Association of somatic DNA methylation variability with progression-free survival and toxicity in ovarian cancer patients

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Background: We have addressed whether inter-individual methylation variation in somatic (white blood cells, WBCs) DNA of ovarian cancer patients provides potential for prognostic and/or pharmacoepigenetic stratification.

Patients and methods: WBC DNA methylation was analysed by bisulphite pyrosequencing at ataxia telangiectasia mutated (ATM), estrogen receptor 1 (ESR1), progesterone receptor (PGR), mutl homologue 1 (MLH1), breast cancer susceptibility gene (BRCA1), secreted frizzled-related protein 1 (SFRP1), stratatin (SFN), retinoic acid receptor beta (RARB) loci and the repetitive element LINE1 in 880 SCOTROC1 trial patients [paclitaxel (Taxol)–carboplatin versus docetaxel (Taxotere)–carboplatin as primary chemotherapy for stage Ic–IV epithelial ovarian cancer].

Results: We observed no significant associations (P < 0.005, after correction for multiple testing) for progression-free survival (PFS) using test and validation sets. However, we did identify mean SFN methylation associated with PFS (hazard ratio, HR = 1.01 per 1% increase in methylation, q = 0.028); particularly in the paclitaxel (HR = 1.01, q = 0.006), but not in the docetaxel arm in stratified analyses. Furthermore, higher methylation within the ESR1 gene was associated with CA125 response (odds ratio, OR = 1.06, q = 0.04) and with neuropathy (HR = 0.95, q = 0.002), but only in the paclitaxel arm of the trial.

Conclusions: This is the first study linking DNA methylation variability in WBC to clinical outcomes for any tumour type; the data generated on novel prognostic and pharmacoepigenetic DNA methylation biomarkers in the circulation now need independent further evaluation.

Key words: clinical trial, DNA methylation, ovarian cancer, pharmacoepigenetics, prognosis, toxicity
introduction

Ovarian cancer is the most lethal gynaecological cancer, having a 5-year survival rate of only 36% in the UK [1]. The most widely used primary treatment is cytoreductive surgery and platinum/taxane chemotherapy. Over 80% of patients respond to primary chemotherapy; however, the majority relapse within 18 months [2]. The factors which underlie variations in outcome are unclear, but are likely to be both tumour and host-related. What is clear is that the identification of biological markers of response and survival, measurable at presentation, could play a major role in optimising treatment strategies. This becomes increasingly important as more molecularly targeted therapies show activity in ovarian cancer, since these will need evaluation within the context of platinum/taxane chemotherapy of ovarian cancer.

The SCOTROC1 phase III randomised clinical trial compared docetaxel (Taxotere)–carboplatin with paclitaxel (Taxol)–carboplatin in ovarian cancer patients at first presentation [3]. The trial showed no difference in progression-free or overall survival (OS) between treatment arms, but did show greater levels of neurotoxicity in the paclitaxel arm (30% versus 11%) and a higher incidence of high-grade neutropenia in the docetaxel arm (94% versus 84%). Pharmacogenetic studies in the SCOTROC1 cohort have so far identified no significant associations with genotypes in candidate genes [4], and have failed to replicate two genome-wide association studies (GWASs) identified survival-associated SNPs in the phase III replication stage of the GWAS [5].

There is increasing evidence from cancer epidemiological studies that DNA methylation in normal somatic cells such as white blood cells (WBCs) can be informative on environmental carcinogen exposures and cancer risk [6, 7]. We have previously demonstrated that epigenetic variability at candidate loci in peripheral blood DNA may be useful as biomarkers of breast cancer risk, and have replicated the risk association of one marker in the ataxia-telangiectasia mutated (ATM) gene in pre-diagnostic blood samples [8, 9]. In the present study, we hypothesise that epigenetic variation detected in non-cancer somatic cells, white blood cell (WBC) DNA isolated before chemotherapy, might also be useful in predicting clinical outcomes following chemotherapy.

The primary objective of the study was to identify an association between WBC DNA methylation of candidate genes (at presentation) and progression-free survival (PFS) in ovarian cancer patients. Secondary objectives were to identify an association with OS, RECIST response, CA125 response or toxicity (haematological toxicity, GI toxicity and neurotoxicity). Candidate genomic targets were chosen based on the known methylation variability or a potential role in drug sensitivity, including (i) known methylation variable intragenic regions across the ATM gene that had previously been associated with breast cancer risk [8, 9]; (ii) LINE1 repetitive elements as a surrogate for genome-wide methylation levels; (iii) genes known to be hypermethylated in ovarian cancers estrogen receptor 1 (ESR1), progesterone receptor (PGR), breast cancer susceptibility gene 1 (BRCA1), secreted frizzled-related protein 1 (SFRP1), stratifin (SFN), retinoic acid receptor beta (RARB) (http://www.pubmeth.org/) and (iv) genes where methylation in ovarian tumours is known to associate with drug sensitivity and PFS from previous studies, mutL homologue 1 (MLH1) [10, 11]. To our knowledge, this is the first such study to systematically investigate candidate gene DNA methylation biomarkers in WBC DNA taken at presentation for any cancer type.

materials and methods

patient samples

Peripheral blood DNA was available from whole blood from 880 of the 1077 patients enrolled in the SCOTROC1 clinical trial, which has been described in more detail elsewhere [3, 4]. Patient characteristics of samples analysed are summarized in Supplementary Table S1, available at Annals of Oncology online. All patients gave written informed consent for blood samples to be collected, and appropriate ethical review boards approved the study. All laboratory analyses were conducted blinded to clinical outcomes, and the statistical analysis plan was prospectively designed following REMARK recommendations [12]. For completeness of reporting, all data are presented in Supplementary Table 3, available at Annals of Oncology online.

laboratory methods

DNA extracted from whole blood were analysed by bisulphite DNA pyrosequencing using primers described in Supplementary Table 2, available at Annals of Oncology online. Any sample failing quality control was removed from the analysis as listed in Supplementary Table 4, available at Annals of Oncology online. Full details of the methods are given in Supplementary Methods, available at Annals of Oncology online.

statistical analysis

Following a statistical power analysis to determine the number of samples required for test and validation sets unadjusted univariate models were first run. The methylation markers were initially run in the models as continuous values, but for methylation markers with markedly skewed distributions (i.e. SFRP1, both regions of the ATM markers, MLH1, SFN and the second region assayed of ESR1), cubic transformation was used to help improve model fit. Logistic regression models were used to investigate the association between each of the markers and CA125 response, response to therapy (progressive disease versus stable disease) or complete or partial response using RECIST criteria [13], serious haematological toxicity, GI toxicity (three or four grade GI toxicity versus zero to two), and neurotoxicity (two to four grade neurotoxicity versus zero or one). All multivariable cox and logistic models were adjusted for figo stage, age, bulk of residual disease, ECOG performance status, histological type and platelets to ensure independence from these known prognostic factors. Multiple testing was accounted for using Benjamini Hochberg correction. Data were analyzed using R, version 2.10. Further details of statistical analysis are reported in Supplementary Methods, available at Annals of Oncology online.

results

association with PFS

The patients and study design were described in Supplementary Figure S1, available at Annals of Oncology online. The 11 loci tested included four promoter CpG islands (CGI) (BRCA1, PGR, RARB and MLH1), and were typically unmethylated with rare examples of individuals who were methylated at these promoters (Figure 1). We also assayed six intragenic methylation (IGM) regions, which were highly methylated and typically more variable than CpG islands (two regions of the
ESR1 and ATM genes each, SFRP1 and SFN [14]. Finally, we also assayed for one repetitive element, LINE1, representing genome-wide methylation.

Using mean methylation of the CpG sites, no significant associations with PFS in both test and validation analyses were found for any locus using the multiple correction P value cut-offs (P < 0.005). We did observed four loci that were nominally significant (P < 0.05) in the test set, but these were not significant after correction for multiple testing and were not significant in the validation set (Table 1). However, a single CpG site at SFN identified as nominally significant in the test set retained nominal significance (P = 0.026) with a false discovery rate of q = 0.104 in the validation set.

A CpG site in SFN associated with PFS and neurotoxicity
Mean methylation at the stratfin gene (SFN) locus was associated with PFS (HR = 1.03 for every 3% increase in methylation, q = 0.028) in the full dataset (Table 2). Using a dichotomous categorical quantile split on the combined dataset (n = 712), using the mean SFN methylation, we observed that the three highest quantiles (n = 534) of methylation had significantly poorer PFS compared with the lowest quantile (n = 178) [HR = 1.3 (1.1–1.6), P value = 0.01, log-rank test P = 0.04] resulting in a mean PFS time of 18 months in the lowest quantile of methylation compared with 14 months for the remaining individuals. Interestingly, a single CpG site in SFN was associated with PFS [HR = 1.01 (1.00, 1.03), P = 0.016] and with high-grade (2/3/4) neuropathy [OR = 1.01 (1.00–1.04), P = 0.029]. In the SCOTROC1 trial, significantly higher neuropathy was observed in the paclitaxel compared with the docetaxel arm [3]. Interestingly, the association between SFN methylation and PFS was only significant in the paclitaxel arm (q = 0.006) and not the docetaxel arm (q = 0.732), with a trend for association (q = 0.07) with high-grade neuropathy in the paclitaxel arm (Table 2).

methylation at ESR1 associates with response to chemotherapy and neuropathy in the paclitaxel arm
A significant association was observed for one region of ESR1 with CA125 response and high-grade neuropathy. Using a categorical split (above and below the median), higher methylation of ESR1p3a was associated with a 2-fold increase in CA125 response [OR = 2.1 (1.4–3.1), P = 0.0004]. Of note, we also observed an interaction between ESR1p3a mean methylation and high-grade neuropathy, again with a stronger association in the paclitaxel arm of the trial [OR = 0.95 for each 1% increase in methylation (0.93–0.98), q = 0.002, p interaction between study arm and ESR1p3a mean methylation P = 0.006] which was not significant in the docetaxel arm of the trial (Table 2).

discussion
It is clear that epigenetic alterations, detected in tumour DNA, play an important role in carcinogenesis and drug resistance in many tumour types, including ovarian cancer [15]. For instance, variation in tumour DNA methylation at CpG islands associated with the promoter of WNT pathway genes is independently associated with PFS and response to chemotherapy in high-grade serous ovarian cancer [16]. Changes in DNA methylation in tumour DNA has also been observed during the acquisition of drug resistance in ovarian cancer [10, 11]. Thus, there is substantial evidence for DNA methylation and epigenetic regulation in tumours influencing sensitivity to platinum-based chemotherapy and patient survival.
in ovarian cancer. What is not yet clear is whether the normal epigenetic diversity in the population, or epigenetic variation in the normal cell DNA of individuals, plays a role in patients’ response to therapy. Recent studies have shown that DNA methylation in WBCs can be informative about environmental carcinogen exposures and cancer risk [6, 7], although potential prognostic or pharmacoepigenetic relevance of DNA methylation has not been examined. To this end, we have carried out a pharmacoepigenetic study to systematically investigate candidate gene DNA methylation biomarkers in WBC DNA taken at presentation from a phase III ovarian cancer clinical trial, SCOTROC1 [3].

In the SCOTROC1 trial, significantly higher neuropathy was observed in the paclitaxel compared with the docetaxel arm, while the docetaxel arm had higher haematological toxicity [3]. Interestingly, the association between SFN methylation and PFS we observe was only significant in the paclitaxel arm and not in the docetaxel arm. Neurotoxicity was significantly associated with taxane dose reductions during the SCOTROC1 trial (OR = 3.47, 95% CI = 1.99–6.14, P < 0.001). This raises the speculation that the associations with PFS and neuropathy may be linked and could be a consequence of a systemic effect of methylation on pharmacological response to chemotherapy in these patients rather than tumour response per se. We also observe an interaction between ESR1p3a mean methylation and high-grade neuropathy, with a stronger association in the paclitaxel arm of the trial.

Stratfin (SFN, 14-3-3 sigma) is a member of the 14-3-3 family that interacts with p53, to initiate cell cycle checkpoints after DNA damage [17]. In a small study of neuroblastomas [n = 47 (test); n = 58 (validation)], hypermethylation of the SFN promoter in tumour DNA was associated with poorer progression-free and OS [18]. However, in our study we have not examined methylation of the promoter region, but rather an intragenic CpG site previously demonstrated to show DNA methylation variability between individuals [8]. That a single CpG site can be important for patient survival was recently demonstrated in chronic lymphocytic leukaemia [19].

A link between estrogen and ovarian cancer risk has long been suspected and recent, large case–control studies have shown an increased risk of ovarian cancer mortality associated with HRT use [20]. The mechanism of this increased mortality is not known; however, ESR1 expression in tumours is lower in patients with poorer survival [21] and CA125 response [22]. High levels of estrogen inhibit paclitaxel-induced apoptosis in cancer cell lines and inhibits paclitaxel efficacy in mouse xenograft ER-positive tumours [23, 24]. We hypothesise, therefore, that high levels of ESR1 methylation observed in blood DNA may be protective against paclitaxel related neurotoxicity due to higher ESR1 expression and more interference with paclitaxel-induced apoptosis.

The limitations of this study include the small effect sizes observed when methylation is analysed as a continuous measure. However, these must be interpreted within their context: hazard ratios are represented per 1% increase in methylation or per 1 standard deviation and so may appear to be small but may still represent large increases in risk. It is also important to note that the distribution of methylation for markers (presented in Figure 1) shows that most of the markers

<table>
<thead>
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<th>Gene</th>
<th>Outcome</th>
<th>CG</th>
<th>Site</th>
<th>SD Transform</th>
<th>n</th>
<th>HR</th>
<th>95% CI</th>
<th>P</th>
<th>q</th>
<th>HR</th>
<th>95% CI</th>
<th>P</th>
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<tbody>
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<td>SFN</td>
<td>PFS</td>
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<td>1.0</td>
<td>1.09</td>
<td>420</td>
<td>1.13</td>
<td>1.12 (1.02, 1.23)</td>
<td>0.018</td>
<td>0.018</td>
<td>0.425</td>
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Survival models were run using cox proportional hazard models and logistic models were run using generalized linear models specifying the binomial family. All models were adjusted for age, stage, bulk of residual disease, ECOG performance status, histological type and plate.

Table 1. Primary analysis for progression-free survival (PFS) in test, validation and full dataset.
we have investigated have methylation ranges of 20%–30% between individuals which does have a significant impact on gene expression as we have shown for the ATM markers [8]. A further limitation is that epithelial ovarian cancer is now considered to consist of at least five different subtypes of ovarian cancer, with different molecular pathology and clinical outcomes [23]. SCOTROC1 included all histological subtypes of epithelial ovarian cancer, and the methylation differences we observe could reflect association with particular subtypes with distinct clinical outcomes. We avoided further subgroup analysis based on the histological subtype as this would further reduce the statistical power of the analysis. However, the analyses presented were adjusted for histological type (serous versus non-serous), and it is unlikely that the toxicity associations would be due to histological subtype of ovarian cancer. Since this study was done within patients selected for eligibility for recruitment to the SCOTROC1 clinical trial it is possible that there is restricted generalisability to a non-selected ovarian cancer population.

Whole blood DNA is a mixture of numerous different cell types that may have differential methylation at the candidate gene loci assessed in this study. However, we have previously shown that the intragenic ATM markers tested here are not differentially methylated in B cells, T cells or monocytes [8], and recent data of blood cell fractions interrogated on the 27K Illumina showed no differential methylation for the gene loci represented on the array (BRCA1, MLH1, RARB and PGR) [26]. Furthermore, there was no significant correlation between blood counts at the time of methylation analysis (WBC, haemoglobin, neutrophils and platelets) and methylation of SFN, ESRI and RARB (data not shown).

In conclusion, we have shown for the first time that analysis of DNA methylation of WBCs may identify markers of clinical outcomes and toxicity in ovarian cancer patients. DNA methylation status associated with markers of toxicity, which if validated in further independent clinical studies, may identify those patients at risk of toxicity to paclitaxel or docetaxel. Such markers could be considered for patient stratification in future trials involving paclitaxel–carboplatin, which would enrich for patients having the best prospects for longer PFS and least toxicity.

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disclosure

The authors have declared no conflicts of interest.

references


