Clinical Cancer Research

Epigenetic Regulation of the Homeobox Gene *MSX1* Associates with Platinum-Resistant Disease in High-Grade Serous Epithelial Ovarian Cancer

Nair A. Bonito[1], Jane Borley[1], Charlotte S. Wilhelm-Benartzi[1], Sadaf Ghaem-Maghami[1], and Robert Brown[1,2]

Abstract

Purpose: Although high-grade serous ovarian cancer (HGSOC) is frequently chemoresponsive, a proportion of patients do not respond to platinum-based chemotherapy at presentation or have progression-free survival (PFS) of less than 6 months. Validated predictive biomarkers of lack of response would enable alternative treatment stratification for these patients and identify novel mechanisms of intrinsic resistance. Our aim was to identify DNA methylation biomarkers of poor response to chemotherapy and demonstrate involvement of the associated gene in platinum drug cell sensitivity.

Experimental Design: DNA methylation was investigated in independent tumor cohorts using Illumina HumanMethylation arrays and gene expression by Affymetrix arrays and qRT-PCR. The role of Msh homeobox 1 (*MSX1*) in drug sensitivity was investigated by gene reintroduction and siRNA knockdown of ovarian cancer cell lines.

Results: CpG sites at contiguous genomic locations within the *MSX1* gene have significantly lower levels of methylation in independent cohorts of HGSOC patients, which recur by 6 months compared with after 12 months ($P < 0.05$, $q < 0.05$, $n = 78$), have poor RECIST response ($P < 0.05$, $q < 0.05$, $n = 61$), and are associated with PFS in an independent cohort ($n = 146$). A decrease in methylation at these CpG sites correlates with decreased *MSX1* gene expression. *MSX1* expression is associated with PFS (HR, 0.92; 95% CI, 0.85–0.99; $P = 0.029$; $n = 309$). Cisplatin-resistant ovarian cancer cell lines have reduced *MSX1* expression, and *MSX1* overexpression leads to cisplatin sensitization, increased apoptosis, and increased cisplatin-induced p21 expression.

Conclusions: Hypomethylation of CpG sites within the *MSX1* gene is associated with resistant HGSOC disease at presentation and identifies expression of *MSX1* as conferring platinum drug sensitivity. *Clin Cancer Res; 22(12); 3097–104. ©2016 AACR.*

Introduction

Epithelial ovarian cancer (EOC) is the second commonest, but most lethal, of the gynecologic malignancies, with overall 5-year survival rates around 45% [reviewed in (1,2)]. Adjuvant chemotherapy is recommended for those with stage IC disease or higher and normally comprises of a platinum-based agent with or without taxol (3). Despite this, approximately 20% of patients will be resistant to first-line platinum agents, and the prognosis in these resistant patients is particularly poor (2). Currently, there are no reliable methods to determine or predict platinum resistance, and thus patients who will gain little clinical benefit undergo treatment regimens associated with high morbidity and side effects. Defective homologous recombination shows promise as a marker of platinum response, although has limited sensitivity and specificity (4), and ultimately, combinations of independent predictive biomarkers of response are likely to be required. Biomarkers that predict treatment response may identify novel targets for therapies aimed at reversal of resistance and will facilitate stratification of patients who should be considered for alternative treatment with noncytotoxic, molecularly targeted agents.

The use of DNA methylation as a biomarker has several advantages over other molecular endpoints, including stability of DNA methylation *ex vivo* compared with RNA. Furthermore, DNA methylation measurements can be compared with absolute reference points (for instance, completely methylated or completely unmethylated DNA), which provides a quantitative assay (5). In addition, tumor DNA methylation can be detected in body fluids of patients, potentially leading to measurement of tumor-derived biomarkers noninvasively (5). There are now numerous examples of the potential of DNA methylation as a therapy stratification biomarker or as marker of acquired resistance, although few methylation biomarkers have been clinically validated or used as stratification biomarkers in clinical studies (6).

The initial aim was to identify DNA methylation at loci associated with progressive disease (PD) in high grade serous ovarian cancer (HGSOC) using Illumina 27K HumanMethylation array data. This analysis identified methylation at the Msh homeobox 1 gene (*MSX1*) as associated with platinum-resistant disease, which we aimed to validate in independent HGSOC cohorts for

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Translational Relevance

Although considered a chemoresponsive disease, a proportion of high-grade serous ovarian cancer (HGSOC) patients do not respond to platinum-based chemotherapy at initial presentation. Clinically validated response biomarkers would enable alternative treatment stratification for these patients and identify novel resistance mechanisms. We have identified CpG sites in DNA at contiguous genomic locations within the Msh homeobox 1 gene (*MSX1*) as significantly associated with lack of response of HGSOC patients in discovery, test, and validation patient cohorts. A decrease in methylation was significantly correlated with decreased *MSX1* gene expression, and the expression is associated with progression-free survival of patients. Increased expression of *MSX1* in three platinum-resistant ovarian cancer lines led to cisplatin sensitization and increased p21 expression. In conclusion, hypomethylation and low expression of *MSX1* is a biomarker of resistant HGSOC disease at presentation and identifies expression of MSX1 as conferring platinum drug sensitivity.

associations with response and progression-free survival (PFS). Dysregulation of HOX genes has been associated with a variety of human cancers [summarised in (7)]. *MSX1*, also known as *HOX7*, is a homeobox gene with critical roles in the control of cellular differentiation in development (8,9). MSX1 has been shown to cause a change in cell morphology and a reduction in cell growth by the induction of apoptosis in HeLa cancer cells (10). We have examined the role of *MSX1* in chemosensitivity by gene reintroduction into platinum-resistant ovarian tumor cell lines that have reduced *MSX1* expression compared with matched parental, nonplatinum exposed, lines.

Materials and Methods

Patient samples

Fresh-frozen EOC tissue was collected and stored at −80°C at Imperial College NHS Trust Tissue Bank, Hammersmith Hospital ("Hammersmith Cohort"). Tumors selected were from primary debulking surgery (no previous chemotherapy), advanced stage disease [Federation Internationale des Gynaecologistes et Obstetristes (FIGO) stage III and IV] with serous histology. Tumor samples selected were from patients treated between 1999 and 2010, although 76% of patient samples were from 2006 onward. All surgeries were performed at the West London Gynaecology Cancer Centre, London, United Kingdom. Tumors were selected sequentially from the tissue bank. Patients were excluded with mixed cell or borderline pathology. To assess the quality of the individual fresh-frozen tissue samples used for DNA extraction, adjacent tissue was histopathologically examined and samples excluded with low tumor cell content.

Overall survival (OS) and PFS were defined as the interval from the date of initial surgical resection to the date of last known contact or death for OS and the interval from the date of initial surgical resection to the date of progression or recurrence for PFS. Follow-up was calculated up to June 2011. Stage was defined using FIGO criteria.

The response to chemotherapy was defined by RECIST 1.1 (11) criteria using scans done at two distinct time points, CT chest, abdomen, and pelvis scan postsurgery but prior to adjuvant chemotherapy and after 6 cycles of chemotherapy. Patients were categorized into those who responded to chemotherapy (complete or partial response), stable disease, or PD.

The clinical characteristics of the Hammersmith cohort and the The Cancer Genome Atlas (TCGA) samples used for validation ("TCGA Cohort") are shown in Supplementary Table S1. REMARK criteria have been used throughout the analysis, and a diagram of flow of patients through the DNA methylation study is shown in Supplementary Fig. S1.

Bisulfite conversion

Up to 500 mg of tumor tissue was used per sample and DNA extracted by the chlorinated Nucleon extraction method (Gen-Probe Life Sciences Ltd.) and resuspended in TE buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 8). Tumor DNA was bisulfite converted using the EZ-96 DNA Methylation Kit (Zymo Research) as per manufacturer's protocol. 1 μg of genomic DNA was used for each sample. Successful bisulfite conversion was confirmed by successful conversion of cytosines at the calponin locus (12).

Infinium HumanMethylation27 BeadChip

Samples were processed as per the Infinium Assay Methylation Protocol Guide (Illumina; http://www.illumina.com/products/infinium_humanmethylation27_beadchip_kits.ilmn#documentation). Following bisulphite conversion, 200 ng per samples were resuspended at 50 ng/μL. Methylation data were summarized as β values, calculated as M/(M+U), where M is signal from methylated beads, and U is signal from unmethylated beads at the targeted CpG site. β values were adjusted for background, and data were log transformed to achieve a normal distribution. Probes with a detection $P > 0.05$ were removed. Quality control checks were performed through Genome Studio. The 27K Illumina data have been deposited into GEO under GSE75414.

TCGA dataset analysis

Illumina Human Methylation27 BeadChip data on HGSOC from TCGA data portal (https://tcga-data.nci.nih.gov/tcga/; "TCGA Cohort" were used for independent validation of correlations observed in the Hammersmith cohort. Level 2 expression data on Affymetrix HGU133A microarrays, level 3 methylation data, and annotated clinical data were obtained. The expression microarray data were preprocessed and normalized across samples.

Plasmid transfection

Cell lines used are described in Supplementary Table S2. Cell lines were obtained and used within 6 months from the Department of Surgery and Cancer, Imperial College London, UK cell stocks. STR profiling was used to authenticate cell lines prior to freezing and to confirm that pairs of lines were related. All cell lines were mycoplasma free. Cells were stable transfected either with *MSX1* expression plasmid obtained from OriGene (RC205682) or the corresponding empty vector (PS100001) using FuGENE HD Transfection Reagent (Promega) as per respective protocols. Cells were grown and maintained under 500 μg/mL of geneticin (Life Technologies). The siRNA oligos (Qiagen) were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Real-time PCR and gene expression profiling

To analyze gene expression, 2-step RT-PCR was performed. Total RNA was extracted using the QIAshredder and RNeasy Mini Kits (Qiagen) according to manufacturer's protocol. Total RNA was converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) using specific primers. Each reaction was performed following the manufacturer's instructions using 10 ng of converted RNA. The data was analyzed using the $\Delta\Delta C_t$ method.

Protein analysis

Proteins were isolated using RIPA lysis buffer: 93.2 mmol/L disodium hydrogen phosphate, 6.8 mmol/L sodium dihydrogen phosphate, 0.5% (v/v) Triton X-100, 0.5 mmol/L EDTA pH 8, 1 mmol/L sodium orthovanadate, 100 U/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonylfluoride. Western blots were incubated with MSX1 antibody (G116; Cell Signaling Technology). β-Actin (Abcam) was used as loading control.

Annexin V assay

Cells were seeded the day before treatment at 2×10^5 cells per well and treated for 24 hours, after which they were washed twice with PBS and detached from the plate using TrypLE Express Reagent (Thermo Fisher Scientific). Cells were then stained with FITC-Annexin V/PI Kit (BD Pharmingen) according to the manufacturer's instructions. Cells were read using FACSCanto (BD Biosciences) immediately after staining and analyzed using FlowJo software v8.8.9.

Caspase-Glo 3/7 assay

To quantify caspase activity, we have used Caspase-Glo 3/7 Assay (Promega) following the manufacturer's instructions. Briefly, 1×10^4 cells were seeded overnight, after which they were treated with the indicated cisplatin concentrations for 24 hours, after which we incubated cells with caspase reagent. Luminescence was read after 1 hour of incubation at room temperature using LUMIstar Optima Plate Reader (BMG LABTECH).

Cell proliferation assays

Cells were seeded at 1×10^4 cells per well in triplicate; after 24 hours, cells were treated with cisplatin at concentrations of 0 to 50 μmol/L for 24 hours. Cell proliferation was quantified with MTT assay after a further 48 hours of culture after 24 hours of cisplatin treatment using CellTiter 96 AQueous One Solution Proliferation Assay (Promega).

Statistical analysis

All statistical analyses were performed using the R statistical package (version 2.10 at http://www.r-project.org). Individual generalized linear regression models using continuous data were performed adjusting for array batch, grade, and residual disease to determine significant differentially methylated loci and associated differential gene expression of Affymetrix gene expression data with RECIST chemotherapy response, and PFS < 6 months versus >12 months in both datasets. A significant relationship between TCGA DNA methylation and gene expression in the tumor tissue was determined through Spearman correlation.

For gene expression using Fast SYBR Green, SEM was calculated for three independent experiments. To determine the statistical significance of the differences observed, an unpaired Student t test was used. The differences were considered significant when a two-sided P value was below 0.05. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$).

Results

Hypomethylation of *MSX1* in HGSOC is associated with PD and short PFS

Discovery analysis across all loci on the 27K Illumina bead array, comparing HGSOC patients from a Hammersmith cohort ($n = 78$) with PD versus those who responded to chemotherapy or had stable disease, identified multiple CpG sites at the *MSX1* gene, which demonstrated significant differential methylation, even after taking multiple comparisons into account ($P < 0.05$, FDR < 5%). *MSX1* was the only locus on the 27K array showing multiple CpG sites with significant association with response and was therefore selected for further investigation (Table 1; Fig. 1). We further evaluated this relationship in a cohort ($n = 61$) of Hammersmith patients with response data defined by RECIST 1.1 criteria (Table 1). We observe 8 contiguous CpG sites spanning the first intron and 2nd exon of *MSX1* to be significantly ($P < 0.05$) associated with RECIST response (Fig. 1). For 7 of 8 significant probes, methylation was significantly lower in the group with PD versus those with response to chemotherapy or stable disease, with a methylation difference of >15% (Supplementary Fig. S2). PFS of less than 6 months is widely used to clinically define platinum-resistant disease, whereas PFS greater than 12 months is often used to define platinum-sensitive disease (13,24). Six of the 8 CpG sites within the *MSX1* gene identified as associated with response have significantly lower levels of methylation in HGSOC from the Hammersmith cohort, which recur by 6 months compared with after 12 months ($P < 0.05$, $q < 0.05$), with the remaining two showing a similar trend (Table 1, Supplementary Fig. S3).

Primary chemotherapy response was not defined by RECIST criteria in the TCGA dataset and therefore could not be used to validate the association with response observed in the Hammersmith cohort. However, PFS data were available in the TCGA dataset and methylation data available for 7 of the 8 *MSX1* probes identified from the Hammersmith cohort (Table 1; Fig. 1). Linear regression models adjusting for array batch, grade, and residual disease found methylation levels at 4 probes to be significantly associated with <6 months versus >12 months PFS and a further 2 showing strong trend (Table 1). Consistent with the Hammersmith dataset, methylation at these CpGs demonstrated a corresponding lower level of methylation in patients with PFS < 6 months.

MSX1 gene expression is associated with DNA methylation and PFS

DNA methylation and gene expression data were available for 252 samples in the TCGA data. This data demonstrated that a decrease in methylation at 6 of the identified CpG sites were significantly correlated ($P < 0.05$) to a decrease in gene expression (Table 1; Fig. 1). *MSX1* expression and PFS were significantly associated in the TCGA cohort dataset using univariate Cox proportional hazard regression analysis (HR, 0.92; 95% CI, 0.85–0.99; $P = 0.029$; $n = 309$). Patients with low expression of *MSX1* had poorer PFS than those with higher *MSX1* expression. A significant association was retained in multivariable Cox

Table 1. Summary of statistical analysis of association between MSX1 methylation, patient survival and gene expression

	RECIST Chemotherapy response, $n = 61$, HH					PFS <6 months, vs. >12 months, $n = 39$, HH					PFS < 6 months, vs. >12 months, $n = 146$, TCGA			TCGA, $n = 252$
	Linear regression model		Median methylation (β value)			Linear regression model (adjusting for chip, grade, and residual disease)		Median methylation (β value)			Linear regression model (adjusting for chip, grade, and residual disease)			DNA methylation to gene expression correlation
Probe ID	*P*	*q* value	Chemotherapy response	PD	Δ Methylation	β Coefficient	*P*	*q* value	PFS > 12 months	PFS < 6 months	Δ Methylation	β Coefficient	*P*	*q* value	*P*	ρ
cg22609784	<0.001	0.002	0.65	0.27	0.39	−0.402	0.009	0.032	0.65	0.53	0.12	−0.179	0.023	0.114	0.058	0.119
cg09573795	0.018	0.045	0.76	0.78	−0.02	−0.202	0.082	0.159	0.76	0.67	0.08	−0.124	0.039	0.115	0.013	0.157
cg27038439	<0.001	0.002	0.91	0.73	0.18	−0.167	0.011	0.034	0.91	0.75	0.16	NA	NA	NA	NA	NA
cg24840099	<0.001	<0.001	0.78	0.34	0.45	−0.526	<0.001	0.001	0.78	0.34	0.45	−0.147	0.053	0.115	0.003	0.184
cg09748975	<0.001	<0.001	0.88	0.55	0.33	−0.315	<0.001	0.001	0.88	0.56	0.32	−0.124	0.042	0.115	0.019	0.147
cg03843978	0.028	0.055	0.61	0.46	0.15	−0.121	0.089	0.159	0.61	0.48	0.13	−0.057	0.188	0.283	0.019	0.148
cg20891301	0.021	0.046	0.75	0.57	0.18	−0.197	0.002	0.010	0.75	0.57	0.17	−0.082	0.05	0.115	0.031	0.136
cg01785568	0.005	0.015	0.69	0.43	0.26	−0.387	0.002	0.010	0.71	0.45	0.27	−0.208	0.067	0.126	<0.001	0.497

Abbreviations: HH, Hammersmith cohort; NA, data not available; TCGA, TCGA cohort.

analysis when age, grade, stage, and residual disease were included in the model ($P = 0.014$), demonstrating that *MSX1* expression is an independent biomarker from clinical factors known to be associated with PFS.

MSX1 expression sensitizes cells to cisplatin by increased apoptosis

Relative *MSX1* gene expression was examined by qRT-PCR on three matched pairs of cisplatin-sensitive and -resistant ovarian cancer cell lines: PEA1/PEA2, PEO1/PEO4, and PEO14/PEO23 pairs derived from HGSOC patients before treatment and at relapse after platinum-based chemotherapy (14) and A2780/A2780cp70–sensitive and *in vitro*–derived resistant line (15). Gene expression was markedly lower in the platinum-resistant cell lines derived from patients following chemotherapy or *in vitro* derived compared with their platinum-sensitive pair as determined by qRT-PCR and Western blot analysis (Fig. 2). However, no significant difference in DNA methylation could be detected between the pairs of cell lines (data not shown). To examine whether increased *MSX1* expression would increase the cell sensitivity to cisplatin, the ovarian cell lines A2780cp70, PEA2, and PEO4 were stably transfected either with an *MSX1*-containing plasmid (MSX1) or with the corresponding empty vector (EV). As shown in Fig. 3, all three ovarian cancer cell lines transfected with MSX1 showed an increase in cisplatin sensitivity compared with the empty vector control. Growth curves of the MSX1 transfectants and empty vector in the absence of cisplatin showed no significant differences, suggesting a direct effect on drug resistance rather than changes in proliferation affecting drug sensitivity (data not shown).

To examine whether *MSX1* expression affected levels of target genes involved in cell-cycle regulation and apoptosis, the HGSOC cell lines PEA2 and PEO4 overexpressing either *MSX1* or the corresponding EV were treated with cisplatin, and the expression of p21 and *MSX1* was analyzed by qRT-PCR. Overexpression of *MSX1* increases the expression of p21 after cisplatin treatment, when compared with the EV transfectants (Fig. 3). However, cell-cycle analysis of the *MSX1*-overexpressing PEA2 and PEO4 cell lines showed no difference in the proportion of cells in G_1, S, or G_2 phases of the cell cycle following cisplatin treatment (Supplementary Fig. S3). Using Annexin V staining and uptake of propidium iodide, apoptotic, necrotic, and live cells were identified following cisplatin treatment (Fig. 4A-F). This showed a clear increase in the proportion of apoptotic cells in *MSX1*-overexpressing cells. This was further confirmed using caspase activity assays (Fig. 4G).

To further support the involvement of *MSX1* in cisplatin resistance, we examined the effect of siRNA knockdown on cisplatin-induced apoptosis. Transient siRNA knockdown of *MSX1* had no effect on proliferation in the absence of cisplatin (data not shown) but did decrease cisplatin-induced apoptosis significantly in the A2780 and PEA1 cell lines, with a trend in the PEO1 line (Fig. 5).

Discussion

In independent tumor cohorts, differential methylation of multiple CpG sites at an intragenic region of the homeobox gene *MSX1* spanning the intron and second exon is significantly associated with primary chemotherapy resistance and poor PFS in HGSOC. Increased methylation significantly correlates with increased gene expression. *MSX1* encodes a member of the

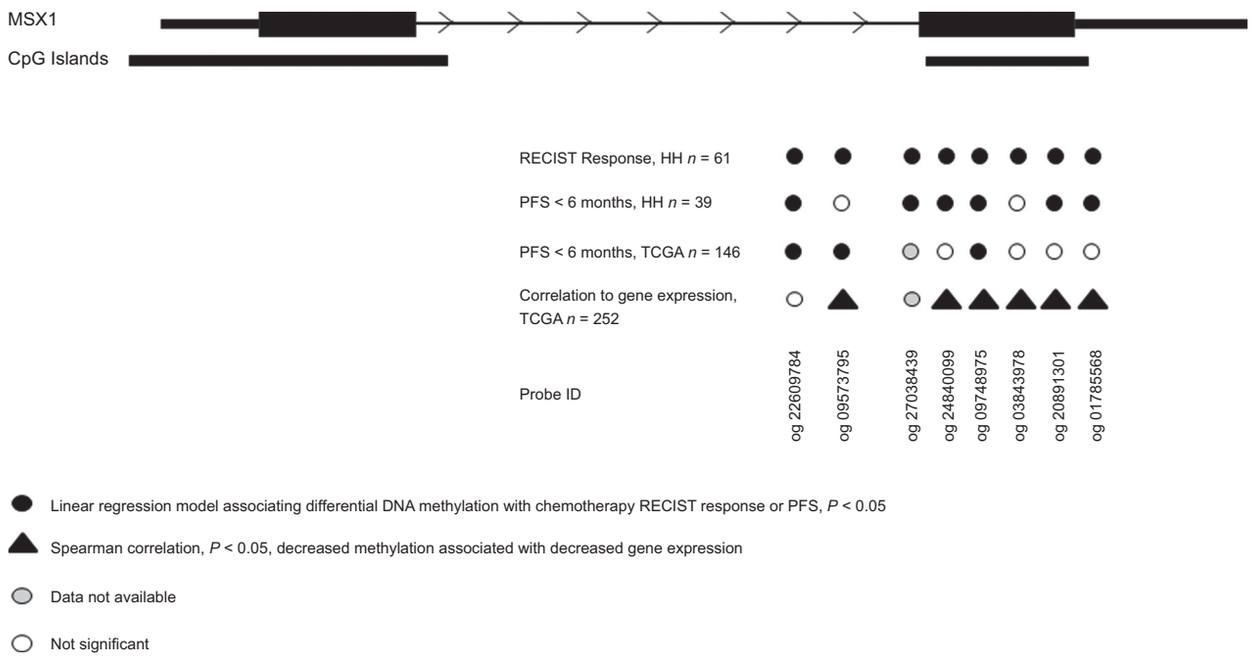

Figure 1.
Schematic of *MSX1* locus showing CpG sites associated with clinical outcome. HH, Hammersmith cohort.

muscle segment homeobox gene family, and its expression is associated with epithelial–mesenchymal interactions during embryogenesis (17). Intriguingly, the same 8 CpG sites we observe associated with response to chemotherapy in *MSX1* have been associated with fetal heart malformation, and again, increased methylation is associated with an increase in *MSX1* expression (18). Given the suggestion by the authors that DNA methylation at these CpG sites impairs MSX1-dependent mesenchymal embryogenesis, it is tempting to speculate that MSX1 in ovarian cancer may influence epithelial–mesenchymal transition, which has been implicated in the resistance of ovarian tumors to chemotherapy (19).

Binding of MSX1 in the genome of myoblasts promotes enrichment of the repressive H3K9me2 mark via recruitment of EHMT2 (G9a) histone methyltransferase (20), the enzyme responsible for maintaining this histone mark. Given the involvement of epigenetic regulation in drug resistance (6), it is therefore possible that MSX1 is mediating its effects on chemosensitivity through epigenetic regulation. An alternative explanation of MSX1 effects on chemosensitivity could be through p53 stabilization. Previous studies have suggested that MSX1 decreased cell growth and induced apoptosis in HeLa cell lines through stabilization of p53, decreased degradation, and increased nuclear localization (10). Interestingly, EHMT2 methylates lysine 373 in p53 (21). Potential hypotheses to be examined in future studies would be whether MSX1 cooperates with G9a/EHMT2 in methylating p53 (and related p63 and p73 proteins), leading to changes in cisplatin sensitivity, or whether MSX1/EHMT2-mediated changes in histone H3K9 methylation can directly influence expression of genes associated with drug resistance.

Both tumor cohorts use fresh-frozen tumor that is not microdissected, and heterogeneous cell types within each sample may confound overall gene expression data, although will be expected to have less impact on DNA methylation data. There was no correlation between methylation data in the Hammersmith cohort and the percentage of tumor nuclei, therefore there is no bias in the proportions of tumor and normal cells in biopsies influencing the association observed (data not shown).

A limitation of the TCGA data is that the primary chemotherapy response was not defined by RECIST criteria and so

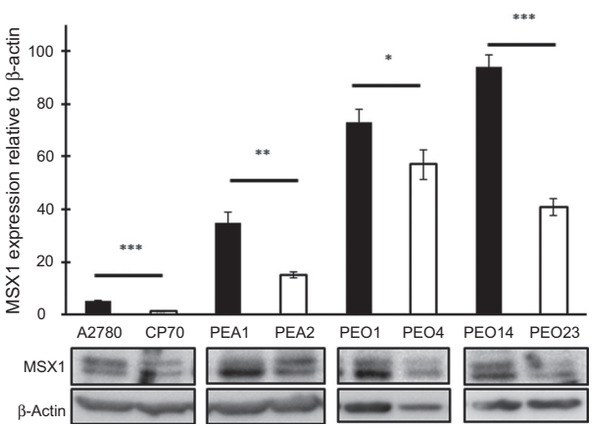

Figure 2.
MSX1 gene expression in cisplatin-sensitive (black bar) and -resistant (white bar) cell line pairs. RNA was isolated from the different cell lines, and *MSX1* gene expression was analyzed and normalized against *β-actin*. Results from qRT-PCR are shown in the top graph and from Western blot analysis of protein expression beneath. Statistical analysis was performed using SEM and Student *t* test (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.005$).

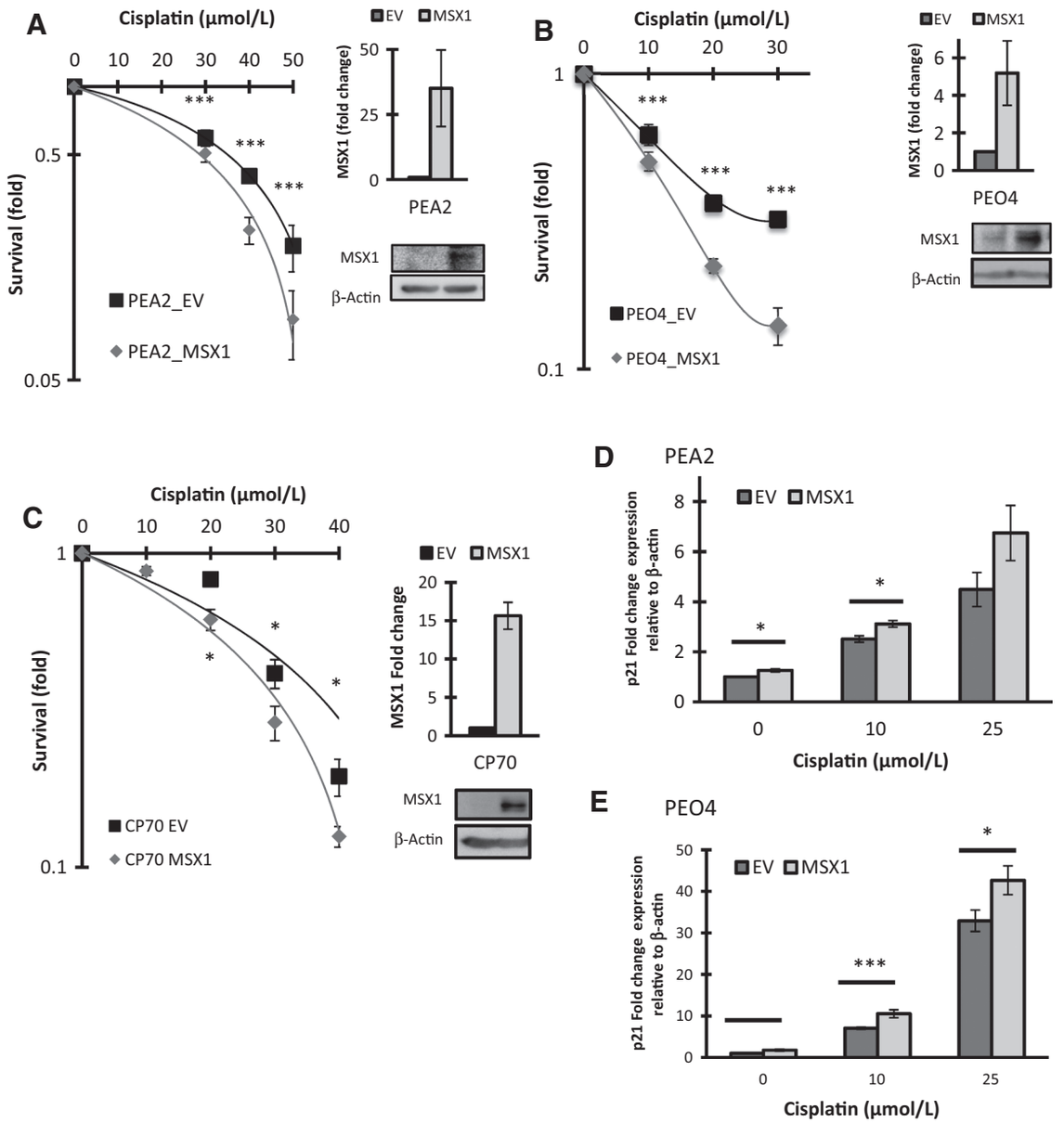

Figure 3.
MSX1 overexpression resensitizes cells to cisplatin treatment. MSX1 stable-transfected cell lines compared with EV transfectants of PEA2 (A), PEO4 (B), and CP70 (C) were treated with cisplatin for 24 hours. MTT assay was performed 48 hours after treatment. Data are representative of at least three independent experiments. RNA and protein levels showing MSX1 overexpression shown inset. D, PEA2 and E, PEO4 cells were treated with the indicated cisplatin concentrations for 24 hours. p21 RNA levels were analyzed relative to β-actin. Statistical analysis was performed using SEM and Student *t* test (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.005$).

could not be used to validate the association with response observed in the Hammersmith cohort. Instead, we have used PFS to validate the associations observed. PFS of less than 6 months is widely used to clinically define platinum-resistant disease, whereas PFS greater than 12 months is often used to define platinum-sensitive disease (13, 22, 23). Analysis of the TCGA data validated the association with PFS observed in the Hammersmith cohort at 4 CpG *MSX1* sites, with others showing similar trend. Consistent with the Hammersmith cohort, CpG sites demonstrated a corresponding lower level of methylation in patients with PFS < 6 months. It should be noted that the TCGA data represent analysis of tumors collected across multiple clinical sites, and the robustness and reliability of clinical data from multiple centers may not be as well controlled as in the single site Hammersmith data. Indeed, analysis of the TCGA data does not show some of the expected clinical correlations, such as association between survival and surgical debulking (data not shown).

We have identified methylation and expression of MSX1 as a biomarker of lack of response to primary chemotherapy

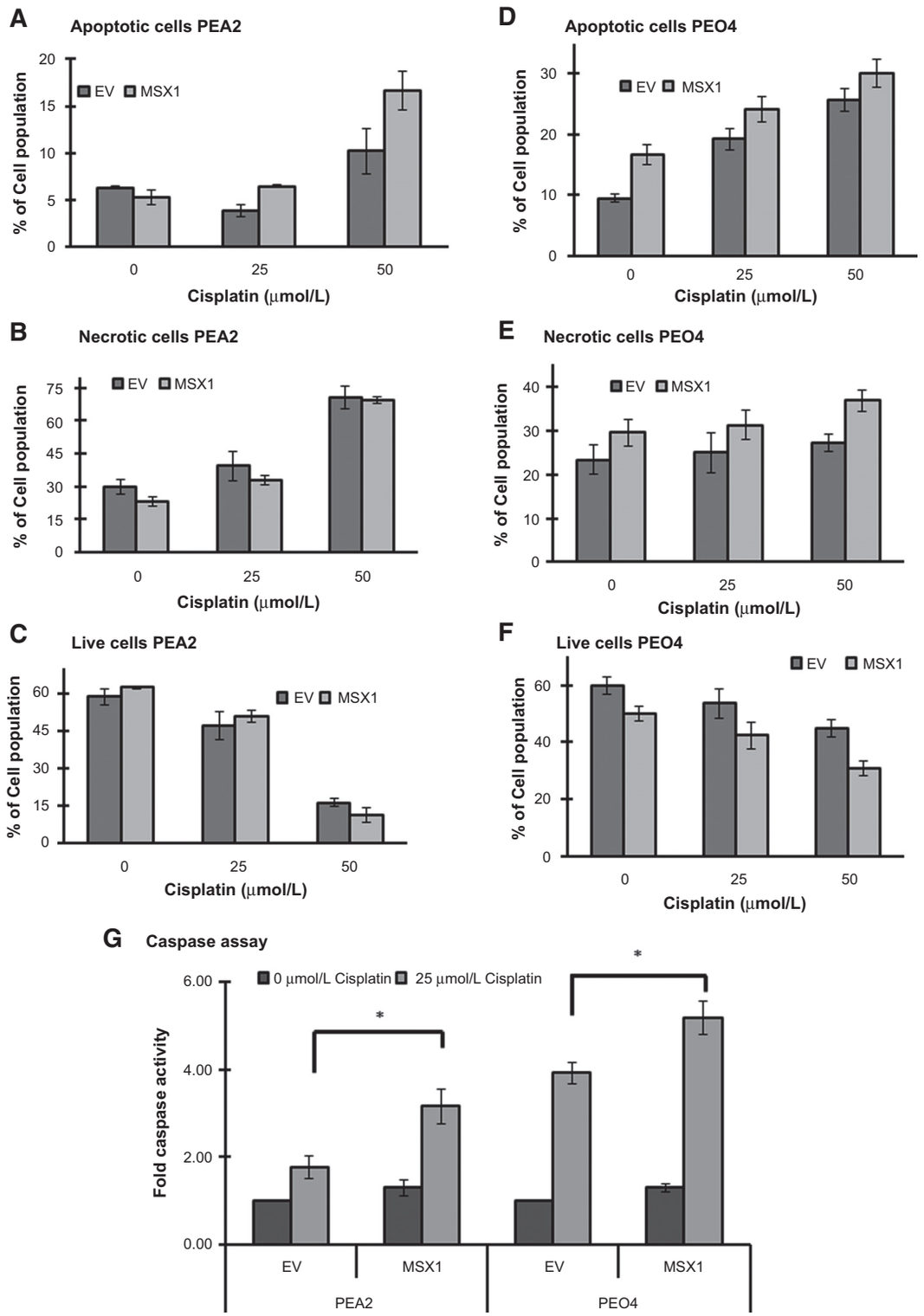

Figure 4.
Cisplatin-induced apoptosis in MSX1-overexpressing lines. PEA2 (A, B, and C) and PEO4 (D, E, and F) were treated with cisplatin for 24 hours at the represented concentrations. A–F, cells were collected and stained with FITC-Annexin V and with PI and read by flow cytometer. Early apoptotic cells were positively stained to FITC-Annexin V and negative to PI (A and D), whereas necrosis were positive to both (B and E), and live cells were positive to both (C and F). G, caspase activity was measured after one hour incubating cells with Caspase-Glo 3/7 Reagent. The caspase activity is represented as fold increase to the untreated control. Statistical analysis was performed using SEM and Student *t* test (*, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.005$).

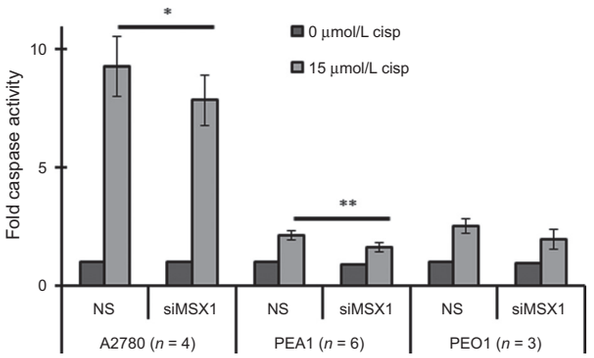

Figure 5.
Cisplatin (cisp)-induced apoptosis following MSX1 knockdown. Caspase activity following cisplatin treatment of cells transfected with AllStar siRNA nonspecific control (NS) or MSX1 siRNA (siMSX1) (*, $P < 0.05$; **, $P < 0.01$).

in HGSOC. Clinical stratification based on such biomarkers could enable alternative treatment stratification for these patients at presentation or of maintenance therapies during remission. The functional role of MSX1 and its role potentially in epigenetic regulation or methylation of cellular proteins such as p53 need further in-depth study.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: N.A. Bonito, J. Borley, S. Ghaem-Maghami, R. Brown
Development of methodology: N.A. Bonito, C.S. Wilhelm-Benartzi, S. Ghaem-Maghami, R. Brown
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N.A. Bonito, J. Borley, S. Ghaem-Maghami
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N.A. Bonito, J. Borley, C.S. Wilhelm-Benartzi, R. Brown
Writing, review, and/or revision of the manuscript: N.A. Bonito, J. Borley, C.S. Wilhelm-Benartzi, S. Ghaem-Maghami, R. Brown
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Ghaem-Maghami, R. Brown
Study supervision: S. Ghaem-Maghami, R. Brown

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