Epigenetic regulation of the homeobox gene MSX1 associates with platinum resistant disease in high grade serous epithelial ovarian cancer


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Running Title: MSX1 DNA methylation and platinum resistant ovarian cancer

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**Translational Relevance**

Although considered a chemo-responsive 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 homeobox $MSX1$ gene 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 expression is associated with progression-free survival of patients. Increased expression of $MSX1$ in three platinum-resistant ovarian cancer lines led to cisplatin sensitisation 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.
Abstract

Purpose: Although High Grade Serous Ovarian Cancer (HGSOC) is frequently chemo-responsive, a proportion of patients do not respond to platinum-based chemotherapy at presentation or have progression-free survival 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 tumour 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 to 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 progression-free survival (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 over-expression leads to cisplatin sensitisation, 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.
Introduction

Epithelial ovarian cancer (EOC) is the second commonest, but most lethal, of the gynaecological malignancies, with overall 5 year survival rates around 45% [Reviewed in (1, 2)]. Adjuvant chemotherapy is recommended for those with stage 1C 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 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 regimes 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 which 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 non-cytotoxic, 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 to RNA. Furthermore, DNA methylation measurements can be compared to absolute reference points (for instance, completely methylated or completely unmethylated DNA), which provides a quantitative assays (5). Additionally, tumour DNA methylation can be detected in body fluids of patients, potentially leading to measurement of tumour-derived biomarkers non-invasively (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 in high grade serous ovarian cancer (HGSOC) using Illumina 27K HumanMethylation array data. This analysis identified methylation at the Homebox gene MSX1 as associated with platinum resistant disease which we aimed to validate in independent HGSOC cohorts for associations with response and progression free survival. Dysregulation of HOX genes have been associated with a variety of human cancers [summarised in (7)]. Msh homeobox 1 (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 induction of apoptosis in HeLa cancer cells (10). We have examined the role of MSX1 in chemosensitivity by gene reintroduction into

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platinum resistant ovarian tumour cell lines that have reduced MSX1 expression compared to matched parental, non-platinum exposed, lines.
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”). Tumours selected were from primary debulking surgery (no previous chemotherapy), advanced stage disease (FIGO stage 3 and 4) with serous histology. Tumour samples selected were from patient treated between 1999 and 2010, although 76% of patient samples were from 2006 onwards. All surgery was performed at the West London Gynaecology Cancer Centre, UK. Tumours were selected sequentially from the Tissue Bank. Patients were excluded with mixed cell or borderline pathology. In order 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 tumour cell content.

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

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 post-surgery but prior to adjuvant chemotherapy, and after 6 cycles of chemotherapy. Patients were categorised into those who responded to chemotherapy (complete or partial response), stable disease or progressive disease.

The clinical characteristics of the Hammersmith Cohort and the The Cancer Genome Atlas 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 are shown in Supplementary Figure S1.

Bisulphite conversion

Up to 500mg of tumour tissue was used per sample and DNA extracted by the chlorinated Nucleon extraction method (Gen-Probe Life Sciences Ltd.) and resuspended in TE buffer (10mM Tris, 1mM EDTA, pH8). Tumour DNA was bisulphite converted using the EZ-96 DNA Methylation™ Kit (Zymo) as per manufacturers protocol. 1μg of genomic DNA was used for each sample. Successful bisulphite 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 50ng/μl. Methylation data was 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 was log transformed to achieve a normal distribution. Probes with a detection p value of >0.05 were removed. Quality control checks were performed through Genome Studio. The 27K Illumina data has been deposited into GEO under GSE75414.

TCGA dataset analysis

Illumina Human Methylation27 Beadchip data on HGSOC from The Cancer Genome Atlas data portal (http://cancergenome.nih.gov/dataportal) (“TCGA Cohort”) was 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 was pre-processed and normalised across samples.

Plasmid transfection

Cell lines used are described in Supplementary Table 2. Cell lines were obtained and used within 6 months from Department of Surgery and Cancer 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 obtain 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 500µg/ml of Geneticin® (Life technologies). The siRNA oligos (Qiagen) were transfected using Lipofectamine RNAiMAX (Invitrogen, Paisley, UK) according to the manufacturer’s instructions.

Real Time-PCR and gene expression profiling

To analyse gene expression, two-step reverse transcription PCR was performed. Total RNA was extracted using the QIAshredder and RNAeasy Mini Kits (Qiagen) according to
manufacturers 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 recommendations using 10 ng of converted RNA. The data was analysed using the ∆∆Ct method.

Protein analysis

Proteins were isolated using RIPA lysis buffer: 93.2mM di-sodium hydrogen phosphate, 6.8mM sodium di-hydrogen phosphate, 0.5% (v/v) Triton X-100, 0.5mM EDTA pH8, 1mM sodium orthovanadate, 100 U/ml aprotinin, 10 µg/ml leupeptin and 1mM phenylmethylsulphonyl fluoride. Western blots were incubated with MSX (G116- Cell Signal technology). β-actin (abcam) was used as loading control.

Annexin V assay

Cells were seeded the day before treatment at 200,000 cells/well and treated for 24h after which they were washed twice with PBS and detached from the plate using TrypLE™ Express reagent (ThermoFisher). Cells were then stain with FITC-Annexin V/PI kit (BD Pharmingen) according with the suggested protocol. Cells were read using FACS Canto (BD bioscience) immediately after staining and analysed using FlowJo software v8.8.9.

Caspase 3/7 Glo assay

To quantify caspase activity we have used Caspase Glo 3/7 assay (Promega) following the manufacture’s instructions. Briefly, 10,000 cells were let seed overnight after which they were treated with the indicated cisplatin concentrations for 24h 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 lab tech).

Cell proliferation assays

Cells were seeded at 10,000 cells per well in triplicate, after 24 hours cells were treated with cisplatin at concentrations of 0 µM to 50 µM for 24 hours. Cell proliferation was quantified with MTT assay after a further 48 hours of culture after 24 hours of cisplatin treatment using CellTitre 96 Aqueous One Solution Proliferation Assay (Promega).
Statistics

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 in order to determine significant differentially methylated loci and associated differential gene expression of Affymetrix gene expression data with RECIST chemotherapy response, and PFS <6months versus >12months in both datasets. A significant relationship between TCGA DNA methylation and gene expression in the tumour tissue was determined through Spearman correlation.

For gene expression using FastSYBR Green, the standard error of mean (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 progressive disease and short progression free survival

Discovery analysis across all loci on the 27K Illumina bead array, comparing HGSOC patients from a Hammersmith Cohort (n=78) with progressive disease (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 and Figure 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 (Figure 1). For 7 out of 8 significant probes methylation was significantly lower in the group with progressive disease versus those with response to chemotherapy or stable disease, with a methylation difference of >15% (Supplementary Figure S2). Progression-free survival (PFS) of less than 6 months is widely used to clinically define platinum resistant disease, while 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 to after 12 months (p<0.05, q<0.05), with the remaining two showing a similar trend (Table 1 and Supplementary Figure 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 was available in the TCGA dataset and methylation data available for 7 of the 8 MSX1 probes identified from the Hammersmith Cohort (Table 1 and Figure 1). Linear regression models adjusting for array batch, grade and residual disease found methylation levels at 4 probes to be significantly associated with <6 month versus >12 month 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 progression-free survival

DNA methylation and gene expression data was 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 and Figure 1). MSX1 expression and progression free survival (PFS) were significantly associated in the TCGA cohort dataset using univariate Cox proportional hazard regression analysis (Hazard Ratio, 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 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 sensitises 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 to their platinum sensitive pair as determined by qRT-PCR and Western analysis (Figure 2). However, no significant difference in DNA methylation could be detected between the pairs of cell lines (data not shown). In order to examine whether increased MSX1 expression would increase the cells 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 Figure 3, all three ovarian cancer cell lines transfected with MSX1 showed an increase in cisplatin sensitivity compared to 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 analysed by qRT-PCR. Overexpression of MSX1 increases the expression of p21 after cisplatin treatment, when compared to the EV transfectants (Figure 3). However,
cell cycle analysis of the MSX1 over-expressing PEA2 and PEO4 cell lines showed no difference in proportion of cells in G1, S or G2 phases of the cell cycle following cisplatin treatment (Supplementary Figure S3). Using Annexin V staining and uptake of propidium iodide apoptotic, necrotic and live cells were identified following cisplatin treatment (Figure 4A-F). This showed a clear increase in the proportion of apoptotic cells in MSX1 over-expressing cells. This was further confirmed using caspase activity assays (Figure 4G).

To further support the involvement of MSX1 in cisplatin resistance, we examined the effect of siRNA knockdown on cisplatin induced apoptosis. Transient siRNA knock-down 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 (Figure 5).
Discussion

In independent tumour 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 progression free survival in HGSOC. Increased methylation significantly correlates with increased gene expression. MSX1 encodes a member of the muscle segment homeobox gene family and its expression is associated with epithelia-mesenchymal interactions during embryogenesis (18). 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 (19). 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 (EMT) which has been implicated in resistance of ovarian tumours to chemotherapy (20).

Binding of MSX1 in the genome of myoblasts promotes enrichment of the repressive H3K9me2 mark via recruitment of EHMT2 (G9a) histone methyltransferase (21), 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 stabilisation. Previous studies have suggested that MSX1 decreased cell growth and induced apoptosis in HeLa cell lines through stabilisation of p53, decreased degradation and increasing nuclear localisation (10). Interestingly, EHMT2 methylates lysine 373 in p53 (22). 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 tumour cohorts use fresh frozen tumour that is not microdissected and heterogenous 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 tumour nuclei, therefore there is no bias in the proportions of tumour 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 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, while PFS greater than 12 months is often used to define platinum sensitive disease (13, 24). 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 represents analysis of tumours collected across multiple clinical sites and the robustness and reliability of clinical data from multiple centres 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 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 needs further in-depth study.
References


Figure Legends

Figure 1: Schematic of MSX1 locus showing CpG sites associated with clinical outcome.

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 analysed and normalised against β-actin. Results from qRT-PCR are shown in the top graph and from Western analysis of protein expression beneath. Statistical analysis was performed using standard error of the mean (SEM) and Student t-test (* - P<0.05, ** - P<0.01 and *** - P<0.005).

Figure 3. *MSX1* overexpression re-sensitise cells to cisplatin treatment. *MSX1* stable transfected cell lines compared to empty vector (EV) transfectants of PEA2 (A.) PEO4 (B.) CP70 (C.) were treated with cisplatin for 24h. MTT assay was performed 48h after treatment. Data is representative of at least three independent experiments. RNA and protein levels showing *MSX1* over-expression shown in insert. D. PEA2 and PEO4 cell were treated with the indicated cisplatin concentrations for 24 hours. p21 RNA levels were analysed relative to β-actin. Statistical analysis was performed using standard error of the mean (SEM) and Student t-test (* - P<0.05, ** - P<0.01 and *** - P<0.005).

Figure 4 Cisplatin induced apoptosis in MSX1 over-expressing lines. PEA2 (A. B. C.) and PEO4 (D. E. F.) were treated with cisplatin for 24h 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 are positively stained to FITC-Annexin V and Negative to PI (A. D.) while Necrosis are positive to both (B. E.) and live cells were positive to both (C. F.). G. Caspase activity was measured after one hour incubating cells with Caspase Glo reagent. The caspase activity is represented as fold increase to the untreated control. Statistical analysis was performed using standard error of the mean (SEM) and Student t-test (* - P<0.05, ** - P<0.01 and *** - P<0.005).

Figure 5. Cisplatin induced apoptosis following MSX1 knockdown. Caspase activity following cisplatin treatment of cells transfected with Allstar siRNA non-specific control (NS) or MSX1 siRNA (siMSX1).
Figure 1. Schematic of MSX1 locus showing CpG sites associated with clinical outcome

- **Linear regression model associating differential DNA methylation with chemotherapy REClST response or PFS, p<0.05**
- **Spearman correlation, p<0.05, decreased methylation associated with decreased gene expression**
- **Data not available**
- **Not significant**
Figure 2. MSX1 gene expression in cisplatin sensitive (Black bar) and resistant (white bar) cell line pairs.
Figure 3. MSX1 overexpression re-sensitise cells to cisplatin treatment

A

Cisplatin [µM]

Survival (fold)

0.05

0.5

PEA2_EV

PEA2_MSX1

B

Cisplatin [µM]

Survival (fold)

0.1

PEO4_EV

PEO4_MSX1

C

Cisplatin [µM]

Survival (fold)

0.1

CP70 EV

CP70 MSX1

D

PEA2

Cisplatin [µM]

p21 fold change expression relative to β-actin

0

2

4

6

8

EV

MSX1

E

PEO4

Cisplatin [µM]

p21 fold change expression relative to β-actin

0

10

20

30

40

50

EV

MSX1

***

*

*
Figure 4 Cisplatin induced apoptosis in MSX1 over-expressing lines

A. Apoptotic cells PEA2

B. Necrotic cells PEA2

C. Live cells PEA2

D. Apoptotic cells PEO4

E. Necrotic cells PEO4

F. Live cells PEO4

G. Caspase assay

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 5. Cisplatin induced apoptosis following MSX1 knockdown.
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NA Data not available

HH: Hammersmith Cohort

TCGA: The Cancer Genome Atlas Cohort
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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.