asked whether this acquired *hMLH1* methylation was associated with patient survival. All laboratory analyses were conducted blinded to clinical outcome. Data from 131 patients were suitable for survival analysis, and of these patients, 78 had died, giving the methylation study 74% power to detect a hazard ratio of 2 for the effect of acquired methylation on survival time from progression. A Kaplan-Meier survival curve of patients whose plasma sample acquires *hMLH1* methylation and those that have not is shown in Fig. 4. Acquisition of *hMLH1* CpG island methylation significantly correlates with poor survival after progression for these epithelial ovarian cancer patients (hazard ratio, 1.83; $P = 0.017$; $n = 131$). Time to progression after primary chemotherapy can influence response to subsequent chemotherapy (24). Indeed, there is a slightly higher proportion of patients who acquire *hMLH1* methylation with a treatment-free interval of <6 months (45% *versus* 39%) and a lower proportion of patients who acquire *hMLH1* methylation with a treatment-free interval of >12 months (16% *versus* 24%) in comparison with patients who do not acquire *hMLH1* methylation. Patient age has also been suggested to influence the propensity for tumors to become methylated (25). However, using multiple Cox regression analysis, the association of acquired *hMLH1* methylation with survival was independent of age and time to progression (hazard ratio, 1.99; $P = 0.007$; $n = 131$).

Any link between acquisition of methylation and histopathological subtype of ovarian cancer could influence these clinical data. The majority of tumors were either serous cystandenocarcinoma or papillary (adeno)carcinoma [38% (50 of 131) and 18% (24 of 131), respectively]. We have not examined each subtype individually because such an analysis would result in small subgroup analysis and greatly weaken the power of the statistical analysis. However, there were 11 clear cell carcinomas and 2 mucinous cystadenocarcinomas, subtypes that have been reported to be associated with particularly poor prognosis in ovarian cancer (26, 27). After adjusting the results of the Cox regression analysis to stratify for these subtypes, a significant association of acquired *hMLH1* methylation with survival was still seen, suggesting that the effect on survival is independent of these ovarian tumor subtypes.



Number At risk:-		0	6	12	18	24	30	36
	Did not become methylated	100	81	41	11	3	1	0
	Became methylated	31	19	7	2	0	0	0

Fig. 4 Kaplan-Meier curves of survival after progression/relapse.

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DISCUSSION

The increased *hMLH1* methylation we observe in plasma samples at relapse after carboplatin/taxoid chemotherapy of epithelial ovarian cancer patients is consistent with *in vitro* observations in ovarian cell line models that cisplatin and carboplatin select for loss of an apoptotic response and acquisition of drug resistance, which is associated with loss of expression of MMR proteins and methylation of *hMLH1* (2, 11). Expression of MLH1 prechemotherapy in ovarian cancer does not appear to be associated with survival (28), although previous small studies have demonstrated acquisition of *hMLH1* methylation and loss of MLH1 protein expression after chemotherapy in ovarian cancer (2). The association of acquired *hMLH1* methylation with patient survival we observe is also consistent with a small retrospective study in breast cancer that associated reduced expression of MLH1 protein in matched patient samples taken after neoadjuvant chemotherapy, but not before chemotherapy, with survival (29). It has been argued that because MMR proteins can recognize and bind to platinum cross-links in DNA, this is necessary for MMR-dependent engagement of DNA damage responses such as activation of p53, p73, and other downstream apoptosis signaling pathways (30, 31). Hence, loss of MLH1 expression may lead to reduced engagement of apoptosis either due to reduced cycles of futile repair (32) or reduced stalling (or increased bypass) of lesions in DNA during DNA replication (12).

It is possible that other genes potentially involved in drug sensitivity may also become methylated at relapse. We have shown previously in advanced ovarian cancer that many hundreds of genes can become aberrantly methylated (33). Thus far, we have not examined methylation of other genes in the plasma DNA samples because examination of MMR status by *hMLH1* methylation and MSI was the stated prospective objective, and the study was powered accordingly for statistical analysis. Nevertheless, concordant methylation of genes in ovarian cancer has been observed previously (17), and future analysis of acquired methylation of genes implicated in drug resistance and MMR-dependent apoptosis will be of interest.

The data presented help to validate methylation of *hMLH1* and loss of DNA MMR as clinically relevant mechanisms of acquired drug resistance in epithelial ovarian cancer. A variety of novel epigenetic therapies capable of reversing epigenetic transcriptional silencing are currently undergoing clinical trial, both alone and in combination with cytotoxic therapies. A concern that has been raised regarding such epigenetic therapies is that they may confer resistance to cytotoxic agents such as carboplatin in some tumors (14). However, it should be noted that data presented thus far show sensitization or no effect induced by epigenetic therapies such as DNA methyltransferase inhibitors on ovarian tumor cells *in vitro* or *in vivo*, rather than induced resistance (13). Nevertheless, the data presented here open the possibility of using a relatively noninvasive blood test to stratify and identify those patients who relapse after standard first-line chemotherapy who may benefit most from novel epigenetic therapies alone or in combination with conventional chemotherapy.

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