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ORIGINAL ARTICLE

Epigenetic silencing of HSulf-1 in ovarian cancer:implications in chemoresistance

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To investigate the mechanism by which HSulf-1 expression is downregulated in ovarian cancer, DNA methylation and histone acetylation of HSulf-1 was analysed in ovarian cancer cell lines and primary tumors. Treatment of OV207 and SKOV3 by 5-aza-2′-deoxycytidine resulted in increased transcription of HSulf-1. Sequence analysis of bisulfite-modified genomic DNA from ovarian cell lines and primary tumors without HSulf-1 expression revealed an increase in the frequency of methylation of 12 CpG sites in exon 1A. Chromatin immunoprecipitation assays showed an increase in histone H3 methylation in cell lines without HSulf-1 expression. To assess the significance of HSulf-1 downregulation in ovarian cancer, OV167 and OV202 cells were transfected with HSulf-1 siRNA. Downregulation of HSulf-1 expression in OV167 and OV202 cells lead to an attenuation of cisplatin-induced cytotoxicity. Moreover, patients with ovarian tumors expressing higher levels of HSulf-1 showed a 90% response rate (27/30) to chemotherapy compared to a response rate of 63% (19/30) in those with weak or moderate levels ($P = 0.0146$, χ^2 test). Collectively, these data indicate that *HSulf-1* is epigenetically silenced in ovarian cancer and that epigenetic therapy targeting HSulf-1 might sensitize ovarian tumors to conventional first-line therapies.
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Keywords: methylation; histone modification; tumor suppressor; chemoresponse; apoptosis

Introduction

DNA methylation and covalent modification of histone proteins are two epigenetic modifications important in transcriptional control (Jones and Baylin, 2002). Hypermethylation of CpG-rich sequences present in the promoters of genes is associated with gene silencing. Epigenetic silencing of tumor suppressor genes is at least as common as mutation as a mechanism of gene inactivation. A broad spectrum of genes belonging to different classes of activity such as DNA repair (Esteller *et al*., 2002), cell cycle control (Xing *et al*., 2004), signal transduction (Terasawa *et al*., 2004), angiogenesis (Yang *et al*., 2003) and invasion (Seidl *et al*., 2004) are inactivated by DNA methylation in cancer cells. The second layer of epigenetic transcriptional control is histone modification such as acetylation, methylation, phosphorylation and ubiquitination (LaVoie, 2005). Of these modifications, acetylation is controlled by histone acetyltransferases and histone deacetylases (HDACs) which control gene expression by remodeling the nucleosomes leading to either transcriptional activation or repression, respectively (Kondo *et al*., 2004). Two chemical agents commonly used to modulate the expression of silenced genes in cancer cells are 5-aza-2′-deoxycytitdine (5-aza-dC), which is an inhibitor of DNA methylation and trichostatin A (TSA), an inhibitor of histone deacetylation (Marks *et al*., 2001; Johnstone, 2002; Yamashita *et al*., 2002). Gene expression studies revealed that the effects of 5-aza-dC are similar to those of TSA exposure than either of the somatic cell DNA methyltransferase knockouts (*DNMT* knockouts), implying a converging mechanism for these agents (Gius *et al*., 2004).

We have identified recently HSulf-1 as a downregulated gene in several tumor types including ovarian and breast cancer cell lines and primary ovarian tumors (Lai *et al*., 2003, 2004a, b). Our initial analysis indicated that loss of heterozygosity (LOH) may represent a mechanism of HSulf-1 inactivation in ovarian tumors. However, this mechanism alone could not account for complete loss of *HSulf-1* expression in cell lines and in some primary tumors (Lai *et al*., 2003) as it would require deletion of both alleles. Therefore, additional mechanisms of inactivation could also play a role in inactivating HSulf-1 expression. Our sequence analysis of *HSulf-1* promoter revealed putative CpG-rich sequences in Exon 1A (Lai *et al*., 2003). This fragment

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(Z58846) was also identified as a putative CpG-rich sequence in previous study (Cross *et al*., 1994). In this study, we report on the methylation status of this putative CpG island in ovarian cell lines and primary ovarian tumors by direct sequencing of the bisulfite-modified DNA. In addition, we also performed chromatin immunoprecipitation (ChIP) (Orlando, 2000) assays to analyse the acetylation status of HSulf-1 promoter. Our results also implicate HSulf-1 in modulating the chemoresponse to cisplatin and provide the evidence of CpG and histone methylation as a mechanism of *HSulf-1* inactivation.

Results

Treatment with a demethylating agent and/or a histone deacytelase inhibitor can reactivate HSulf-1 expression in ovarian cancer cell lines

To examine the relative contributions of DNA methylation and histone deacetylation in silencing of HSulf-1 expression, we determined the effects of treatment with a demethylating agent 5-aza-dC, in ovarian cancer cell lines SKOV3 and OV207 without HSulf-1 expression (Figure 1a). Semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR) results indicated that treatment with 5-aza-dC resulted in reactivation of HSulf-1 transcription in SKOV3 and OV207 cells (Figure 1b and c). In addition, treatment of OV207 cells with 5-aza-dC in combination with TSA resulted in an increase in the reactivation of HSulf-1 expression compared to 5-aza-dC treatment alone (Figure 1d). These results suggest that gene silencing as a result of CpG methylation also involves recruitment of chromatin modifying factors such as HDACs.

Methylation-associated silencing of HSulf-1 in ovarian cell lines and primary ovarian tumors

Primers encompassing 12 CpG sites within the CpG-rich region in Exon 1A of HSulf-1 (Cross *et al*., 1994; Lai *et al*., 2003) were designed to amplify and sequence the bisulfite-modified DNA as described previously (Herman *et al*., 1996; Shridhar *et al*., 2001). Overall methylation was determined for a subset of cell lines and primary ovarian tumors (fresh frozen) with or without HSulf-1 expression. HSulf-1-deficient cell lines such as OV207, SKOV3OVCAR3 and OVCAR5 and primary ovarian tumors (OV34, 45, 97, 102, 121, 235, 259, 518 and 731) exhibited extensive methylation of the CpG island (Table1 and Figure 2a and b). However, CpG sites 7–11 were unmethylated in the HSulf-1-expressing cell lines (VOSE, OV167, OV202), and frequently hemimethylated in primary ovarian tumors (OV522, OV715 and OV220) (Table 1). Subsequently, sequencing of bisulfite-modified DNA in nonexpressing cells treated with 5-aza-dC revealed methylation of sites 1–4 and complete demethylation of sites 9 and 11 (in OV207), sites 6–8 (in SKOV3) and site 10 (in OVCAR5). These results suggest that methylation status of CpG sites 5–12 could reflect the transcriptional silencing of HSulf-1. Both semi-quantitative and RT–PCR results of tumors and cell lines are also included in Table 1.

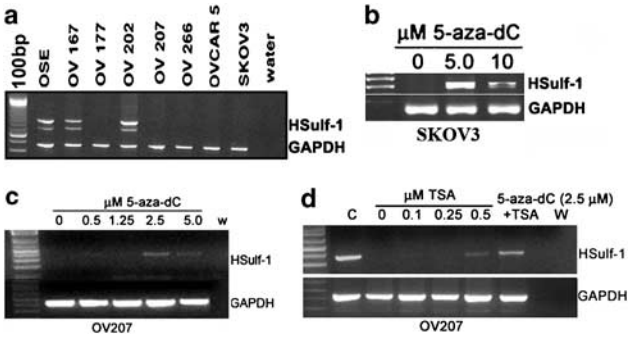

Figure 1 HSulf-1 expression in ovarian cell lines and in OV207 treated with 5-aza-dC and TSA. (**a**) RT–PCR products of HSulf-1 and control GAPDH were visualized by agarose gel electrophoresis with ethidium bromide in ovarian cancer cell lines. (**b**–**c**) Expression of the HSulf-1 gene is induced with 2.5 μM 5-aza-dC and 0.5μM TSA treatments. A synergistic effect of 2.5 μM 5-aza-dC and 0.5 μM TSA combined treatment results in increased expression of HSulf-1. w, water.

Methylation of the CpG island was associated with chromatin change

In OV207, treatment with 5-aza-dC plus TSA resulted in robust HSulf-1 expression (Figure 1c), indicating the potential involvement of DNA and histone methylation. To investigate this, ChIP assays were used to assess the acetylation/methylation status before and after 5-aza-dC or TSA treatment. The diagrammatic representation of HSulf-1 CpG island is shown in Figure 3a. ChIP analysis of OV207 cells treated with 5-aza-dC or TSA exhibited increased histone acetylation, compared with untreated OV207 (Figure 3b). Quantification of the histone acetylation levels is indicated in the histogram (Figure 3c). To compare the acetylation and methylation status in cell lines, ChIP analyses were performed in OV207, SKOV3 and OV202 cells using three set of primers encompassing the CpG island. OV207 and SKOV3 cell lines, which do not express HSulf-1, showed an increase in the levels of histone H3 K9 methylation (Figure 3d and e) compared to OV202 cell line (Figure 3f). HSulf-1 expressing OV202 showed >4-fold decrease in methylation of chromatin (histone H3 at K9) compared to OV207 or SKOV3 (Figure 3g). PCR-optimized ChIP analysis of H3 acetylation across cell lines revealed >4 increase in H3 acetylation in OV202 compared to SKOV3 cells (Figure 3h and i). Collectively these results indicate that HSulf-1 expression is under epigenetic control.

Re-expression of HSulf-1 by demethylating agent sensitizes cancer cells to cisplatin treatment

Our previous study showed that HSulf-1 expression conferred sensitivity to cisplatin and taxol (Lai *et al*., 2003, 2004b). HSulf-1 represents a potential target of epigenetic therapy to re-sensitize cancer cells to cisplatin and taxol. Epigenetic therapy has been successfully used *in vitro* to overcome drug resistance (Balch *et al*., 2004).

Table 1 HSulf-1 methylation analysis in ovarian cell lines and primary ovarian tumors

Sample	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*RT–PCR*	*PCR* ΔC_t	*LOH*
(A) Analysis of the methylation status of sites 1–12 within HSulf-1 exon 1A in ovarian cell lines and primary ovarian tumors and its corresponding expression levels															
Ovarian cell lines															
VOSE	●	●	●	●	⊖	○	○	○	○	○	○	●	+	4.6	ND
OV 167	●	●	●	●	○	○	○	○	○	○	○	●	+	5.49	ND
OV 202	●	●	●	●	○	○	○	○	○	○	○	●	+	5.43	ND
OV 207	●	●	●	●	⊖	●	⊖	●	●	○	●	●	−	18.1	ND
OVCAR 3	●	●	●	●	●	●	●	●	●	●	●	●	−	10.33	ND
OVCAR 5	●	●	●	●	●	●	●	●	●	●	●	●	−	15.19	ND
SKOV3	●	●	●	●	●	●	⊖	○	●	●	●	●	−	14.44	ND
Primary ovarian tumors															
OV 102	●	●	●	●	●	●	●	○	●	●	●	●	−	10.45	+
OV 518	●	●	●	●	●	●	●	●	●	●	●	●	ND	ND	ND
OV 522	●	●	●	●	⊖	●	⊖	⊖	⊖	⊖	⊖	⊖	+	3.26	ND
OV 687	●	●	●	●	●	●	●	●	●	⊖	⊖	●	−	8.15	ND
OV 715	●	●	●	●	●	●	●	⊖	⊖	⊖	⊖	⊖	+	3.82	ND
OV 731	●	●	●	●	●	●	●	●	●	⊖	●	●	−	ND	ND
OV 734	●	●	●	●	●	●	●	●	●	⊖	⊖	●	−	9.91	ND
OV 34	●	●	●	●	●	●	●	●	●	●	●	●	−	6.17	ND
OV 45	●	●	●	●	●	●	●	○	●	●	●	●	−	7.88	ND
OV 97	●	●	●	●	⊖	●	●	⊖	●	●	⊖	●	−	10.73	−
OV 98	●	●	●	●	⊖	●	●	⊖	●	⊖	⊖	●	weak	5.78	+
OV 121	●	●	●	●	●	●	●	●	●	●	●	●	−	13.08	−
OV 183	●	●	●	●	●	●	●	⊖	●	●	●	●	−	6.99	+
OV 220	●	●	●	●	⊖	●	⊖	⊖	⊖	●	●	⊖	+	5.07	−
OV 235	●	●	●	●	●	●	●	●	●	⊖	⊖	●	−	7.66	ND
OV 259	●	●	●	●	●	●	●	●	●	●	●	●	−	10.98	ND
(B) Analysis of the methylation status of sites 1–12 in OV207, OVCAR5 and SKOV3 before and after treatment with 5-Aza-dC. Empty circles – unmethylated, filled circles-methylated and circles with a line-hemimethylated. HSulf-1 mRNA expression by semi-quantitative RT–PCR and real-time PCR and HSulf-1 LOH status are also indicated.															
OV 207	●	●	●	●	⊖	●	⊖	●	●	○	●	●	−		
OV 207 + 5-Aza-dC	●	●	●	●	⊖	⊖	⊖	●	○	○	○	⊖	+		
OVCAR 5	●	●	●	●	●	●	●	●	●	●	●	●	−		
OVCAR5 + 5-AZA-dC	●	●	●	●	●	●	●	●	⊖	○	●	●	weak		
SKOV3	●	●	●	●	●	●	⊖	○	●	●	●	●	−		
SKOV3 + 5-Aza-dC	●	●	●	●	●	○	○	○	●	●	●	●	+		

Abbreviations: LOH, loss of heterozygosity; ND, not determined; RT–PCR, reverse transcription – polymerase chain reaction

Therefore, we tested whether re-expression of HSulf-1 by epigenetic therapy could overcome drug resistance. HSulf-1 expression was induced by 5-aza-dC treatment in OV207 and followed by cisplatin treatment. Surviving cells were assayed for (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) reduction 3 days following cisplatin treatment. As shown in Figure 4a, induction of HSulf-1 in OV207 cells sensitized these cells to cisplatin-induced cytotoxicity.

Specific contribution of HSulf-1 in conferring chemosensitivity to ovarian cancer cells

As 5-aza-dC treatment also induces several epigenetically silenced genes, we could not fully evaluate the contribution of HSulf-1 re-expression on chemosensitivity. Therefore, we opted to silence HSulf-1 in OV167 and OV202 cells with endogenous HSulf-1 expression. Scrambled (scr) small interfering RNA (siRNA) served as controls (Figure 4b and c). These experiments were repeated twice with the same results. As shown in Figure 4b and c, there was an increase in the survival following cisplatin treatment in the cells pretreated with HSulf-1 siRNA compared with control cells treated with scr siRNA. The specific contribution of HSulf-1 was evaluated by re-expressing HSulf-1 following siRNA-mediated downregulation in OV167 cells. Re-expression of HSulf-1 rescued cisplatin sensitivity in OV167 cells.

Effect of HSulf-1 expression on clinical chemosensitivity

On the basis of our observation that HSulf-1 confers chemosensitivity *in vitro*, we tested whether HSulf-1 expression would correlate with chemoresponse *in vivo*. HSulf-1 expression was determined by RNA *in situ* hybridization using HSulf-1 riboprobes on a tissue microarray (TMA) containing 60 primary epithelial ovarian tumors. Patient characteristics are described in Table 2. All patients had advanced-stage disease, and the majority received optimal debulking surgery. Representative staining intensities corresponding to low, moderate and high levels of HSulf-1 expression are shown in Figure 5. Independently, tumor response to

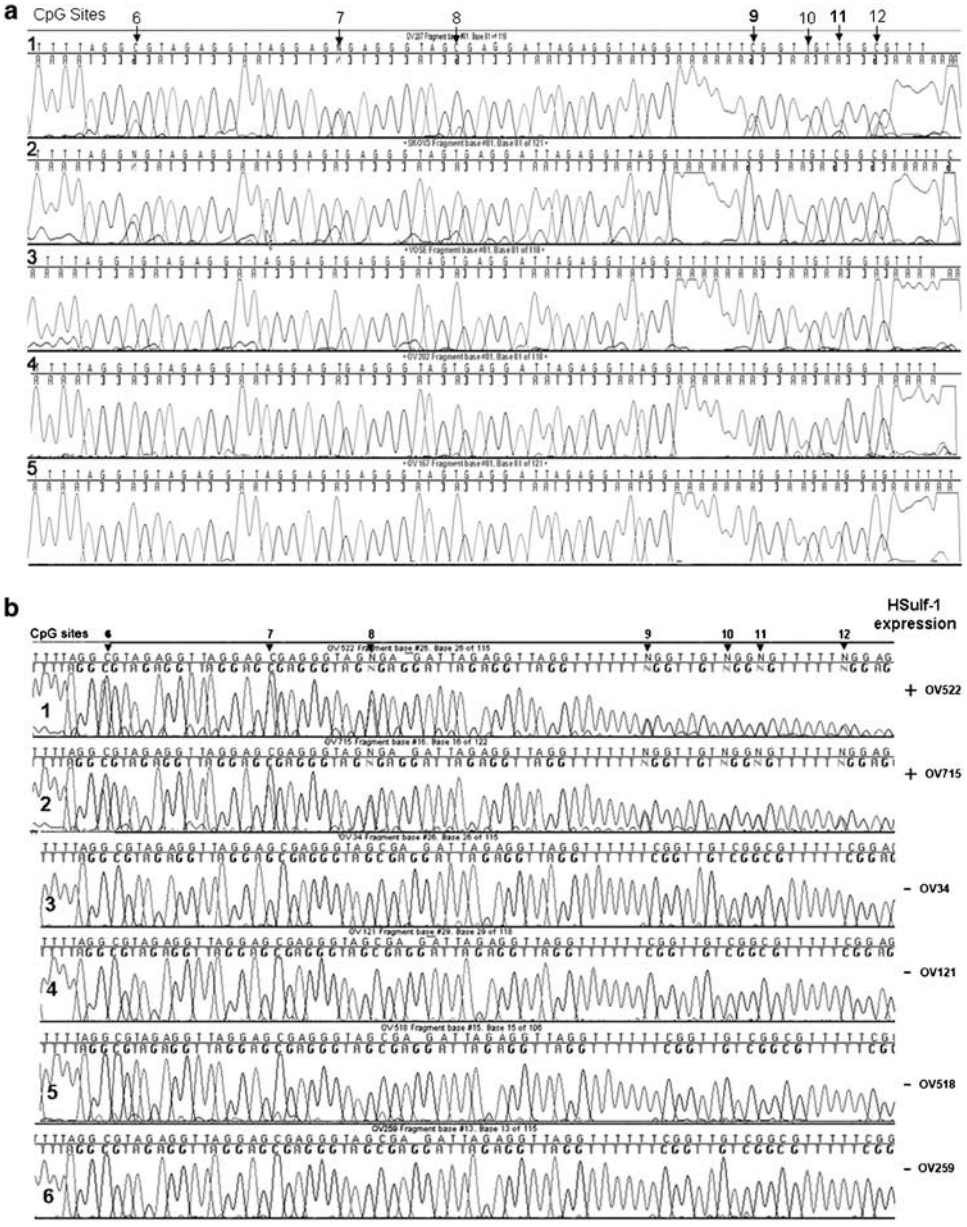

Figure 2 Methylation analysis of HSulf-1 by genomic sequencing of bisulfite modified DNA in ovarian cell lines and primary ovarian tumors. (**a**) Sequence chromatograms of CpG sites 6–12 of bisulfite modified DNA from ovarian cell lines. OV207 and SKOV 3 (1–2) without HSulf-1 expression show methylation of several CpG sites whereas none of these site are methylated in VOSE, OV202 and OV167 (3–5) with HSulf-1 expression. HSulf-1 expression in these cell lines are indicated as + for expression and – for no expression. Sequence chromatograms of CpG sites 6–12 of bisulfite-modified DNA from primary ovarian tumors. Chromatograms 1–2: tumors OV522 and OV715 with HSulf-1 expression by RT–PCR show hemimethylation of several cytosine residues after sodium bisulfite treatment as shown by N in the sequence chromatograms. Chromatograms 3–6: tumor samples (OV34, OV121, OV518 and OV259) without HSulf-1 expression show methylation of all six CpG sites 6–12.

chemotherapy was defined as complete response (CR), partial response (PR) and no response (NR) as described previously (Chien *et al*., 2006). Responders were defined as those patients who showed CR or PR to chemotherapy. Nonresponders were defined as those patients who showed NR to chemotherapy as described above. As there is no statistically significant difference between low-staining group vs moderate-staining group, these two groups were combined and reanalysed against high level staining group by χ^2 test. As shown in Figure 5c, tumors with high levels of HSulf-1 showed 90% (27/30) response rate compared to 63% (19/30) response rate in tumors with low to moderate levels of HSulf-1. The odds ratio for not responding to therapy in cohort with low levels of HSulf-1 was 6.0 (95% confidence interval, 1.481–24.31). These results suggest that HSulf-1 expression influences tumor response to chemotherapy in patients. As all patients analysed had

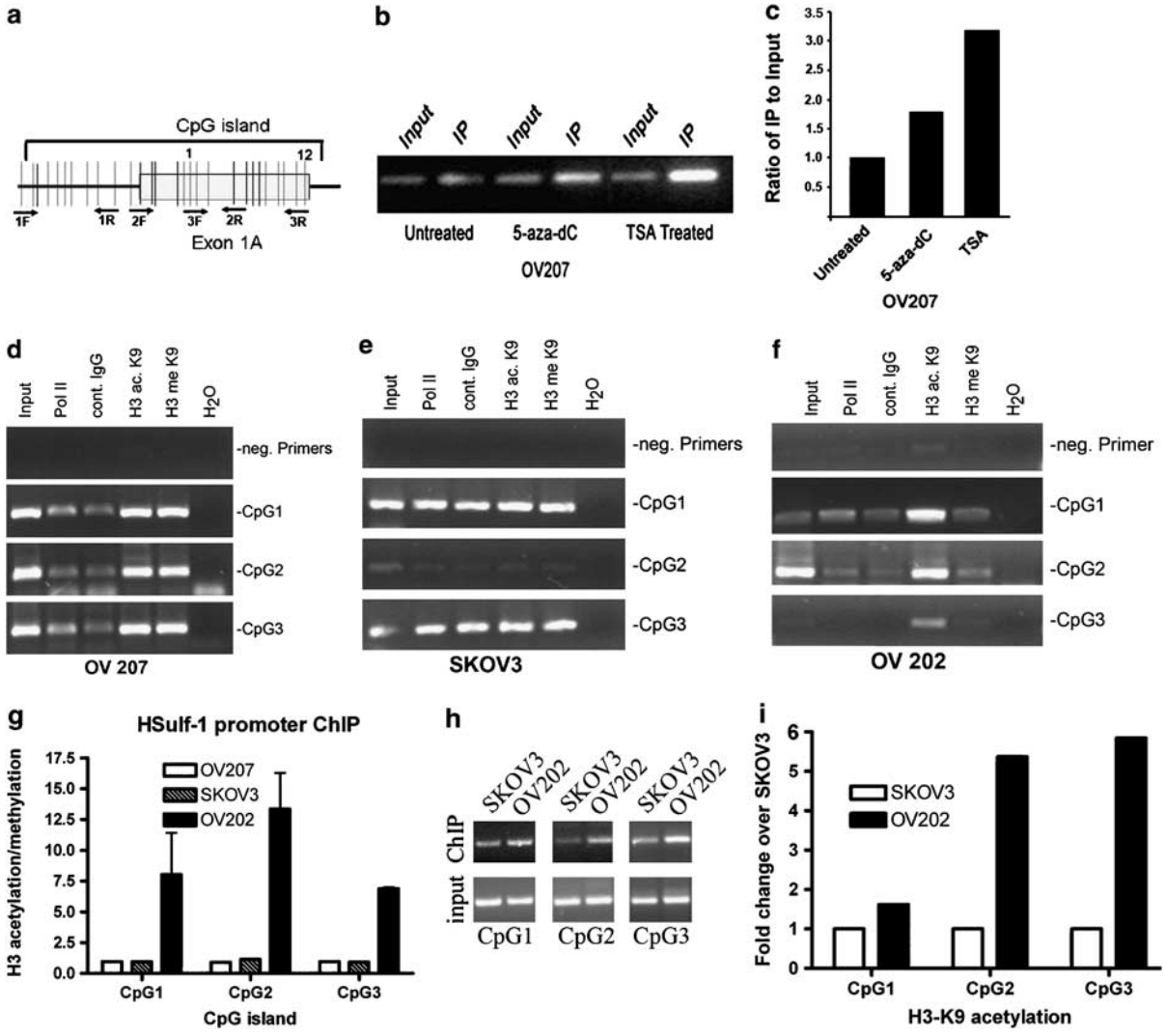

Figure 3 ChIP analysis of histone H3 acetylation associated with HSulf-1 CpG island in ovarian cell lines. (**a**) Diagrammatic representation of HSulf-1 CpG island showing CpG sites (vertical lines), location of 12 CpG sites in Exon 1A and location of primers used in ChIP analysis. (**b**) OV207 cells treated with 5-aza-dC and TSA show increased acetylation as indicated by increased band intensities following ChIP with CpG3 primers. (**c**) Acetylation levels are expressed as the ratio of signal intensity of immunoprecipitated product (acetylated DNA-IP) to input as described in the Materials and methods section. OV207 treated with 5-aza-dC and TSA show increased ratios compared to untreated parental control. (**d**–**e**) ChIP assays using CpG1, CpG2 and CpG3 primers encompassing the HSulf-1 CpG island in OV207 and SKOV3 show the equivalent level of histone H3 acetylation (H3 ac. K9) over methylation (H3 me K9) of chromatin associated with HSulf-1 CpG island. (**f**) ChIP assays indicate higher levels of H3 acetylation compared to methylation in OV202. ChIP assays with anti-RNA polymerase II (Pol II) and control IgG (cont. IgG) represents positive and negative controls, respectively. PCR using negative primers (neg. Primers) flanking a region of genomic DNA between GAPDH gene and CNAP1 gene represents specificity of ChIP reactions. Independent ChIP experiments were repeated at least three times to confirm the reproducibility of these results. (G) The ratios of acetylation/methylation of histone H3 at K9 in OV207, SKOV3 and OV202 were determined by ChIP using CpG1, CpG2 and CpG3 primers encompassing the HSulf-1 CpG island. (H) ChIP analysis using CpG1, CpG2 and CpG3 primers encompassing the HSulf-1 CpG island in OV202 and SKOV3 indicates higher levels of acetylated histone H3 K9 in HSulf-1 expressing OV202 cells compared to HSulf-1-deficient SKOV3 cells. (**i**) Histogram showing fold change in acetylated histone H3 K9 in OV202 cells over SKOV3 cells as determined by ChIP using CpG1, CpG2 and CpG3 primers encompassing the HSulf-1 CpG island.

advanced stage disease with mostly serous histology, no correlation of HSulf-1 expression to stage, grade or histology was performed. The specificity of the riboprobe used in RNA *in situ* was determined on the TMA using a sense probe. Figure 5e and f show the hybridization signal from one of the sections on the TMA with the antisense and the sense probe respectively.

Discussion

Epigenetics is a widely accepted mechanism of silencing gene expression in cancer. Epigenetic modifications of DNA and histones are involved in gene regulation by altering chromatin structure (Fujii *et al*., 2003). In this report we demonstrated that DNA methylation and histone modifications regulate HSulf-1 expression and

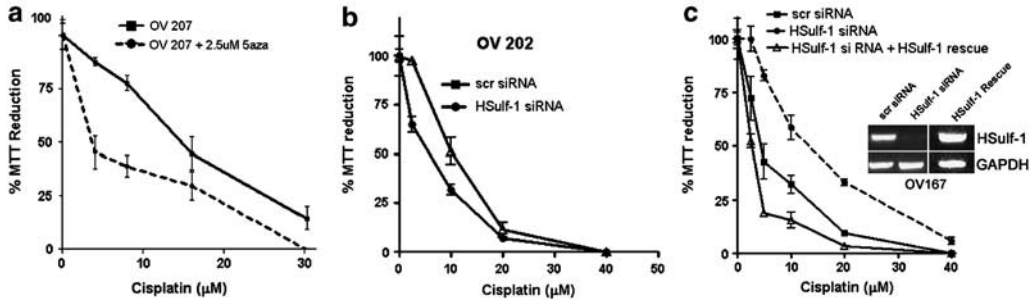

Figure 4 Re-expression of HSulf-1 promotes cisplatin toxicity *in vitro*. (**a**) OV207 cells treated for 48 h with 5-aza-dC, the cells were exposed to varying concentrations of cisplatin for 24 h and assayed by MTT reduction assay. 5-aza-dC-treated OV207 cells were less sensitive to cisplatin treatment compared to untreated controls. (**b** and **c**) Following HSulf-1 suppression by siRNA, OV202 (**b**) and OV167 (**c**) cells were treated with varying concentrations of cisplatin ranging from 2.5 to 40 μM for 24 h, and viable cells assayed by MTT. Suppression of HSulf-1 results in significant attenuation of cisplatin toxicity in OV202 and OV167 cells (HSulf-1 siRNA) (•) compared to scr siRNA-transfected cells (■) or in OV167 cells (**c**) expressing HSulf-1 siRNA + HSulf-1 ORF plasmid (Δ). As siRNA was targeted towards 3′ UTR of HSulf-1, siRNA transfection did not affect exogenous expression of HSulf-1 (Rescue) by plasmid containing HSulf-1 open reading frame. Inset in (**c**) analysis of HSulf-1 expression by RT–PCR indicates that transient expression of siRNA against HSulf-1 effectively suppressed endogenous expression of HtrA1 in OV167 cells (top panel, lane 2). Scr siRNA did not affect endogenous expression of HSulf-1 (lane 1). Level of HSulf-1 following rescue (top panel, lane 3. Lower panel shows GAPDH loading controls.

Table 2 Ovarian cancer patient characteristics

No. subjects	60	
Median age at diagnosis	61	(38–80)
No. alive at the time of analysis	16	(26.66%)
Average length of follow-up[a] (months)	43.7	
Chemotherapy, *n*		
Cytoxan	13	(21.67%)
Taxol	47	(78.33%)
Primary surgery, *n*		
Optimal debulking	53	(88.33)
Suboptimal	7	(11.67)
Stage, *n*		
3	46	(76.66%)
4	14	(23.34%)
Grade, *n*		
2	1	(1.67%)
3	30	(50%)
4	29	(48.33%)
Histology, *n*		
Mixed	1	(1.67%)
Endometrioid	8	(13.33%)
Serous	51	(85%)
Pre-CA125, median (range)	830	(8.2–86 000)
Post-CA125, median (range)	12.05	(4.7–947)

[a]Follow-up refers to time from date of initial surgery to death or to most recent follow-up visit. Pre-CA125, ovarian carcinoma antigen CA125 levels before surgery; post-CA125, ovarian carcinoma antigen CA125 levels after chemotherapy, measured in IU.

provided the first evidence of methylation in combination with chromatin histone modification as a mechanism of HSulf-1 inactivation in ovarian cancer. Re-expression of *HSulf-1* has been shown to inhibit tumor growth (Dai *et al*., 2005; Lai *et al*., 2006; Narita *et al*., 2006). In this study, we analysed the relationship between HSulf-1 induction with 5-aza-dC and chemoresponse of ovarian cancer cell lines *in vitro* and correlated HSulf-1 expression in patient samples to a better therapeutic index.

Nonpromoter CpG-island methylation has been shown to be more susceptible to aberrant methylation (Douglas *et al*., 2004). In addition, the density and the number of methylated CpG dinucleotides can exert transcriptional repression over long distances (Nguyen *et al*., 2001). We therefore analysed the methylation status of the CpG island within Exon 1A. On the basis of data on primary tumors and cell lines before treatment, we conclude that CpG sites 5–12 could play a role in HSulf-1 transcriptional control. We observed an increase in the frequency of methylated CpGs in tumors and cell lines with HSulf-1 loss. On the basis of the data on primary tumors and cell lines before after 5-aza-dC treatment, we conclude that CpG sites 5–12 could play a role in HSulf-1 transcriptional control. Data from Galm *et al*., (2002) suggest that increasing the 5′aza-2′-deoxycytidine (DAC) dose beyond a certain level does not necessarily result in higher demethylating capacity within a given time course, but rather reaches a plateau phase of demethylation .

In addition to this epigenetic modification, we also analysed histone acetylation as another mechanism of epigenetic control in this region as histone hypoacetylation is another commonly occurring epigenetic modification in silenced genes. In many tumors, including ovarian cancers, deacetylation-induced silencing of genes such as *p21CIP1/WAF1* (Blagosklonny *et al*., 2002) and *gelsolin* (Han *et al*., 2000) is well documented (Balch *et al*., 2004). Highly acetylated histones are usually associated with transcriptionally active sequences and hypoacetylated histones with silenced genes. Consistent with these results, we observed higher levels of histone H3 (K9) acetylation over methylation in HSulf-1 expressing OV202. In contrast, higher levels of histone H3 (9) methylation were observed in HSulf-1 silenced OV207 and SKOV3 (Figure 3c). These results suggest that gene silencing as a result of DNA

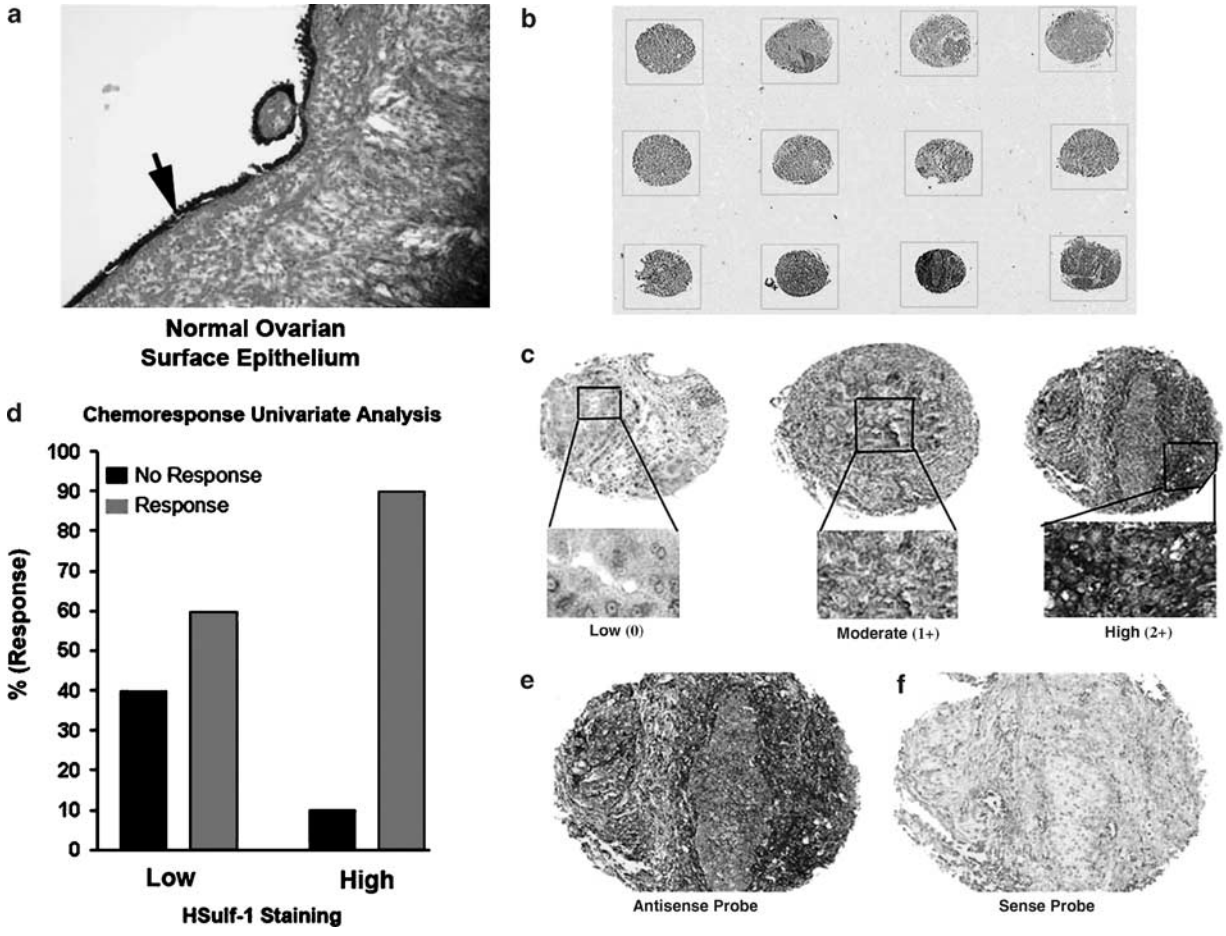

Figure 5 Expression of HSulf-1 correlates with patient response to chemotherapy. (**a**) Normal ovarian surface epithelium shows specific staining for HSulf-1 by RNA *in situ*. (**b**) RNA *in situ* staining pattern in ovarian tumors on a TMA showing low (0), moderate (1+) and high (2+) levels of HSulf-1. (**c**) A small section of TMA with three representative HSulf-1 expression levels is shown. (**d**) Univariate analysis of chemoresponse showing significant association between high levels of HSulf-1 expression and chemoresponse. Tumors with high levels of HSulf-1 responded significantly better than those with low levels as analysed by univariate analysis ($P = 0.0146$). (**e**) A representative tumor on the TMA probed with the antisense HSulf-1 riboprobe. (**f**) The same tumor in E probed with sense riboprobe for HSulf-1. The absence of staining in the sense-probed section indicates the specificity of the hybridization.

methylation also involves recruitment of chromatin modifying factors such as HDACs.

Ovarian cancer, although initially responsive to conventional chemotherapeutic regimens, relapse after a median period of 18 months (Greenlee *et al*., 2001). The ability of a cancer cell to respond to a chemotherapeutic agent is believed to be due, in part, to its apoptotic capacity (Johnstone *et al*., 2002). It is now evident that chemoresistant cells have downregulated proapoptotic genes and/or overexpression of antiapoptotic genes. Several genes involved in apoptotic pathways related to chemotherapeutic action in ovarian cancer are downregulated by epigenetic mechanisms (Makarla *et al*., 2005). Methylation of specific genes such as *hMLH1* has been shown to correlate with the onset of tumor relapse after carboplatin/taxol therapy in ovarian cancer patients (Gifford *et al*., 2004). In addition, treatment of cisplatin-resistant A2780/CP ovarian cancer cells with 5-aza-dC induced the expression of hMLH1 and resensitized these cells to cisplatin in a mouse xenograft model (Plumb *et al*., 2000). Epigenetic downregulation of MCJ (DNAJC15) (Shridhar *et al*., 2001; Strathdee *et al*., 2005), p16 (Katsaros *et al*., 2004), RASSF1A (Yoon *et al*., 2001; Makarla *et al*., 2005) and hMLH1 possibly contributes to resistance in ovarian and other cancers.

Although epigenetic therapies have been shown to induce the re-expression of several tumor suppressor genes and resensitize resistant tumors to conventional drugs (Kopelovich *et al*., 2003), with the use of siRNA-mediated gene silencing followed by rescue of the protein expression, we have clearly shown that the presence of HSulf-1 specifically contributes to cisplatin-induced cytotoxicity. We initially tested the effect of cisplatin cytotoxicity in HSulf-1 null OV207 cell line following 5-aza-dC treatment. Our MTT assay clearly indicated that 5-aza-dC treatment of these cells conferred cisplatin sensitivity to these cells (Figure 4a). However, as mentioned earlier, as several tumor suppressor genes could be induced by this treatment, we opted to downregulate HSulf-1 by siRNA-mediated technology and assessed whether downregulation of

HSulf-1 would result in resistance to cisplatin in a HSulf-1 high expressing cell line, OV167. Whereas this experiment clearly indicates that HSulf-1 downregulation could confer resistance to cisplatin (Figure 4c), in order to more specifically prove the contribution of HSulf-1 in chemoresponse, we rescued the protein expression following siRNA-downregulation in these cells. The results from these experiments clearly demonstrate that while full-length HSulf-1 could rescue the cisplatin-induced cytotoxicity, the vector only control did not (Figure 4c) and therefore implicate the contribution of HSulf-1 in chemoresponse.

We emphasize that the increased resistance observed with loss of HSulf-1 or an increased sensitivity observed following rescue of HSulf-1 expression do not confer absolute resistance or sensitivity to therapy. Rather, these are relative changes compared to those observed in control cells. However, these studies suggest that expression of HSulf-1 may contribute to a better therapeutic index.

Consistent with the *in vitro* data, our results also implicate HSulf-1 in modulating chemoresponse to the commonly used chemotherapeutic agents *in vivo*. Our *in vivo* data (Figure 5) show 90% of patients (27/30) with tumors expressing high levels of HSulf-1 responded to chemotherapy with complete or partial clinical remissions, whereas only 63% (19/30) of those with weak/moderate levels of HSulf-1 expression respond favorably to treatment ($P=0.0146$, χ^2 test). These results have considerable implications in the treatment options for patients whose tumors no longer express HSulf-1. Owing to the unavailability of fresh frozen samples that is represented on the TMA, bisulfite sequencing of these samples was not performed. We also emphasize that the patients were selected at random, and most of the patient tumors were optimally debulked to eliminate the impact of residual disease on outcomes. Our data demonstrate that HSulf-1 expression is correlated with response to chemotherapy and thus may be useful in conjunction with other molecular markers, in predicting chemosensitivity. Given the observation that the presence of HSulf-1 also inhibits angiogenesis *in vivo* (Narita *et al*., 2006), a combination therapy that includes demethylating agents and or HDACs inhibitors with classical antineoplastic drugs such as carboplatin or taxol or antiangiogenic drug may benefit patients with tumors that lack HSulf-1 expression by promoting the expression of epigenetically controlled genes such as HSulf-1 involved in inhibiting growth and angiogenesis.

Materials and methods

Cell culture

Five of eight ovarian-carcinoma cell lines (OV 167, OV 177, OV 202, OV 207 and OV 266) were low-passage primary lines established at the Mayo Clinic (Conover *et al*., 1998), whereas OVCAR-5 and SKOV-3 were from American Type Culture Collection (Manassas, VA, USA). All cells were grown according to the providers' recommendations.

Expression of HSulf-1 in primary ovarian tumors and cell lines by real-time PCR

Duplex PCR amplification was carried out using the primers HSulf-1-F (5′-AGACCTAAGAAT CTTGATGTTGGAA -3′), HSulf-1-R (5′-CCATCCCATAACTGTCCTCTG -3′), glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-F (5-ACC CACTCCTCCACCTTTGA-3′), GAPDH-R (5′-ATGAGGT-CCACCACCCTGTT-3′). Reactions were run in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) in the presence of SYBR-Green dye according to the following condition: 2 min at 50°C and then 10 min at 95°C for initial denaturation, followed by 40 cycles at 95°C (15 s), 60°C (1 min), followed by the measurement of fluorescence at the end of each cycle. Each run included a negative control and a known GAPDH control. Results from real-time PCR were calculated as threshold cycles normalized to that of the GAPDH gene according to the method of Overbergh *et al*., (1999) and expressed as ΔC_t values. Expression of HSulf-1 by semi-quantitative RT–PCR was as previously described (Lai *et al*., 2003). Two different sets of primers were used. Primers 1929F (5′-GAGCCATCTTCACCCATTC AA-3′), 2754R (5′- TTCCCACCTTATGCCTTGGGT-3′) were used to analyse HSulf-1 expression in ovarian cell lines (Figure 1a). Primers HSulf-1 F (5′- CTCACAGTCCGG CAGAGCACGCGGAAC-3′) and HSulf-1 R (5′- CACGGC GTTGCTGCTATCTGCCAGCATCC-3′) were used to analyse induction of HSulf-1 following drug treatment (Figure 1b–d).

Treatment of cells with TSA and 5-aza-dC

OV 207 cells were seeded at a low density in a 100 mm tissue culture dish and maintained for a total of 72 h. Mock treatments consisted of identical volumes of absolute ethanol or water. For 5-aza-dC (Sigma, St Louis, MO, USA) treatment, drug was added after 24 h in culture and cells were incubated for a total 48 h. Culture medium was exchanged every 24 h for 5-aza-dC treatment. For combined treatment, 5-aza-dC was added at 24 and 48 h time points and TSA (Sigma) at 48 h time points only.

Genomic sequencing of bisulfite modified DNA

The methylation status of HSulf-1 was determined by genomic sequencing of bisulfite-modified DNA as described previously (Shridhar *et al*., 2001). The PCR primers used for bisulfite-modified DNA are: MF: 5′-TTTGGGGTCGGAGTCGGG TCG -3′ and MR:-5′-TTCCTCATCTACCCTAACCTCC-3′. The conditions for amplification were: 94°C for 10 min, then 45 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s in an MJ Research Peltier Thermocycler (Waltham, MA, USA).

ChIP

The ChIP assays for histone acetylation and methylation were performed as described by Chadee *et al*. (1999). The presence of the HSulf-1 CpG island in the immunoprecipitates was detected by PCR using gene-specific primers as described below. Individual ChIP assays were repeated three times to confirm the reproducibility. PCR amplification was performed using immunoprecipitated DNA or a 1:25 dilution of input that had not been immunoprecipitated. Samples were heated initially for 5 min at 95°C followed by 35 cycles of 95°C for 30 s, 59°C for 30 s and 72°C for 30 s followed by a 7 min extension at 72°C. The primer pairs for the HSulf-1 CpG island were as follow: CpG1F, 5′-AGCACTTGGTCTC TGGAACG-3′, CpG1-R, 5′-TCGGGACACGGC TGAA AGGA-3′; CpG2-F, 5′-TGCCTCTGCTCCTCCTCTTC-3′,

CpG2-R, 5′-CCGATCAAGCCG CCCCGGC-3′; CpG3-F, 5′-CAACTAGGAAACCCCAGGCG - 3′; CpG3-R, 5′-ACAG GGAGG AGGCAGAGGAG- 3′. To evaluate the level of histone acetylation following 5-aza-dC or TSA treatment, the ratio was determined by quantifying the intensities of the PCR product in immunoprecipitated DNA vs 1:25 diluted input DNA (total chromatin) amplified by PCR in a linear range. To evaluate the level of histone acetylation over methylation, the ratio was determined by quantifying the intensities of the PCR products from ChIP with acetylation- or methylation-specific histone H3 (monomethyl K9) antibodies (Upstate, Chicago, IL, USA). Each ChIP assay also included RNA polymerase II (Pol II) and control immunoglobulin (Ig)G. Negative control primers flanking a region of genomic DNA between the GAPDH gene and CNAP1 gene (For: 5′-ATGGTTGC CACTGGGGATCT-3′ and Rev: 5′-TGCCAA AGCCTA GGGGAAGA-3′) were also included as a control for each ChIP analysis. PCR conditions for all primers are the same as indicated above. ChIP analysis was repeated three times. To determine the acetylation status of chromatin following 5-aza-dC or TSA treatment, OV207 cells were treated with 2.5 μM 5-aza-dC and/or 1 μg/ml TSA for 48–72 h.

Attenuation of cisplatin-induced cytotoxicity following siRNA-mediated downregulation of HSulf-1 in ovarian cancer cell lines
OV167 and OV202 cells with endogenous expression of HSulf-1 transfected with siRNAs for 2 days were treated with increasing concentrations of cisplatin ranging from 2.5 to 30 μM for 24 h in a 96-well plate. Cells were then incubated in fresh medium for additional 3–4 days. To quantify viable cells, cells were incubated for 2 h with MTT by adding 20 μl of 5 mg/ml MTT into each well. Cells were then washed with phosphate-buffered saline and solubilized with 100 μl dimethyl sulfoxide. Absorbance was read at $\lambda = 570$ nm and survival was calculated as a percentage of vehicle-treated controls.

Rescue of RNA interference (RNAi)
For rescue of RNAi, OV167 cells were initially transfected with HSulf-1siRNA targeted to the 3′-UTR (GCUACCCU GGGUACCUUUG). Scr siRNA (CCUAGGUGCAUCCC GUUUG) served as controls. Twenty-four hours later, cells were transfected with plasmid carrying HSulf-1ORF and empty vector constructs using Lipofectamine Plus (Invitrogen, Carlsbad, CA, USA). As RNAi is targeted toward the 3′ UTR of HSulf-1 mRNA, the mRNA encoded from the plasmid containing HSulf-1 ORF does not contain the 3′UTR and therefore is resistant to RNAi.

In situ hybridization
Specific human HSulf-1 riboprobes were generated by RT–PCR using the following primer pairs: HSulf-1: sense, 5′-ACTGTA CCAATCGGCCAGAG-3′; antisense, 5′-CCTCCTTGAATG GGTGAAGA-3′. RNA *in situ* hybridization was as described previously (Qian *et al*., 2004).

Analysis of chemoresponse in ovarian cancer
Contingency tables containing levels of HSulf-1 staining and tumor responses to chemotherapy were generated and two-sided χ^2 analysis was performed using the Prism statistical software (GraphPad Software Inc., San Diego, CA, USA). Criteria for patient selection and response evaluation were as described previously (Chien *et al*., 2006)

TMA Construction and Digital imaging system were as previously described (Chien *et al*., 2006).

Statistical analysis
Contingency tables containing levels of HSulf-1 staining and tumor responses to chemotherapy were generated, and two-sided χ^2 analysis was performed using the Prism statistical software (GraphPad Software Inc.). $P < 0.05$ ($\alpha = 0.05$) is considered statistically significant.

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