Histone Deacetylase-1-mediated Suppression of FAS in Chemoresistant Ovarian Cancer Cells

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Abstract. *Background: Loss of FAS expression in ovarian cancer cells has recently been associated with resistance to chemotherapeutic drugs. However, the mechanism for suppression of FAS expression is unknown. Materials and Methods: The cell surface and transcript expressions of death receptors in parental chemosensitive (A2780) and their derivative chemoresistant (A2780-AD) ovarian cancer cells were determined by flow cytometry and quantitative real-time polymerase chain reaction, respectively. The epigenetic regulation of FAS promoters in both A2780 and A2780-AD ovarian epithelial cells were determined by chromatin immunoprecipitation assays. Conclusion: This study demonstrated that expression of FAS is suppressed in A2780-AD cells compared to parental A2780 ovarian cells. No difference in DNA methylation was observed at FAS promoters between A2780-AD cells compared to parental cells. However, the level of acetylated histone H3 associated with FAS promoter in A2780-AD cells was significantly lower compared to parental cells, and there was a corresponding increase in histone deacetylase 1 (HDAC1) enzyme associated with the FAS promoter in resistant cells. Knockdown of HDAC1 expression, and pharmacological inhibition of HDAC enzymatic activity significantly increased FAS expression in resistant A2780-AD cells. These results suggest that epigenetic changes in histone modifications may contribute to the loss of FAS expression in chemoresistant ovarian cancer cells and that enhancement of FAS expression could increase tumor cell sensitivity to immune cells.*

Epithelial ovarian cancer remains the most lethal gynecological malignancy, with high mortality rate in patients and a 5-year survival rate of less than 30% in those with advanced-stage disease (1). The high mortality rate is due in large part to the frequent diagnosis of the disease at advanced stages and the development of resistance to chemotherapeutic drugs (2). Studies suggest that different immunotherapy strategies for ovarian cancer might overcome barriers of resistance to standard chemotherapy (3). Death receptors (DRs) are implicated in carcinogenesis, tumor immune surveillance and response to chemotherapy (4, 5). DR4 (TRAIL-R1), DR5 (TRAIL-R2) and FAS (CD95/APO1) are members of the tumor necrosis factor receptor superfamily and are common DRs that are utilized by antitumor immune cells to induce apoptotic signals in tumor cells. Interaction between FAS ligand, and its agonist receptor FAS, plays an important role in triggering apoptosis. However, tumor cells often down-regulate cell-surface expression of death receptors in order to avoid elimination by immune cells (6, 7). Loss of FAS expression on tumor cells impairs the interaction between FAS and FAS ligand during cancer progression (8). Thus, understanding the molecular mechanisms that contribute to suppression of DRs on cancer cells could increase tumor cell sensitivity to cytotoxic T-lymphocyte-mediated killing.

Loss of FAS expression has recently been associated with resistance to chemotherapeutic drugs in ovarian cancer cells, and up-regulation of FAS reverses the development of resistance to cisplatin in ovarian cancer cells (9). However, the mechanism of suppression of FAS expression in ovarian cancer has not been established. To maintain specific growth advantages, a tumor cell undergoes heritable changes in gene function (10). DNA accessibility during transcription is affected by differential packaging of DNA with histone and non-histone proteins into chromatin. Gene silencing by chromatin remodeling is an established mechanism in progression of many types of cancer, including ovarian cancer (11). Transcriptional activity is suppressed by the addition of methyl groups to CpG dinucleotides by DNA methyl transferase (DNMT) enzymes and the removal of acetyl groups on lysine residues in histone proteins by histone deacetylase (HDAC) enzymes (12). It has been shown that expression of DRs can be influenced by DNA methylation and histone acetylation (13, 14). Thus, epigenetic regulation of DRs may also contribute to their dynamic expression in cancer

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Key Words: Ovarian cancer, chemoresistance, death receptors, DNA methylation, histone acetylation.

progression; however, the molecular details at specific gene promoters in chemoresistant ovarian cancer remain unknown.

In the current study, the epigenetic regulation of FAS expression was investigated in ovarian cancer cells. The focus of this study was on cisplatin-resistant A2780-AD ovarian epithelial cells and their parental cells.

Materials and Methods

Cell lines and reagents. The chemosensitive A2780 cell line and the derivative chemoresistant A2780-AD cells were generously provided by Dr. Bob Brown, Imperial College London, UK (15). These cells were maintained in RPMI-1640 medium (Corning Life Sciences, Tewksbury, MA, USA.) supplemented with 10% fetal bovine serum, 5 mM L-glutamine and 5 mM penicillin-streptomycin at 37˚C with 5% CO_2. Chemoresistant A2780-AD cells were further maintained in 3 μM cisplatin.

Cisplatin and trichostatin A (TSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies recognizing histone H3 and acetylated histone H3 were from Millipore (Lake Placid, NY, USA). Anti-HDAC1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-HDAC2, anti-HDAC3, anti-DNMT1, anti-DNMT3a and anti-DNMT3b were obtained from Abcam (Cambridge, MA, USA).

Cell surface staining and flow cytometric analysis. Cell-surface staining of ovarian cancer cells was performed using the following primary labeled antibodies: phycoerythrin-conjugated (PE)-FAS, PE-DR4, allophycocyanin-conjugated (APC)-DR5, and the appropriate isotype matched controls (BioLegend. San Diego, CA, USA). Surface staining was performed in cell staining buffer for 45 min on ice. Stained cells were acquired on a BD Fortessa flow cytometer. Dead cells were excluded from the analysis.

RNA expression and quantitative real-time polymerase chain reaction (qRT-PCR). mRNA was isolated using Qiazol RNA extraction reagent (Qiagen, Valencia, CA, USA) as described in the manufacturer's protocol. RNA was quantified and cDNA was generated from 1 μg of total extracted RNA using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Following cDNA synthesis, quantitative real-time PCR was performed using QuantiFast SYBR Green PCR Kit (Qiagen) and specific primers and probes targeting genes of interest (*FAS*: Hs00163653_m1; *DR5*: Hs00366278_m1; and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*): Hs99999909; Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Transcript expression was assessed using an ABI prism 7900HT Real-Time PCR System (Applied Biosystems). Reactions were normalized against *HPRT1* expression and calculations were performed using standard curves generated.

To determine the effect of TSA exposure on *FAS* transcript expression, 1×10^6 A2780-AD cells were plated per 10 cm^2 tissue culture plate and were incubated overnight. Cells were treated with 125 nM TSA and were further incubated for 48 h. RNA isolation and DNA synthesis were performed as described above.

siRNA constructs and transfection. Short interfering RNA (siRNA) pre-designed for *HDAC1* (Qiagen) was used to knock-down expression of HDAC1. Scrambled All Star Control siRNA (Qiagen) was used as a control. Chemoresistant A2780-AD cells were transfected with 10 nM of *HDAC1*-specific siRNA or control siRNA using HiPerfect transfection reagent (Qiagen) according to the manufacturer's instructions. Following the incubation time, cells were harvested and analyzed for RNA expression.

Chromatin immunoprecipitation (ChIP) assay. ChIP assays were performed as previously described in Cacan *et al*. (16). Isolated DNA was quantified by real-time PCR on an ABI prism 7900HT (Applied Biosystems) using specific primers and probes targeting *FAS* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) promoter regions. *FAS*: forward: 5'-TCG AGG TCC TCA CCT GAA G-3', reverse: 5'-TGC ACA AAT GGG CAT TCC T-3' and probe: 5'-CCA GCC ACT GCA GGA ACG CC-3'; and for *GAPDH*: forward: 5'-AAT GAA TGG GCA GCC GTT A-3', reverse-5'-TAG CCT CGC TCC ACC TGA CT-3' and probe: 5'-CCT GCC GGT GAC TAA CCC TGC GCT CCT-3'. Values generated from real-time PCR reactions were calculated based on standard curves generated, were run in triplicate reactions, and were analyzed using the SDS 2.0 program (Applied Biosystems).

Methylation-specific PCR. Methylation-specific PCR was performed as described elsewhere (16). Briefly, A2780 and A2780-AD cells were plated and genomic DNA was extracted using the EZ-DNA Methylation-Direct™ Kit (Zymo Research, Irvine, CA) according to the manufacturer's protocol. Using the kit, 500 ng of DNA was bisulfite converted per the manufacturer's instructions.

Statistics. Statistical differences between groups were calculated using Student *t*-test and calculated at 95% confidence. Values represent the mean±SEM of three independent experiments. Values of $p<0.05$ were accepted as being statistically significant.

Results

Suppression of FAS expression in ovarian cancer cells. Suppression of some DRs was observed in variety of cancer cell lines (17, 18). To determine whether surface expression of DR4, DR5 and FAS is suppressed in chemoresistant ovarian cancer cells, parental A2780 and their derivative multidrug-resistant A2780-AD cells were compared. Flow cytometric analysis demonstrated that FAS surface protein expression was significantly lower in chemoresistant cells compared to chemosensitive cells (Figure 1A), suggesting that FAS expression is suppressed in chemoresistant ovarian cancer. DR4 and DR5 surface protein expression was also compared in these cells. While no significant change was observed in DR5 expression (Figure 1C), a decrease was observed in DR4 expression (Figure 1B); however, not significantly.

Next, *FAS* and *DR5* transcript expressions were compared in parental A2780 and A2780-AD cells. qRT-PCR analysis showed that relative *FAS* mRNA expression was significantly lower in chemoresistant cells compared to chemosensitive cells (Figure 2A). In contrast, no significant difference was observed in *DR5* transcript expression between cell lines (Figure 2B). Consistent with surface protein expression, *FAS* transcript expression was also down-regulated in chemoresistant A2780-AD cells.

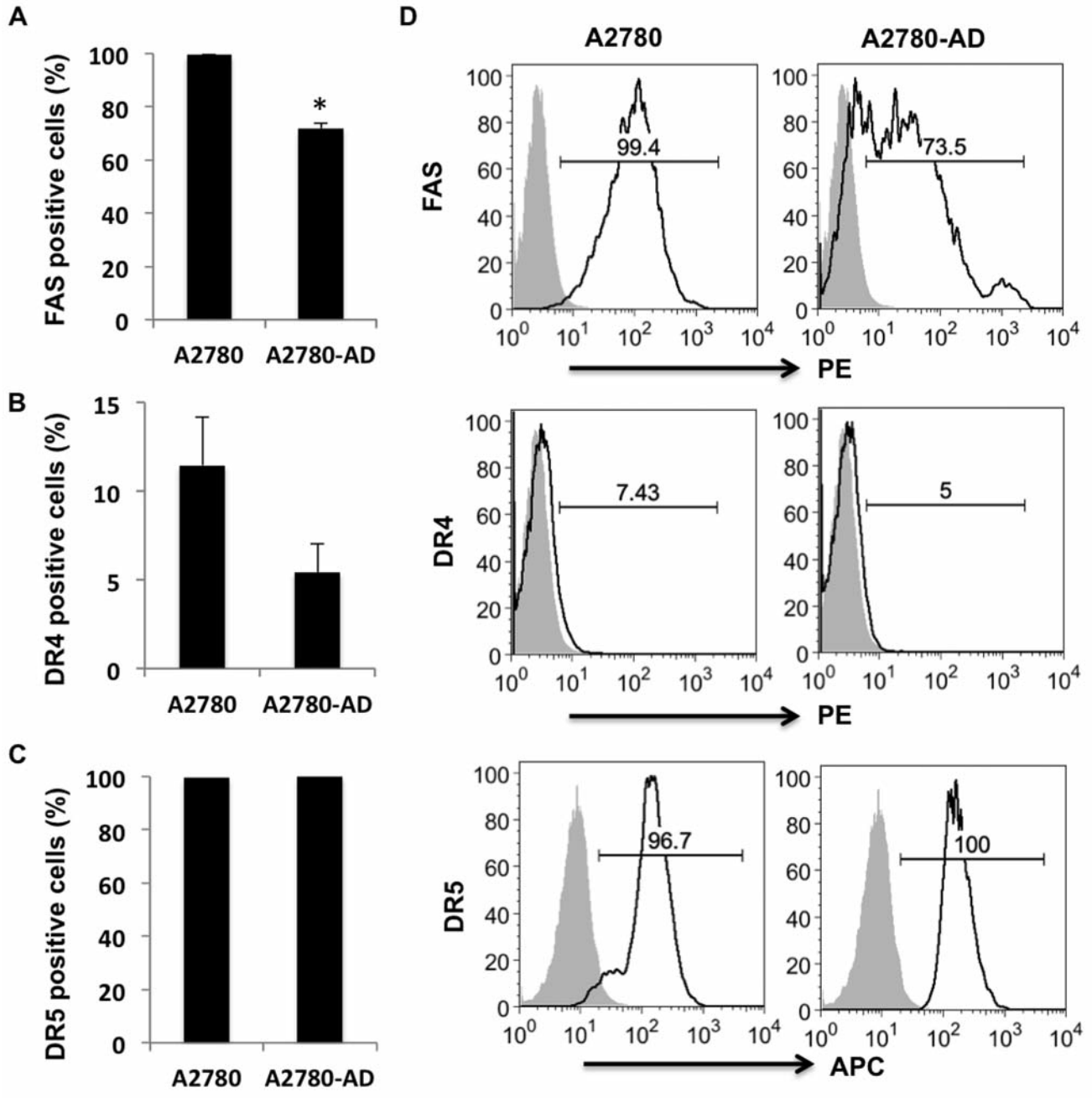

Figure 1. *Cell-surface expression of death receptors in chemosensitive A2780 and chemoresistant A2780-AD ovarian cancer cells. Chemosensitive and chemoresistant ovarian cancer cells were harvested and stained with phycoerythrin (PE)-labeled antibody against human FAS (A), death receptor-4 (DR4) (B), and allophycocyanin (APC)-labeled DR5 (C). Surface expression of DRs was analyzed by flow cytometry. Isotype control stained cells were set to 5% positive. The graph presents the average of three independent experiments, with error bars denoting SEM. *p<0.05. D: Representative fluorescence-activated cell sorting plots showing FAS, DR4 and DR5 expression in chemosensitive and chemoresistant ovarian cancer cells.*

DNA methylation and DNMT binding to FAS. DNMTs are responsible for methylating DNA and for silencing genes. We recently observed that FAS expression was regulated by DNA methylation in colorectal cancer cells (16). However, it is unclear if suppression of this gene is regulated by DNA methylation in chemoresistant ovarian cancer cells. To determine whether the *FAS* promoter is methylated in ovarian cancer cells, methylation-specific PCR was utilized. The data showed similar promoter methylation in both ovarian cancer cell lines (Figure 3A). To determine if there is any difference

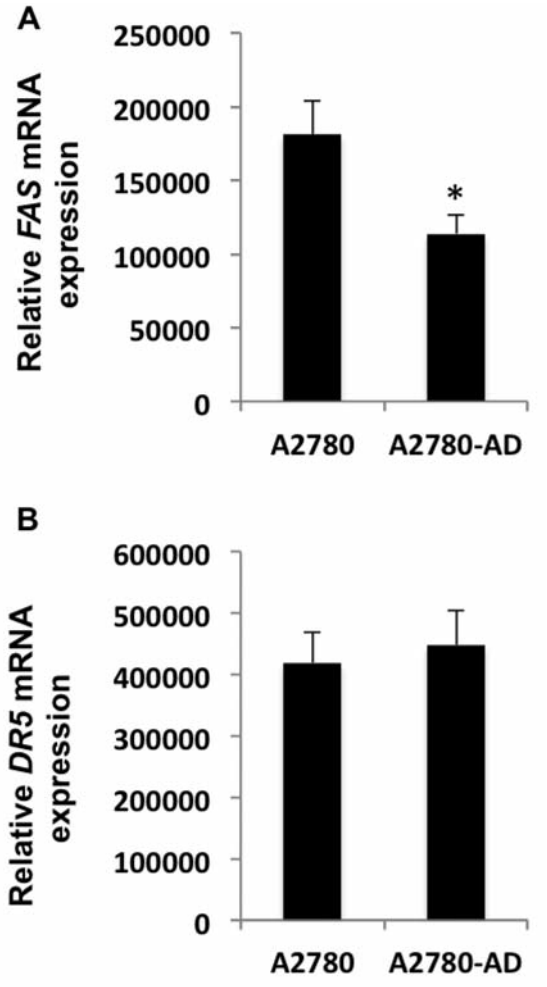

Figure 2. *Relative mRNA expression of FAS (A) and DR5 (B) in chemosensitive A2780 and chemoresistant A2780-AD ovarian cancer cells. Cells were harvested, RNA was extracted and cDNA was generated. Data were quantified using real-time PCR with primers and probes specific for FAS- or DR5-coding regions and the obtained data were normalized to the expression of the housekeeping gene HPRT1. The data are the average of three independent experiments, with error bars denoting SEM. *p<0.05.*

in DNA-methylating enzyme status at *FAS* promoters, ChIP assays were performed to determine binding of DNMTs to *FAS* promoter. However, no difference was observed in the level of DNMT1, DNMT3a or DNMT3b binding to *FAS* promoter in chemosensitive and chemoresistant ovarian cancer cells (Figure 3B-D). These data suggest that DNA methylation does not contribute to regulation of FAS in chemoresistant ovarian cancer cells.

Histone acetylation and HDAC binding to FAS promoter. Epigenetic regulation and suppression of FAS have been reported in lung and melanoma tumor cell lines (19, 20), and we recently reported that expression of FAS appears to be sensitive to epigenetic modifications in colorectal cancer cells (16). To explore whether suppression of FAS is due to histone modifications, ChIP assays were carried out on the chemosensitive ovarian cancer cell line A2780 and in chemoresistant A2780-AD daughter cells. Acetylation at histones associated with the *FAS* promoter in A2780 and A2780-AD cells were compared. While total levels of histone H3 were similar at FAS promoter in chemosensitive and chemoresistant cells (Figure 4A), the level of acetylated histone H3 was significantly lower at *FAS* promoter in A2780-AD cells as compared to chemosensitive A2780 cells (Figure 4B). Together these data suggest that loss of acetylation at *FAS* promoter contributes to the loss of FAS expression in chemoresistant ovarian cancer.

Histone acetylation is dynamically regulated in cells by the opposing actions of histone acetyltransferases (HATs) which add the acetyl functional group to histones, and histone deacetylases (HDACs) which remove them. To determine the potential mechanism that could be responsible for the decrease of histone acetylation in chemoresistant ovarian cancer cells, the binding of HDACs to the promoter region of *FAS* was evaluated since a significant decrease in histone acetylation level at the *FAS* promoter region was observed in the A2780-AD cell line. Class I HDACs are overexpressed in ovarian cancer tissues and are thought to play a significant role in gene silencing during ovarian cancer progression (21). The association of HDAC1 with *FAS* promoter in A2780 and A2780-AD cells was investigated. A striking increase in HDAC1 association with *FAS* promoter in A2780-AD cells was observed compared to parental A2780 cells (Figure 4C). The association of HDAC2 and HDAC3 with FAS promoter in A2780 and A2780-AD cells was also investigated. Despite an increase in the binding of HDAC2 and HDAC3 to *FAS* promoter in chemoresistant ovarian cancer cells, the difference was not significant (Figure 4D-E). These data show that reduced FAS expression in A2780-AD ovarian cancer cells correlates with enhanced HADC1 binding and loss of histone acetylation at the *FAS* promoter compared to A2780 cells.

Expression of FAS is increased following HDAC1 knockdown in chemoresistant ovarian cancer cells. The data indicate that HDAC1 binds with significantly increased frequency to the *FAS* promoter in chemoresistant A2780-AD cells compared to parental chemosensitive A2780 cells. To investigate molecular roles for HDAC1 in regulating FAS expression, a siRNA duplex was utilized to specifically knock-down endogenous *HDAC1* expression in A2780-AD cells. siRNA-mediated knockdown of *HDAC1* resulted in a significant increase in endogenous FAS expression compared to use of control siRNA (Figure 5A), suggesting that HDAC1 plays a critical role in regulating *FAS* transcription. Taken together

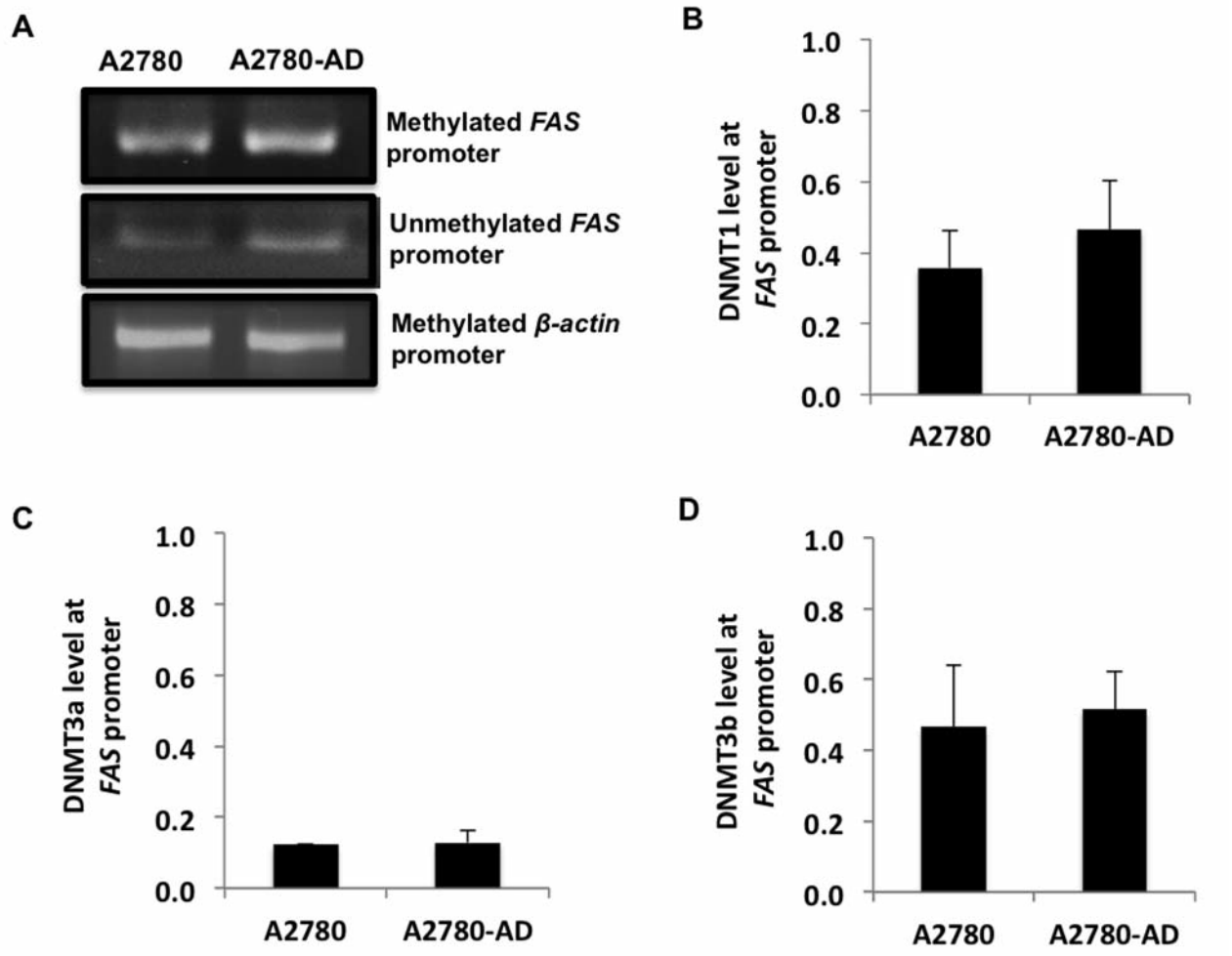

Figure 3. *DNA methylation and DNA methyl transferase (DNMT) binding at FAS promoter in parental A2780 cells and chemoresistant A2780-AD cells. A: Promoter methylation of FAS in A2780 and A2780-AD cells. Methylation-specific PCR analysis of methylation at the FAS promoter was carried out A2780 parental cells and multidrug-resistant A2780-AD cells. Genomic DNA was isolated, bisulfite converted and amplified in PCR with primers specific for non-methylated FAS, methylated FAS, or beta-actin. ChIP assays were carried out on A2780 parental cells and multidrug-resistant A2780-AD to determine levels of DNMT1 (B), DNMT3a (C) and DNMT3b (D) associated with FAS promoters in A2780 and A2780-AD ovarian cancer cells. Lysates were immunoprecipitated with control, anti-DNMT1, anti-DNMT3a, or anti-DNMT3b to determine DNMT binding at FAS promoter. Associated DNA was isolated and analyzed via real-time PCR using primers spanning the FAS and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoters. Real-time PCR values were normalized to the total amount of promoter DNA added (input). Input values represent 5% of the total cell lysate. Values represent the mean±SEM of three independent experiments.*

these data indicate that HDAC1 accumulation at *FAS* promoter likely contributes to suppression of FAS in chemoresistant ovarian cancer cells.

To determine if pharmacological inhibitors of HDACs could alter the expression of FAS in chemoresistant ovarian cancer cells, the HDAC inhibitor TSA was used to inhibit HDAC activity. A2780-AD cells were treated with 125 nM TSA for 2 days and FAS expression was quantified by RT-PCR. The data show that TSA treatment significantly increased expression of FAS as compared to control treated in A2780-AD cells (Figure 5B).

Discussion

Growing evidence suggests that FAS activity is regulated by multiple mechanisms controlling the expression of FAS receptors (16, 22). The current study would seem to mark the first description of the regulation of expression of *FAS* gene by HDAC in chemoresistant ovarian cancer cells.

It has been reported that expression of FAS is suppressed in drug-resistant ovarian cancer cells (9). To probe the mechanisms responsible for suppressing FAS expression, A2780 and A2780-AD cells were compared. The hypothesis

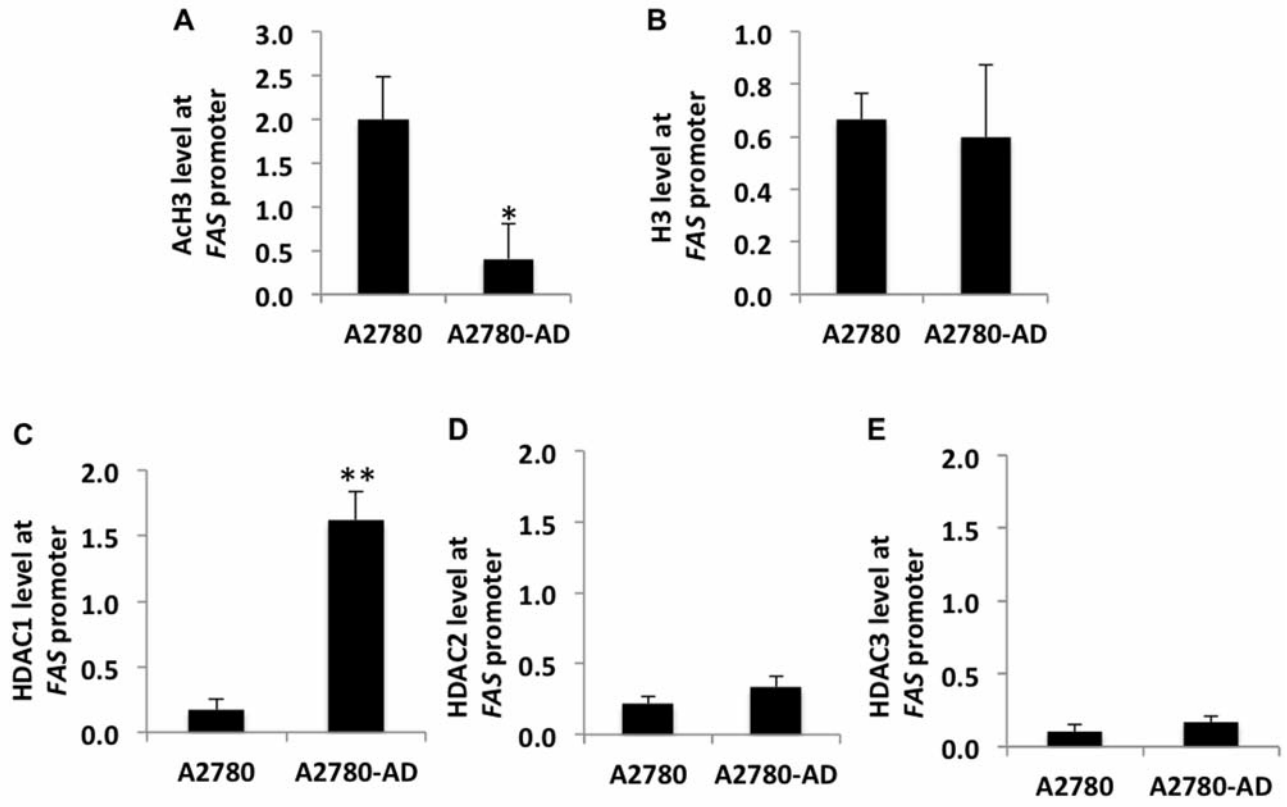

Figure 4. *Histone acetylation and histone deacetylase (HDAC) binding at FAS promoter in parental A2780 cells and chemoresistant A2780-AD cells. ChIP assays were carried out on A2780 parental cells and multidrug-resistant A2780-AD. Lysates were immunoprecipitated with control, anti-acetyl histone H3 (AcH3) (A), anti-histone H3 (B), anti-HDAC1 (C), anti-HDAC2 (D), or anti-HDAC3 (E). Associated DNA was isolated and analyzed via real-time PCR using primers spanning the FAS and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) promoters. Real-time PCR values were normalized to the total amount of promoter DNA added (input). Input values represent 5% of the total cell lysate. Values represent the mean±SEM of three independent experiments. *p<0.05, **p<0.005.*

was that the *FAS* promoter may be epigenetically regulated by DNA methylation because DNA hypermethylation of tumor-suppressor genes is known to be a major mechanism for cancer progression in general, and DNA methylation is also implicated in ovarian cancer chemoresistance (23, 24). Interestingly, no difference in *FAS* promoter methylation was observed between A2780 and A2780-AD cells, suggesting that this mechanism may not specifically correlate to loss of FAS expression in acquired chemoresistance. Recent findings suggest epigenetic control mechanisms for cisplatin resistance in ovarian cancer, and multiple gene targets that may be subject to epigenetic control (25). In the current study, specific contribution of an important epigenetic regulator, HDAC1, to the suppression of FAS expression was observed in chemoresistant ovarian cancer cells. The results clearly demonstrate loss of histone acetylation and gain of HDAC1 binding at the *FAS* promoter in chemoresistant ovarian cancer cells with low FAS expression. This result is consistent with abundant evidence that acetylation of histones H3 and H4 tails is frequently reduced in cancer (24, 26). Furthermore, class I HDACs are overexpressed in ovarian cancer tissues (21), and aberrant HDAC expression is associated with poor responses to chemotherapy (27).

Tumor cells escape from immune responses by down-regulating genes that are essential for effective antitumor immunity (28). Following proper stimulation, cytotoxic T-lymphocytes commonly use DRs to kill tumor cells (29). Interaction between these DRs with their ligands on antitumor immune cells is essential for driving apoptosis in many types of tumor cells (30). Thus, modulation of these molecules is a promising approach for improving the activity of tumor-specific T-cells against resistant cancer cells and for enhancing the efficacy of cancer immunotherapies. FAS interacts with FAS ligand to induce an immune-mediated apoptotic signal; it is possible that enhancing FAS expression will have a therapeutic benefit for ovarian cancer. The results suggest that HDAC enzymes may suppress FAS expression in ovarian cancer cells, and therefore inhibition of HDAC

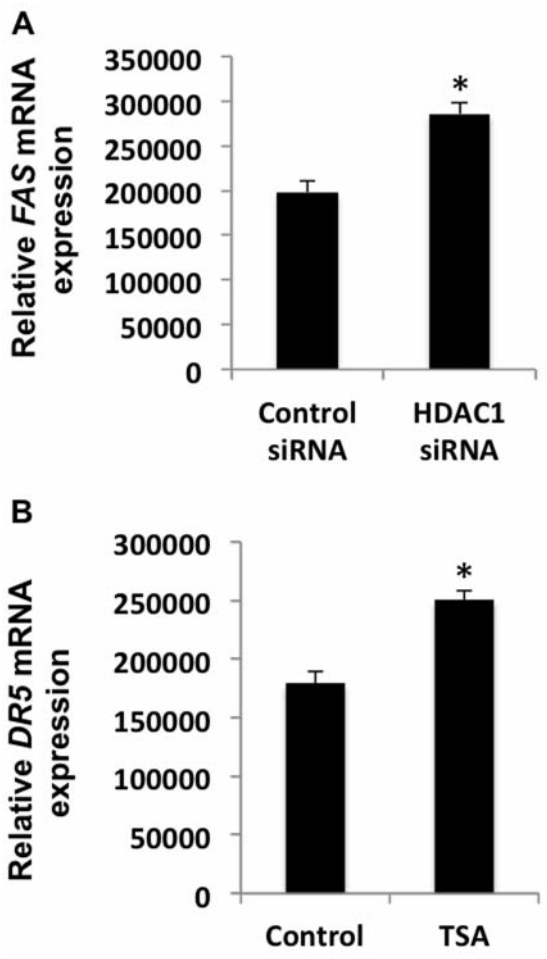

Figure 5. *FAS expression is altered by inhibition of histone deacetylase-1 (HDAC1) in chemoresistant A2780-AD ovarian cancer cells. Relative mRNA expression of FAS was determined following HDAC1 knock-down (A) and TSA treatment (B) in chemoresistant A2780-AD cells. Cells were plated and treated with control siRNA, HDAC1 siRNA, or trichostatin A (TSA) (125 nM). Adherent cells were harvested, RNA was extracted and cDNA was generated. Data were quantified using qRT-PCR with primers and probes specific for FAS-coding regions and the obtained data were normalized to expression of the housekeeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT1). Data are the average of three independent experiments, with error bars denoting the SEM. *p<0.05.*

enzymes should enhance FAS expression. Future studies should define the role that FAS expression regarding the therapeutic effects of HDAC inhibitors in ovarian cancer.

HDAC inhibitors induce a potent anticancer response by inhibiting histone deacetylation, and can inhibit cancer cell growth *in vitro* and *in vivo*, induce apoptosis and enhance cell differentiation (31). To date, three HDAC inhibitors have been approved for cancer treatment by the US Food and Drug Administration. Vorinostat and romidepsin were approved for use in patients with T-cell lymphoma, and belinostat was approved for treatment of patients with relapsed or refractory peripheral T-cell lymphoma (32). The class I selective HDAC inhibitor romidepsin is effective in reducing ovarian cancer cell proliferation (33). Clinical trials showed HDAC inhibitors to be effective antitumor drugs and HDAC inhibitors have recently shown great therapeutic promise against ovarian cancer (34). As a result, HDAC inhibitor-based therapies have gained much attention for cancer treatment and many more HDAC inhibitors are in different stages of clinical development for the treatment of hematological malignancies as well as solid tumors.

This study shows that inhibition of HDACs by TSA induced significant expression of *FAS* transcript in chemoresistant cells. Epigenetically altered expression of *FAS* can result in changes that are sustained within a tumor cell population, and knowledge about such changes could be further exploited to improve combination cancer immunotherapy strategies. Thus, understanding the molecular and genetic mechanisms that drive the development of acquired chemoresistance may lead to strategies to predict and prevent the occurrence of refractory disease.

Conflicts Interests

The Author declares that there is no competing interest in regard to this study.

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Received March 17, 2016
Revised April 18, 2016
Accepted April 20, 2016