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Platinum-based chemotherapy induces methylation changes in blood DNA associated with overall survival in ovarian cancer patients

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Abstract

Purpose: DNA damage repair can lead to epigenetic changes. DNA mismatch repair proteins bind to platinum DNA adducts and at sites of DNA damage can recruit the DNA methylating enzyme DNMT1, resulting in aberrant methylation. We hypothesised that DNA damage repair during platinum-based chemotherapy may cause aberrant DNA methylation in normal tissues of patients such as blood.

Experimental Design: We used Illumina 450k methylation arrays and bisulphite pyrosequencing to investigate methylation at presentation and relapse in blood DNA from ovarian cancer patients enrolled in the SCOTROC1 trial (n=247) and in a cohort of ovarian tumour DNA samples collected at first relapse (n=46). We used an ovarian cancer cell line model to investigate the role of the DNA mismatch repair gene *MLH1* in platinum induced methylation changes.

Results: Specific CpG methylation changes in blood at relapse are observed following platinum-based chemotherapy and are associated with patient survival, independent of other clinical factors (HR=3.7; 95%CI 1.8-7.6, $p=2.8x10^{-4}$). Similar changes occur in ovarian tumours at relapse, also associate with patient survival (HR=2.6; 95%CI 1.0-6.8, p=0.048). Using an ovarian cancer cell line model, we demonstrate that functional mismatch repair (MMR) increases the frequency of platinum-induced methylation.

Conclusion: DNA methylation in blood at relapse following chemotherapy, and not at presentation, is informative about ovarian cancer patient survival. Functional DNA mismatch repair increases the frequency of DNA methylation changes induced by platinum. DNA methylation in blood following chemotherapy could provide a non-invasive means of monitoring patients' epigenetic responses to treatment without requiring a tumour biopsy.

Translational Relevance

Platinum-based chemotherapy is the cornerstone of treatment for a wide variety of cancers. Greater than 80% of ovarian cancer patients respond to platinum-based chemotherapy at first line treatment and is therefore standard of care. However, over 50% of women will not respond to platinum-based chemotherapy at second-line treatment. There are a range of other treatment options at second-line, mainly guided by patient fitness and the platinum free interval. However, there are no biomarkers that guide this choice. We show for the first time that novel DNA methylation biomarkers, measured in blood DNA at the time of first relapse following platinum-based chemotherapy, are prognostic for overall survival of ovarian cancer patients. This opens the potential of a relatively non-invasive prognostic test that could help guide second line treatment options for ovarian cancer patients.

Introduction

Platinum-based chemotherapy remains the cornerstone of treatment for a wide variety of malignancies, including ovarian cancer. However, platinum resistance leads to treatment failure, which can be due to pleiotropic changes in gene expression (1). Epigenetic changes, such as CpG DNA methylation at gene promoters, are one mechanism underlying pleiotropic expression changes. However, the processes leading to aberrant DNA methylation are poorly understood. Recent evidence has suggested that mismatch repair proteins at sites of DNA damage including double strand breaks can recruit the DNA methylating enzyme DNMT1 and result in aberrant methylation (2-5). DNA mismatch repair proteins recognize and bind to sites of platinum-induced DNA damage. Furthermore, numerous DNA damage causing environmental exposures, such as smoking and alcohol, have been reported to alter DNA methylation in blood DNA (6, 7), inducing mainly hypomethylation at methylated intragenic CpG sites. Therefore, we hypothesized that during platinum-based chemotherapy, DNA damage and/or repair causes aberrant hypo- or hyper- DNA methylation, in surrogate as well as tumour tissue which may inform about patient survival.

Chemotherapy-induced DNA methylation changes in blood cells or tumour could have important clinical implications. It is increasingly recognized that DNA methylation changes in blood and tumour cells can influence immune responses (8-10). Thus, systemic changes in patients' immune response induced by chemotherapy may be mediated by epigenetic regulation and influence patient outcomes. Furthermore, if similar DNA methylation changes are induced in tumour as observed in blood, this will create epigenetic diversity in the tumour which can be selected for during further tumour evolution. Indeed, if DNA methylation changes in blood, as a surrogate for tumour changes, is a marker of patient prognosis, this would provide a means to monitor prognosis following chemotherapy and provide a relatively non-invasive test that could help guide second line treatment options for ovarian cancer patients.

Methods and Materials

Clinical Samples

Peripheral blood DNA was available from whole blood from 247 of the 1077 patients enrolled in the SCOTROC1 phase III clinical trial (docetaxel-carboplatin versus paclitaxel-carboplatin for ovarian cancer patients at first presentation) (11, 12). These samples were used in several sub-cohorts including (1) at presentation for array-based discovery (n=107), (2) at relapse for array-based discovery (n=54 matched to 54 presentation blood samples), (3) independent subject blood samples at relapse for survival class pyrosequencing validation (n=89), and (4) for unmatched presentation versus relapse pyrosequencing validation (n=90, 45 independent presentation and 39/45 relapse blood samples overlapping with (3)). A diagram of the overall analysis strategy used is shown in Supplementary Fig.S1. The discovery and first validation study using Infinium 450K arrays were pre-planned, while the second bisulphite pyrosequencing validation was post-hoc. After the initial discovery stage where patients were selected based on progression-free survival, blood samples at relapse were included based solely on availability of sample with no pre-selection. Tumour DNA samples were available from matched presentation and relapse HGSOC tumour DNA samples from 46 patients enrolled in the OCTIPS study (http://www.octips.eu/), described elsewhere(13). All patients gave written informed consent for samples to be collected, and appropriate ethical review boards approved the study. All clinical data used in the study are presented in Supplementary Table S1.

Cell lines

Cell lines A2780, A2780/CP70, CP70/MLH1+ and CP70/MLH1- were obtained from internal lab stocks (14). Cell lines CP70/MLH1+ and CP70/MLH1- will be referred to herein as MLH1+ and MLH1- respectively. All cells were maintained in RPMI media supplemented with 10 % (v/v) foetal calf serum (Sigma), 1 % (w/v) penicillin-streptomycin (Sigma) and 2 mM L-Glutamine (Sigma) and were grown at 37 °C in a humidified 5 % CO_2 atmosphere. Cells consistently tested

negative for mycoplasma infection using the MycoAlert® kit (Lonza). All cell lines were verified using STR based profiling (Genetica DNA Laboratories Inc.).

Western blotting

Western blotting was conducted as previously (15). Antibodies used included MLH1 (Abcam, ab14206), β-actin (Abcam, ab1791) and goat anti-mouse IgG HRP secondary antibody (Santa Cruz Biotechnology, sc-2005).

Cell viability assay

Cisplatin concentration-response curves were determined using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), as used previously (15, 16). Briefly, cells were treated with cisplatin in triplicate at the following concentrations: 0 μM, 5 μM, 10 μM, 20 μM, 30 μM, 40 μM, 50 μM, 60 μM, 70 μM, 80 μM, 90 μM and 100 μM. Percentage cell survival was measured in comparison to untreated control cells.

Cisplatin clonogenic assay and isolation of clones

Cells were seeded at 10^4 cells per well of a 96 well plate in 150 μl growth medium 24 hr prior to cisplatin treatment. Cells at 70-90 % confluence were then treated with cisplatin at 5 μM for 72 hr. Following growth for 18 days, individual colonies were isolated and grown for further 4 weeks prior to DNA, RNA extraction and storage in liquid nitrogen. This treatment was conducted in three biological replicates with approximately 8-9 clones grown from each of three independent treatments (n=25 clones per treatment group).

DNA extraction and bisulphite conversion

DNA samples for clinical blood samples and cell lines were extracted using Qiagen DNA blood Mini Kits and for cell lines we used Qiagen QIA Amp® DNA mini kit. DNA samples from the

OCTIPS cohort of 46 primary and relapse tumours were extracted as described elsewhere (13). We bisulphite converted 500 ng of genomic DNA using EZ-96 DNA-methylation Gold kit (Zymo research, CA) according to manufacturer's instructions.

Pyrosequencing

Pyrosequencing was conducted as previously (6, 12). Primer sequences are detailed in Supplementary Table S2. Wilcoxon signed rank sum test was used to determine significance between groups.

Methylation profiling

We used Illumina Infinium HumanMethylation450 BeadChips for methylation profiling. For the clinical blood samples we selected matched pairs of presentation and relapse blood samples (n=54 subjects) from subjects with poor progression free survival (median PFS time = 10.8 months) and we selected a further n=54 presentation blood samples with good survival time (median PFS time = 48.5 months). For the cell lines we randomly selected 11 clones from each of the four cell line test groups (mock- and cisplatin-treated MLH1+ and MLH1- cells) for methylation profiling. Hybridisation and scanning was conducted by University College London (UCL) Genomics Microarray and High Throughput Sequencing Facility. Microarray pre-processing, quality control and analysis was conducted as previously described using minfi (7, 17). One sample from the presentation group failed initial QC and was excluded from further analysis, no other samples failed QC. Samples were then normalized using the functional normalization method (18) and beta values and M-values were derived. White blood cell proportions were estimated using the Houseman method (19). Using logistic regression comparing presentation/relapse versus each blood cell type we found only a modest increase in monocyte count at relapse (7.0% up to 9.0% at relapse, FDR q value= 0.06), however, we found no alteration in the significant probe associations when adjusting for monocytes. Following QC this resulted in data matrices in the clinical samples of 485,513

probes and 161 samples and in the cell line data of 446,619 probes and 44 samples. As the presence of multiple NAs in the array data hindered some statistical or computational algorithms we used K-nearest neighbour (knn) "Impute" function to fill NAs with estimated β values for those algorithms. Probes that were identified as cross-hybridising or containing SNPs were not excluded *a priori*, but were used to filter any significantly associated markers selected for validation (20). Cell line methylation profile data is deposited in GEO record (Accession Number GSE79312). However, deposition of genomic data from SCOTROC1 clinical trial subjects was not approved at the time of ethical consent.

Statistical Analyses

All analyses were conducted in R version 3.2.2 unless otherwise stated. To compare between good and poor prognosis groups at presentation we used unpaired Student's T-test using M-values and a univariate cox regression in a bootstrapped (x100) selection of test and validation cohorts (n=53 vs 54). To compare the matched presentation and relapse samples we used a paired Student's T-test using M-values. This identified 333 CpG sites significant at FDR<10% and 83 CpG sites at FDR<1%. To investigate the association between each of the 333 markers and progression free survival (PFS), overall survival (OS) or time from first relapse to death (TTD) cox proportional hazards regression models were used. Age-at-relapse was calculated as the age at presentation plus progression free survival time, and the time to death (TTD) was calculated as the time from first relapse to death. Univariate analysis was conducted initially with methylation as a continuous variable reporting hazard ratios for one percent methylation increase and their corresponding 95% Confidence Intervals and p-values were reported for each specific methylation marker. FDR was used to correct for the 333 tests conducted. All multivariable cox models for TTD were adjusted for FIGO stage (dichotomized into stage 3/4 vs stage 1/2), age (continuous variable), bulk of residual disease (dichotomized into > 2cm vs <2 cm or no macroscopic disease), histological type (dichotomized into serous versus other), time to first relapse (continuous variable) and batch (chip).

Hazard ratios for relapse class (1 vs 2) are reported as increased/decreased risk of death in class 1 as compared with patients in class 2 in univariate or multivariable models after adjustment for age, stage, residual disease, histology and time to first relapse. Stepwise cox regression was used by including all 8 CpG sites in the multivariable model along with age, stage, residual disease, histology and time to first relapse and removing non-significant CpG sites until the remaining sites were independently significantly associated with TTD. Hazard ratios for relapse class (1 vs 2) in the tumour DNA samples from OCTIPS cohort are reported as increased/decreased risk of death in class 1 as compared with patients in class 2 in univariate or multivariable models after adjustment for age, stage and time to first relapse. We did not adjust for histology as they were all serous adenocarcinomas and did not adjust for residual disease as this variable was incomplete and was not a true confounder for overall survival in this OCTIPS data (p=0.296) using a univariate analysis of residual disease in relation to survival.

We used the "ConsensusClusterPlus" package using a data matrix of methylation beta values for the 8 selected probes for each sample and used standard parameters using euclidean distance, up to k=6 groups, and n=100 bootstrapped sampling with 80% resampling in each iteration. Kaplan Meier curves were drawn using "survfit" function in the survival package. Receiver Operating Curve (ROC) curve analysis was conducted using the "pROC" package using a categorical variable for survival of TTD<12 months (dichotomized as 0 or 1) compared with continuous methylation index. The methylation index was derived from the three CpG sites that were independent of each other in the multivariable model. For each subject we calculated the sum of beta coefficients multiplied by the methylation value for the three sites. Area under the curve was calculated along with 95% confidence intervals.

To assess the differences in DNA methylation between test groups in the cell line data (cisplatin treated vs mock-treated), we used two-tailed Student's *t*-tests on M-values. We used thresholds of

$p<0.001$ and absolute methylation difference >20% to select significant probes. We used bisulphite pyrosequencing of five selected CpG sites in the independent clones to validate this threshold. Biases, for example in directional change of methylation was determined using "oddsratio" function in the "epitools" package of R, comparing numbers of significant probes that change in each direction with the numbers of non-significant probes. False Discovery Rate (FDR) was used to correct for multiple testing.

Sequence Composition Analysis

For sequence composition analysis, we used genomic positions for the selected probes pulled from bioconductor to generate bed files using a window 25 nucleotides on either side of the CpGs. Nucleotide and dinucleotide frequencies were calculated from FASTA files using HomerTools v4.7.2 (21) and plotted using GraphPad Prism 6. Dinucleotide enrichment in the probes of interest compared to all CpG probes on the 450K array (i.e. excluding nonCpG and SNP probes) was calculated in R version 3.1.2 (2014-10-31) using the hypergeometric distribution function (phyper()).

Results

DNA methylation in blood DNA from SCOTROC1 patients

To determine whether exposure to platinum-based chemotherapy alters DNA methylation, we investigated blood DNA samples from ovarian cancer patients enrolled in the SCOTROC1 phase III clinical trial (11, 12). A diagram of the overall analysis strategy used is shown in Supplementary Fig.S1. Firstly, we compared CpG methylation in blood DNA at presentation, prior to chemotherapy, with matched samples taken at first relapse following treatment (n=54 pairs) using Illumina 450k methylation arrays. We observed 333 CpG sites with significantly changed DNA methylation at time of relapse compared to at presentation (FDR<10%, Supplementary Fig.S2A). We selected 9 probes (based on significance and magnitude of change) to validate the change in methylation levels observed on the arrays using bisulphite pyrosequencing in an independent set of unmatched presentation (n=45) and relapse (n=45) blood samples and all 9 confirmed the expected methylation levels (Supplementary Fig.S2B).

Using univariate cox regression on the sites that change, we identified 8 CpG sites out of the 333 sites that were significantly associated with survival (TTD, Time from first relapse To Death) (FDR < 10%) (Fig.1A). These 8 markers remained significant after adjusting for age, stage, residual disease, histology and for time to first relapse, a known marker of response to platinum-based chemotherapy at second-line treatment (Table 1). Consensus clustering using methylation of these 8 CpG sites identified two classes of relapse samples: class 1 representing the subjects with lower methylation levels and class 2 with higher methylation (Fig.1B, Supplementary Fig.S3). Class 1 had a poorer survival outcome compared to class 2, with a hazard ratio of HR=3.7 (95%CI 1.8-7.6, $p=2.83x10^{-4}$) in multivariable cox regression adjusting as above (Fig.1C, Table 1). Bisulphite pyrosequencing of all 8 methylation markers in an independent validation cohort (n=87 relapse blood samples) confirmed the observation of poorer survival in class 1 compared with class 2

(adjusted HR=1.83 (1.05- 3.18), p=0.032) (Fig.1D, Supplementary Fig.S4, Supplementary Table S3). These results show for the first time that novel DNA methylation biomarkers, measured in blood DNA at the time of first relapse, can predict the overall survival of platinum-treated ovarian cancer patients and are associated with similar changes occurring in tumour DNA following chemotherapy. Given the limited study sample size and restricted genome coverage, it is likely that further methylation changes at relapse associated with survival can be discovered.

Notably, methylation derived clusters of the 8 CpG sites in blood DNA at presentation neither predicts time to first progression (adjusted HR=0.99 (0.58-1.73), p=0.989, Fig.1E) nor overall survival (adjusted HR 1.28 (0.69-2.36)) (Supplementary Fig.S6). Moreover, using a similar discovery approach as above for the relapse blood samples we did not find any significant associations between DNA methylation and survival in blood samples taken at presentation (Supplementary Figure S1). .

Using stepwise cox regression adjusted for clinical variables, and the 8 markers, we found 3 CpG sites independently contributed to the survival association (Supplementary Table S3). These 3 independent markers (cg05529343, cg07960624, cg25953130) were used to derive a methylation index and predict time to death (<12 months vs >12 months) through receiver operating curve analysis, resulting in area under the curve of 0.8 (95%CI 0.7-0.9) for the array and 0.7 (95%CI 0.6-0.8) for the pyrosequencing sets. The genes associated with each CpG is described in Supplementary Table S4.

DNA methylation in ovarian tumours at presentation and relapse

We next analysed methylation in matched primary and relapse ovarian tumour samples (n=46 pairs), to address whether tumour DNA methylation is prognostic at the same 8 CpG sites identified in blood (Fig. 2). In the relapse tumour DNA samples we again observed similar methylation

clusters that predicted increased survival in the higher methylation class 2 compared with class 1 (Adjusted HR = 2.63 (1.01-6.84), p=0.048) (Fig 2B, Supplementary Fig.S5, Supplementary Table S3) adjusted for age, stage and time to first relapse.

Platinum-induced DNA methylation in ovarian tumour cell lines differing in DNA mismatch repair

Mismatch repair of oxidative damage results in aberrant hypermethylation and DNA mismatch repair proteins recognize and bind to sites of platinum-induced DNA damage (2, 4, 22). The DNA mismatch repair gene MLH1 has a crucial role in engaging cell death following platinum damage (22). Therefore, we next examined whether platinum-induced methylation was dependent on expression of the DNA mismatch repair protein MLH1. We used an MLH1 deficient ovarian cell line in which MLH1 expression has been restored (herein described as MLH1+) and its matched control, in which the MLH1 gene is silenced (MLH1-)(14) (Fig 3A). Cell lines were treated with a sub-lethal, non-selective dose of cisplatin (5μM) for 72 hours or mock-treated. Clonal variation was accounted for by analysing multiple clones grown from each of these four treatment groups (n =25 clones per group) (Supplementary Fig.S7A). Cisplatin sensitivity remained unchanged in individual clones (Supplementary Fig.S8). We used the repetitive element LINE1 as a surrogate for genome-wide methylation and showed a significant increase in methylation (+5%, $P=4.09x10^{-5}$) when the MLH1+ clones were treated with cisplatin, but no change in the clones with MLH1 silenced (Fig.3B). Further analysis using Illumina 450k methylation arrays identified significant changes in methylation at 973 and 199 CpG sites in the MLH1+ and MLH1- clones (n=11 clones per treatment) respectively (Fig.3C-3D), using $p<0.001$ and absolute methylation change >20% as a threshold to determine significant changes. We validated 4/5 of these methylation changes in independent clones (n=14 per group, Supplementary Fig.S9). A 5-fold increase in methylation changes (973/199) was observed in response to cisplatin when MLH1 was expressed with a bias towards hypermethylation upon cisplatin treatment (68%, OR=1.85 (95%CI 1.62-2.12), $p=5.6x10^{-}$

[20]). Lastly, we found enrichment for dinucleotides prone to platinum-adduct formation (22) (CpC and GpG, hypergeometric distribution $p<0.001$ and $p<0.05$ respectively) flanking the assayed CpG site, supporting the prediction that damage and subsequent repair are likely to precede these methylation changes (Fig.3F-3G, Supplementary Fig.S10).

Discussion

Several molecular epidemiological population studies have shown that environmental exposures, such as smoking and alcohol, are associated with changes in DNA methylation at specific CpGs in blood DNA (6, 7). In the present study, we show that platinum-based chemotherapy can also induce DNA methylation changes in blood DNA, both increased and decreased methylation changes. Intriguingly, we observe those patients with greater methylation changes at the loci analysed to generally have better overall survival. This could be reflective of differences in DNA repair capacity between individuals. However, the functional consequences of these methylation changes in blood remains unclear although in the case of smoking and methylation at the AHRR gene, DNA methylation and transcriptional changes at AHRR in lung epithelial cells have been shown to be induced by smoking (6) and mediates risk of lung cancer (23), therefore the blood changes may be acting as a surrogate for change occurring in a more relevant tissue for carcinogenesis, such as epithelial tissue. Further investigation of the candidate genes identified in this study (Supplementary Table S4) may provide novel insight into platinum resistance. Indeed, two have previously been implicated in platinum resistant ovarian cancer including down-regulation of DUSP6 associated with platinum resistance in ovarian cancer cell lines (24) and MAD1L1 down regulation in chemo-resistant primary ovarian cancers (25).

Overall, our study shows that methylation changes at specific CpG sites in blood DNA are observed in ovarian cancer patients at time of relapse following platinum-based chemotherapy that mirror changes occurring in tumour DNA at relapse. DNA methylation levels at certain loci can change with age and this is something we have previously investigated for DNA loci in blood taken 6 years apart in a longitudinal study (7). DNA methylation at the 8 CpG sites analysed in this present study does not vary over this 6 year period, with an average intraclass correlation coefficient (ICC) >0.50 and is therefore unlikely to have changed due to age difference of patients at presentation and relapse over less time (median of <3 years, time to recurrence in each group). Furthermore, in cross-

sectional analysis, these 8 markers are also not correlated with age ($R2<0.058$). We observed no association between methylation levels at presentation that predicted overall survival using a similar discovery approach (Supplementary Figure S1), and consistent with previous studies (12, 26). This argues that it is the methylation levels observed following treatment, and not at initial presentation, that are important for patient survival.

Although the prognostic significance of the association of the changes in tumour with survival are of borderline statistical significance (Adjusted HR = 2.63 (1.01-6.84), p=0.048), it should be noted that this is a relatively small number of paired tumours (n=46), the tumours have not been microdissected and not followed up as long as the SCOTROC1 patients. It also should be noted that these markers have been initially identified in blood and while it remains uncertain biologically whether epigenetic changes in blood or tumour may be more relevant for prognosis, using tumour DNA for discovery may produce loci with greater prognostic impact. If the methylation changes have similar mechanisms of being induced in blood and tumour cells, we might expect some level of concordance between the methylation changes observed in blood and in tumour cells. We interrogated the n=333 probes identified in blood (308 probes with 25 failing quality control) in the cancer cell line data and found a significant enrichment for consistent hypermethylation in response to platinum in both datasets (110/308 probes, OR=2.4 (1.5-3.8), p=0.0003). Thus there is concordance, albeit not strong. Indeed we would predict only weak concordance given the different nature of the cells being analysed which will have different chromatin landscape and levels of repair.

Methylation levels in blood DNA, at the time of relapse can predict clinical outcome with an average 15 months increased overall survival in the better prognosis group. This contrasts with the lack of any strong association between methylation and survival when blood samples taken at presentation are analysed (Supplementary Fig.S2) (12, 26). These observations have important

implications for the evolution of epigenetic change and patient outcome across a wide variety of cancers and treatments, particularly those that involve platinum-based chemotherapy or other DNA damaging agents. It is also possible that prognostic DNA methylation changes can already be detected during or immediately after chemotherapy while the patient is in remission. Such blood samples were not available from SCOTROC1 patients, but will be an important avenue for future investigations. If DNA methylation changes following chemotherapy were prognostic this may help identify patients more likely to recur early with platinum resistant disease and who would benefit from molecularly targeted agents as second line treatment or during remission.

Consistent with the hypothesis that the aberrant methylation observed following platinum-based chemotherapy is caused by platinum adducts, we observe 5-fold increased frequency of DNA methylation changes following cisplatin treatment of DNA mismatch repair proficient ovarian cell line models compared to mismatch repair deficient models. However, although DNA mismatch repair proteins recognise and bind to platinum adducts, platinum adducts are not repaired by mismatch repair, rather they cause replication stalling and engagement of cell death following platinum treatment of cells (14, 27). Loss of mismatch repair causes increased replication bypass of platinum lesions and tolerance of cells to platinum-induced DNA damage. Therefore, we propose that replication stalling in cells expressing the mismatch repair protein MLH1 leads to increased DNA methylation at sites of platinum damage (Supplementary Fig.S11).

Authors' contributions

Experiments were performed by AW, CK, JG, NM and KJF. JMF, RB and AW conceived the study design and coordination. JMF, EL, CSWB, AH and KJF provided additional bioinformatics analysis and support. JP, NS, RB, PC, HG, IB, JS, SD-E, AV, IV, DCC-T, CK, GBW and EB, provided samples and clinical annotation. JMF, RB and AW drafted the manuscript. All authors read and approved the final manuscript.

Declaration of Interests

None.

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Figure Legends

Figure 1. Cisplatin induces methylation changes associated with improved ovarian cancer patient survival. (A) Illumina450k arrays were hybridised with bisulphite converted DNA from blood samples at presentation (n=107) and at relapse (n=54, matched to 54 presentation samples). Heatmap for n=333 probes, identified as significantly different (FDR<10%) between matched presentation and relapse blood samples, with 207 (62%) hypermethylated in relapse compared with presentation. Probes are scaled with blue representing low methylation and pink representing high methylation. Univariate cox regression p-values for time to death are shown in the right panel with the 8 significant markers (FDR<10%) (cg05529343, cg12992827, cg16172923, cg07960624, cg25953130, cg13691961, cg01692018, cg07573872) highlighted red. **(B)** Consensus clustering of methylation values for these 8 probes identifies two patient groups (Supplementary Fig.S3). Plot of the correlation matrix heatmap, with the scale presented on the right ranging from white (low correlation) to blue (high correlation) delineating Class 1 (blue) and Class 2 (red). **(C)** Kaplan Meier curve of overall survival (time from relapse to death, TTD) for the two identified relapse classes in the array discovery data set (n=54) shows poorer survival for class 1 (blue dotted line) compared to class 2 (red dotted line) (adjusted HR=3.73 (1.86- 7.60), p=2.8e-4) adjusted for age, residual disease, stage, histology and PFS time in a multivariable cox proportional hazards model. **(D)** Pyrosequencing of 8 CpG sites in WBC DNA at time of ovarian cancer relapse validates the association with survival. Kaplan Meier curve of overall survival (TTD) for the two identified groups shows poorer survival for class 1 (blue) compared with class 2 (red) (adjusted HR=1.83 (1.05- 3.18), p=0.032), adjusted as above. **(E)** Kaplan Meier curve of progression free survival (PFS time) for two identified classes with the 8 CpG sites in WBC DNA taken at presentation shows no association with survival (adjusted HR=0.99 (0.58-1.73), p=0.989). **(F)** Receiver Operating Curves for prediction of TTD<12 months using a methylation index derived from 3 CpG sites that were independently associated with survival. The discovery set AUC = 0.8 (95% CI 0.7-0.9) and the pyrosequencing validation set AUC = 0.7 (95%CI 0.6-0.8).

Figure 2. Methylation profile validation of tumour DNA at time of ovarian cancer relapse predicts overall survival. Consensus clustering of pyrosequencing based methylation values for these 8 probes identifies two groups using the consensus CDF plot and the delta area under the curve (Supplementary Figure S5). A) Plot of the correlation matrix heatmap, with the scale presented on the right ranging from white (low correlation) to blue (high correlation) delineating Class 1 and Class 2. B) Kaplan Meier curve of overall survival for class 1 compared to class 2 (HR=2.63 (1.01- 6.84), p=0.048) adjusted for age, stage, and PFS time in a multivariable cox proportional hazards model. C) Boxplots of methylation beta values in tumour DNA samples of patients at relapse in class 1 (R1, n=27) or class 2 (R2, n=19). Wilcoxon signed rank sum test p-values are shown below the graphs.

Figure 3. Cisplatin induced hypermethylation is dependent on MLH1. Two clonal derivatives of A2780/CP70 cell line were used for the experiments described here, one of which expresses MLH1 (MLH1+) and the other does not (MLH1-). **(A)** Cisplatin dose-response for each parental cell line was determined as the average survival as a percentage of baseline (mock-treated) cells from triplicate MTT assays. The parental A2780 (dotted red), A2780/CP70 (dotted black) and derived cell lines CP70-MLH1+ (solid red) and CP70-MLH1- (solid black) are shown. Error bars show standard error of the mean (SEM). Inset is shown a Western blot for MLH1 with β-actin measured as a loading control. **(B)** MLH+ and MLH- cell lines were treated with 5μM cisplatin or mock-treated for 72 hrs (in three batches) and individual clones (n=25 clones per group) were isolated and cultured for 6 weeks. Percentage methylation of LINE-1 for mock- and 5 μM cisplatin-treated MLH1+ and MLH1- clones (n=22-25 clones per group) were determined using bisulphite pyrosequencing. Two-tailed Student's *T*-tests showed LINE-1 methylation to be significantly higher in MLH1+ clones (p=4.087E^{-5}) and not in MLH1- clones (p=0.63). Error bars represent SEM. **(C-D)** Illumina 450k arrays were hybridised with bisulphite converted DNA from mock and cisplatin treated MLH1- **(C)** and MLH1+ **(D)** clones (n=11 clones per group). Student's T-test p<0.01 and

delta methylation (cisplatin treated minus mock) >20% was used as a threshold to define significant changes (highlighted green). Clonal variation within the treatment groups is shown as grey bars for each probe representing the 95% confidence interval of differences between 6 vs 5 clones (100-fold bootstrapped random sampling) from within the mock-treated group. **(E-F)** Sequence composition analysis of sequence 25 nt up- and downstream of the assayed CpG from significantly changed loci (see C and D) for MLH1- (E, n=199) and MLH1+ (F, n=973). Line plots of dinucleotide frequency are shown for GG (redorange, solid), CC (redorange, broken) and CG dinucleotides (blue) with all other dinucleotides shown in grey. Heatmaps below are -log10(p-values) of enrichment from the hypergeometric test for CC and GG dinucleotides at each position, compared to all CpG loci on the 450k array. Dark blue indicates no significant enrichment ($p > 0.05$), white indicates $0.05 > p > 0.001$, and dark red indicates $p < 0.001$.

Supplementary Information

Supplementary Figures S1-S11

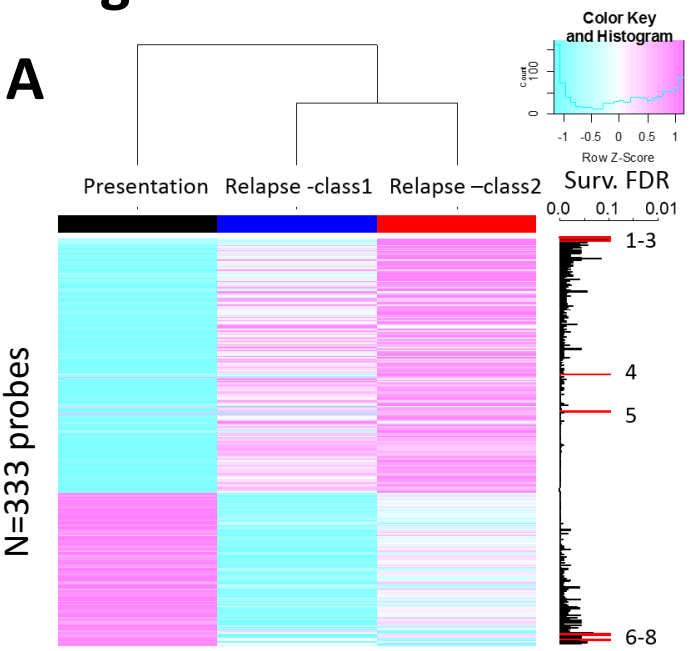
Supplementary Tables T1-T4

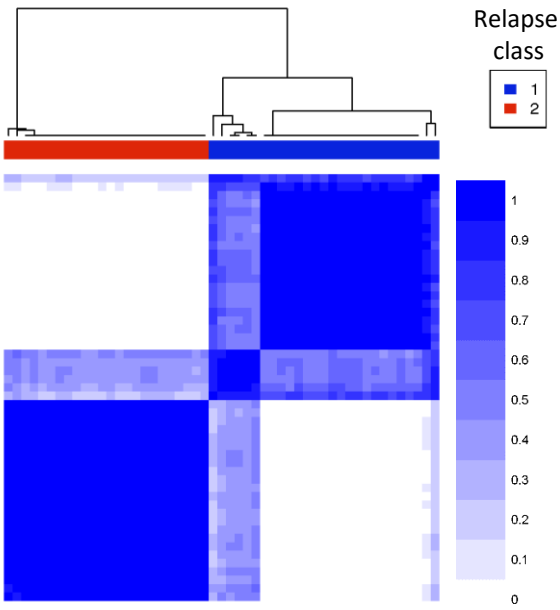
Table 1 - Cox Proportional Hazards Regression models for blood based methylation (SCOTROC1) and tumour DNA methylation (OCTIPS).

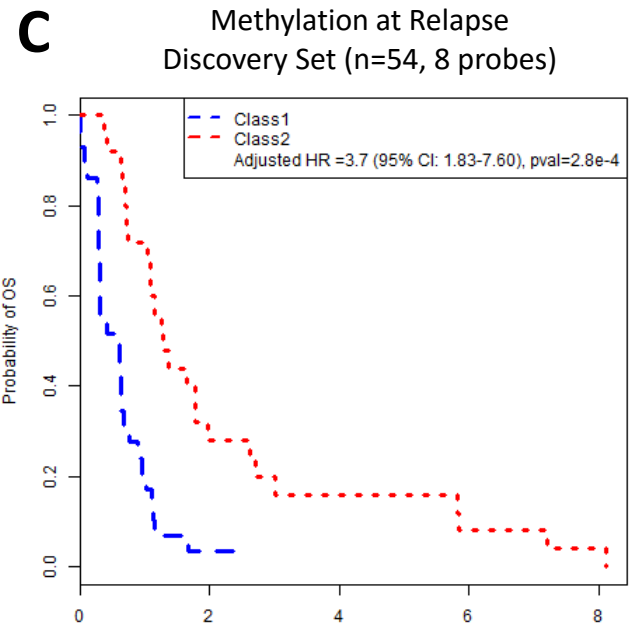
Multivariable models were adjusted for age, residual disease, stage, histology, pfstime in SCOTROC1, and age, stage and pfstime in OCTIPS. Hazard ratios (HR) are reported as the exponential of the coefficient. Asterisks represent statistically significant results *p<0.05; ** p<0.01; *** p<0.001

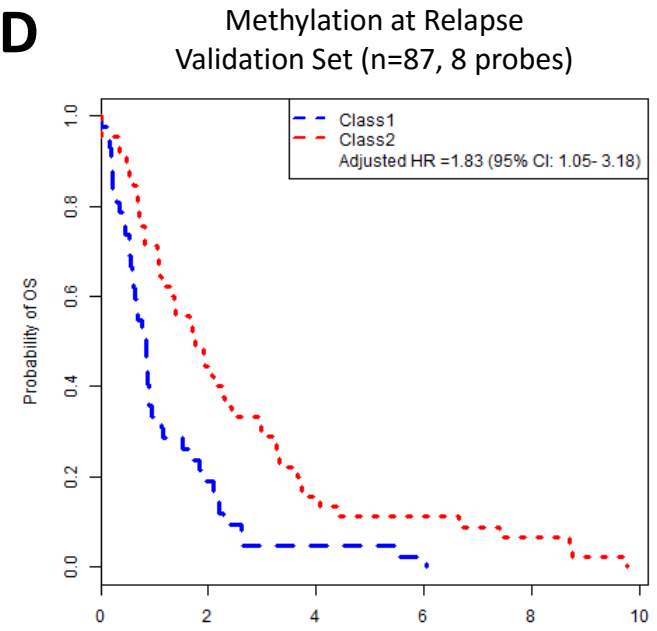
	univariate							**multivariable**						
8 markers discovery (%)	**n**	**coef**	**HR**	**se(coef)**	**z**	**P**	**fdr**	**n**	**coef**	**HR**	**se(coef)**	**z**	**P**	
cg05529343	54	-0.0461	0.9549	0.0145	-3.1857	0.0014	0.093	54	-0.0576	0.9440	0.0188	-3.0680	0.0022	**
cg12992827	54	-0.0760	0.9268	0.0213	-3.5637	0.0004	0.093	54	-0.0642	0.9378	0.0209	-3.0710	0.0021	**
cg16172923	54	-0.0613	0.9405	0.0199	-3.0884	0.0020	0.093	54	-0.0620	0.9398	0.0214	-2.8970	0.0038	**
cg07960624	54	-0.0572	0.9444	0.0187	-3.0580	0.0022	0.093	54	-0.0494	0.9518	0.0188	-2.6220	0.0088	**
cg25953130	54	-0.0627	0.9393	0.0201	-3.1246	0.0018	0.093	54	-0.0544	0.9470	0.0261	-2.0860	0.0370	*
cg13691961	54	-0.0745	0.9283	0.0235	-3.1672	0.0015	0.093	54	-0.0809	0.9223	0.0273	-2.9620	0.0031	**
cg01692018	54	-0.1002	0.9046	0.0325	-3.0849	0.0020	0.093	54	-0.0975	0.9071	0.0382	-2.5500	0.0108	*
cg07573872	54	-0.0412	0.9596	0.0129	-3.1868	0.0014	0.093	54	-0.0345	0.9661	0.0160	-2.1550	0.0312	*
R.class discovery - SCOTROC1	54	-1.3896	0.2492	0.3334	-4.168	3.08E-05	***	54	-1.3169	0.2680	0.3628	-3.63	2.83E-04	***
R.class pyro validation - SCOTROC1	87	-0.6688	0.5123	0.2457	-2.722	0.00648	**	87	-0.6035	0.5469	0.2819	-2.1410	0.0323	*
R.class pyro validation - OCTIPS	46	-1.1418	0.3192	0.4713	-2.4230	0.0154	*	46	-0.9661	0.3806	0.4879	-1.9800	0.0477	*

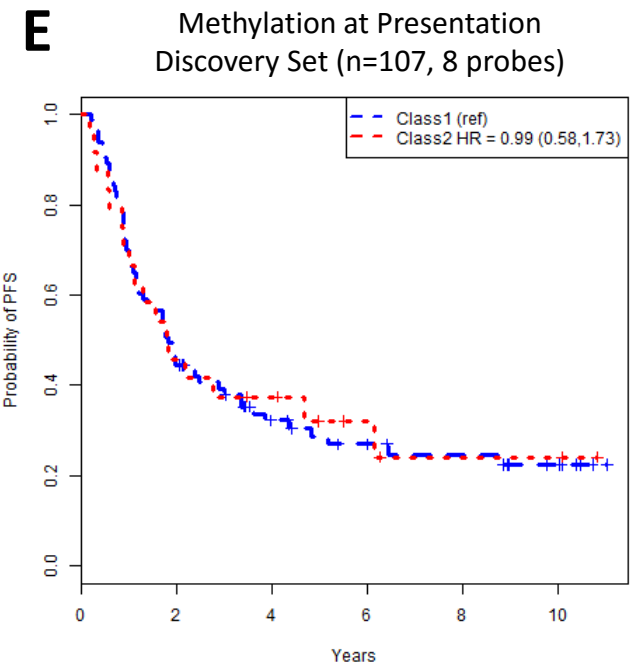
Figure 1


A
Color Key and Histogram
Count
100
0
-1 -0.5 0 0.5 1
Row Z-Score
Presentation
Relapse -class1
Relapse –class2
Surv. FDR
0.0 0.1 0.01
1-3
4
5
6-8
N=333 probes


B
Relapse class
1
2
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0


C
Methylation at Relapse
Discovery Set (n=54, 8 probes)
Class1
Class2
Adjusted HR =3.7 (95% CI: 1.83-7.60), pval=2.8e-4
Probability of OS
0.0
0.2
0.4
0.6
0.8
1.0
0
2
4
6
8


D
Methylation at Relapse
Validation Set (n=87, 8 probes)
Class1
Class2
Adjusted HR =1.83 (95% CI: 1.05- 3.18)
Probability of OS
0.0
0.2
0.4
0.6
0.8
1.0
0
2
4
6
8
10


E
Methylation at Presentation
Discovery Set (n=107, 8 probes)
Class1 (ref)
Class2 HR = 0.99 (0.58,1.73)
Probability of PFS
0.0
0.2
0.4
0.6
0.8
1.0
0
2
4
6
8
10
Years

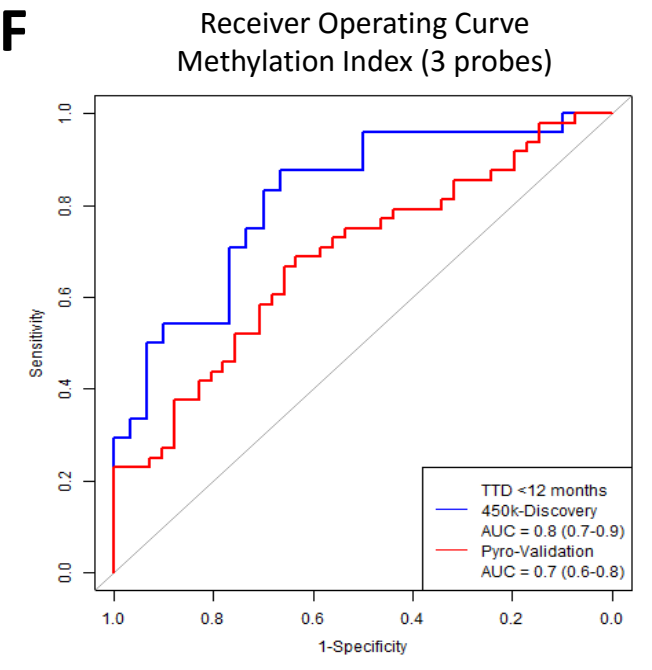
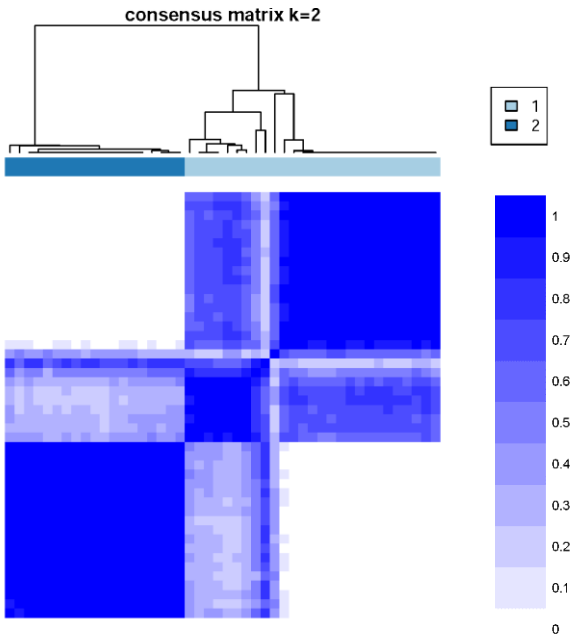
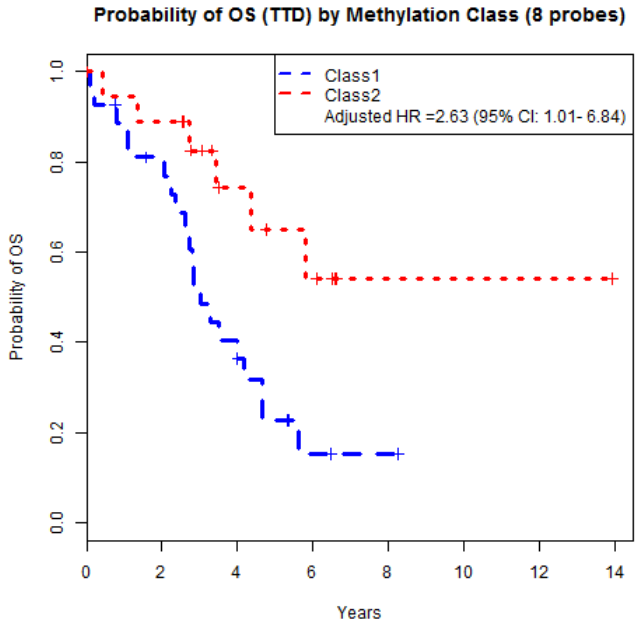

F
Receiver Operating Curve
Methylation Index (3 probes)
Sensitivity
0.0
0.2
0.4
0.6
0.8
1.0
1.0
0.8
0.6
0.4
0.2
0.0
1-Specificity
TTD <12 months
450k-Discovery
AUC = 0.8 (0.7-0.9)
Pyro-Validation
AUC = 0.7 (0.6-0.8)

Figure 2

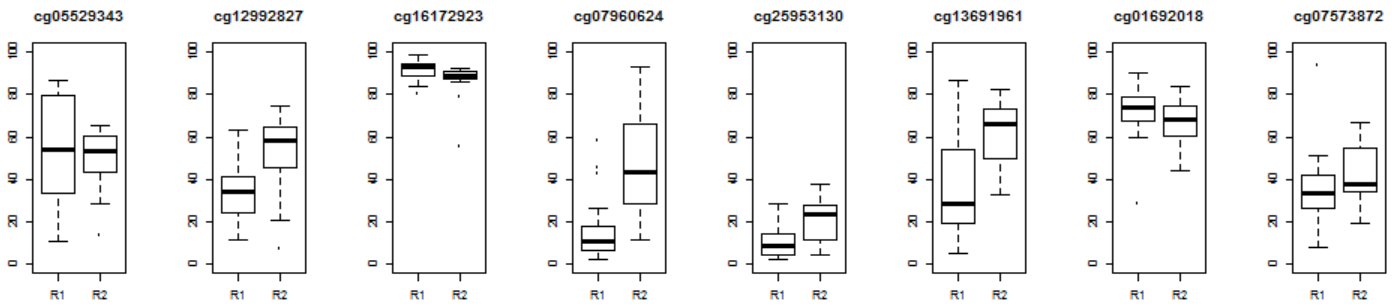
A



B

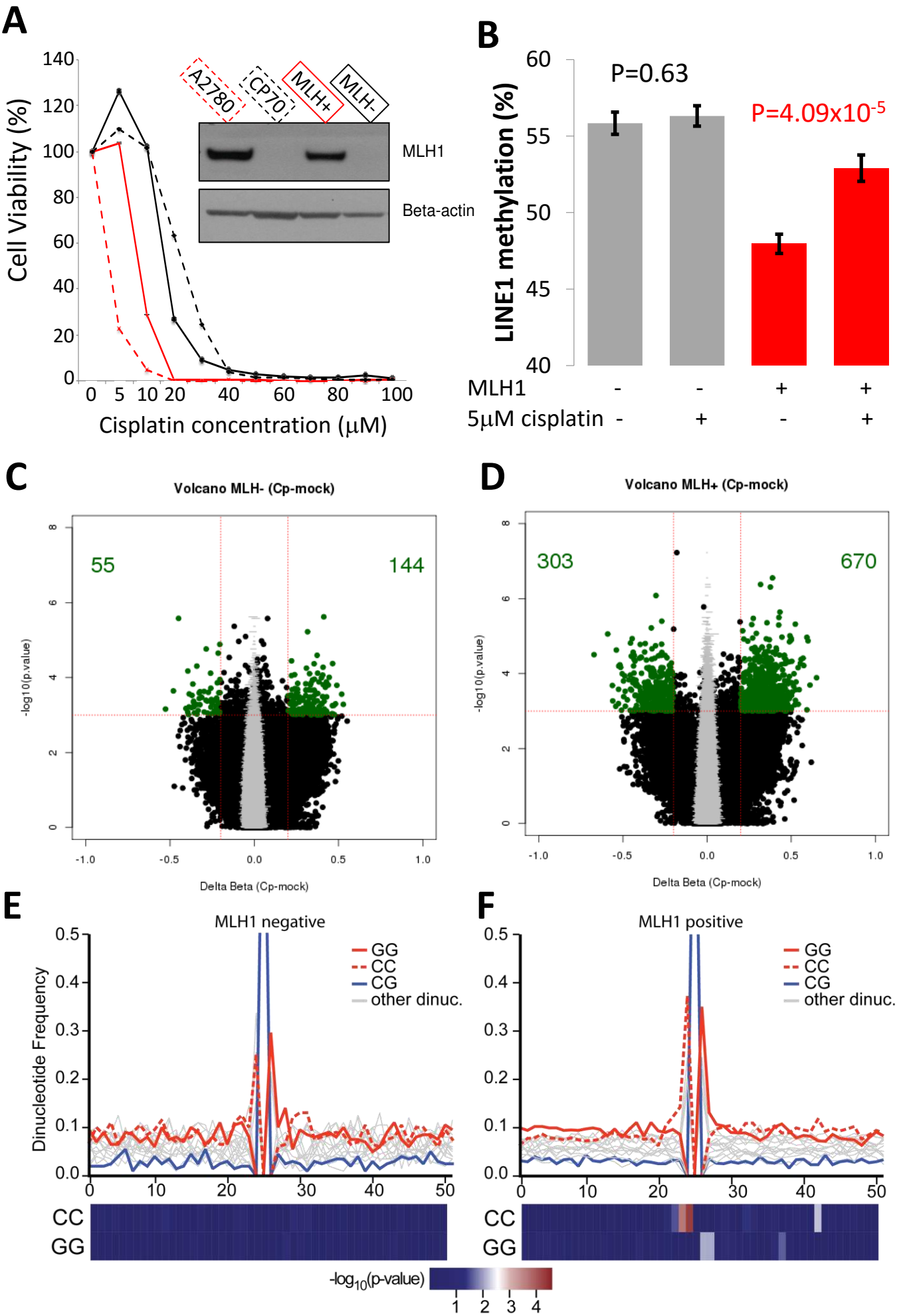


C



P=0.598 P=1.78e-4 P=4.58e-3 P=2.01e-6 P=1.23e-3 P=2.26e-4 P=0.082 P=0.060

Figure 3


A
Cell Viability (%)
Cisplatin concentration (μM)
A2780
CP70
MLH+
MLH-
MLH1
Beta-actin
B
LINE1 methylation (%)
P=0.63
P=4.09x10-5
MLH1
5μM cisplatin
C
Volcano MLH- (Cp-mock)
55
144
-log10(p.value)
Delta Beta (Cp-mock)
D
Volcano MLH+ (Cp-mock)
303
670
-log10(p.value)
Delta Beta (Cp-mock)
E
MLH1 negative
Dinucleotide Frequency
GG
CC
CG
other dinuc.
CC
GG
F
MLH1 positive
GG
CC
CG
other dinuc.
CC
GG
-log10(p-value)