Six1 Overexpression in Ovarian Carcinoma Causes Resistance to TRAIL-Mediated Apoptosis and Is Associated with Poor Survival

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Abstract

Tumorigenesis can arise from inappropriate activation of developmental genes in mature tissues. Here, we show that the developmental regulator Six1 is overexpressed in ovarian carcinoma cell lines (OCC) compared with normal ovarian surface epithelium. As observed in other cancers, Six1 overexpression in OCC leads to increased A-type cyclin expression and increased proliferation. In addition, Six1 overexpression renders OCC resistant to tumor necrosis factor–related apoptosis inducing ligand (TRAIL)–mediated apoptosis, and Six1 knockdown in the TRAIL-resistant SKOV3 ovarian carcinoma line dramatically sensitizes the cells to TRAIL. Because inactivation of the TRAIL response has been linked to metastasis, and because antibodies and recombinant ligand that activate the TRAIL pathway are currently in clinical trials against ovarian carcinoma, we screened normal ovarian and carcinoma specimens for Six1 mRNA. Six1 was overexpressed in 50% of the early-stage (stage I) and 63% of the late-stage (stages II, III, and IV) ovarian carcinomas examined, with late-stage carcinomas expressing ~3-fold higher Six1 mRNA levels on average compared with early-stage tumors. Importantly, in patients with late-stage disease, high Six1 expression was associated with significantly shortened survival (P = 0.0015). These data suggest that Six1 may contribute to ovarian epithelial carcinogenesis by simultaneously increasing proliferation and decreasing TRAIL-mediated apoptosis and imply that Six1 may be an important determinant of TRAIL therapy response that should be considered in patient selection for TRAIL-related clinical trials. [Cancer Res 2007;67(7):3036–42]

Introduction

Ovarian carcinoma is the deadliest gynecologic malignancy and the fourth leading cause of death due to cancer in women (1). Because ovarian cancer is often diagnosed only after the disease has reached an advanced stage, the majority of patients require additional treatment after surgical removal of the tumor. Although more than 70% of patients with advanced ovarian cancer respond to primary chemotherapy, most of them ultimately develop resistance, leading to an overall 5-year survival rate below 20% (2). For this reason, novel approaches are being sought to overcome chemoresistance and to develop more effective therapies.

Currently, biological therapies are being considered as the next approach in the fight against ovarian cancer. These therapies have the potential to selectively target tumors, to minimize toxicity, and to overcome the resistance often observed with conventional therapies (3). One such therapy involves activating the tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) pathway that selectively induces apoptosis in tumor cells while sparing normal cells. Specifically, TRAIL triggers apoptosis rapidly through the extrinsic apoptotic pathway mediated by the death receptors DR4 and DR5 on the cell membrane (4–6). Upon TRAIL-mediated activation of the death receptor, its intracellular death domain attracts the Fas-associated death domain adaptor molecule (7, 8), which further recruits the initiator caspase-8 and caspase-10 to the death receptor to form the death-inducing signaling complex. This process ultimately results in the activation of the terminal executioner caspase-3, caspase-6, and caspase-7, thereby leading to cell death (9). Importantly, cell culture and mouse xenograft experiments have shown that TRAIL can exert selective cytotoxic activity against ovarian carcinoma cells with limited effects to normal cells (10).

TRAIL has been implicated in several aspects of tumorigenesis, including innate immunosurveillance against tumors (11) and inhibition of tumor initiation and metastases (11), and in response to conventional chemotherapy (12, 13). For example, TRAIL-deficient mice show increased tumor susceptibility in response to the chemical carcinogen methylchloanthrene and increased experimental and spontaneous metastasis (11). In addition, mutations in TRAIL receptors have been linked to metastatic breast cancer (14). Thus, activating the TRAIL pathway clinically could induce cell death and prevent metastatic disease, and those tumors that devise mechanisms to escape TRAIL-mediated apoptosis might be more metastatic.

In this study, we show that the homeoprotein Six1, an important developmental regulator (15–20), is a modulator of the TRAIL pathway. Six1 is expressed during embryogenesis (21) but lost in most differentiated tissues (22) and has been implicated in the etiology of numerous cancers, including breast cancer (22–24) and rhabdomyosarcoma (25, 26). During normal development, Six1 stimulates the proliferation and survival of progenitor cells (17–20), and when expressed out of context, Six1 can aberrantly promote proliferation, contributing to tumorigenesis (23, 26). These observations have led us to postulate that inappropriate
Six1 expression in adult differentiated tissues results in the acquisition of an embryonic program of cell expansion that contributes to cancer. Here, we show that Six1 expression is related to the tumorigenicity of ovarian carcinoma, and that it not only increases ovarian carcinoma cell proliferation, but that it also protects ovarian carcinoma cells from TRAIL-mediated apoptosis. As resistance to TRAIL is associated with metastatic disease, these findings suggest that Six1 contributes to late-stage ovarian carcinoma (characterized by loco-regional peritoneal dissemination) by conferring resistance to TRAIL-mediated apoptosis. Furthermore, these finding have important implications for the use of TRAIL reagents in ovarian cancer therapy.

Materials and Methods

Cell lines and cell culture. The ovarian carcinoma cell lines (OCC) CaOV3 and SKOV3 were obtained from the American Type Culture Collection (Rockville, MD). The cell line SNU251 (27) was provided by Dr. Jeffrey Holt (University of Colorado at Denver and Health Sciences, Denver, CO). Cell lines were maintained in DMEM (CaOV3) or McCoy's 5-a (SKOV3) or RPMI 1640 + 1% insulin-transferrin-selenium A (SNU251), all with 10% fetal bovine serum (FBS) at 37°C and 5% CO₂. Ovarian surface epithelial (OSE) cells were harvested from patients undergoing oophorectomy as previously described (28). Epithelial origin was verified by pan-cytokeratin staining.

Transfection for Six1 overexpression and knockdown. Full-length Six1 cDNA (SIXFL; ref. 22) or control pcDNA3.1 (CAT) plasmids were transfected into 1 × 10⁶ CaOV3 or SNU251 cells using Superfect according to manufacturer specifications (Qiagen, Valencia, CA). Transfectants were selected with 400 µg/mL G418. Approximately 2 weeks later, individual clones were isolated and propagated. Six1 expression was verified by quantitative reverse transcription-PCR, Northern blot, and Western blot.

Transfected cells were lysed on ice in radioimmunoprecipitation assay buffer (29). Nuclear proteins were extracted on ice using a Dounce homogenizer. Protein concentrations were determined by the Lowry method (31). Thirty micrograms of each protein sample were loaded on a 12% SDS polyacrylamide gel and transferred to polyvinylidene difluoride. Membranes were probed with antibodies to Six1 (32) and to cyclin A1 (BD Biosciences, Franklin Lakes, NJ) and were normalized to β-actin (Sigma-Aldrich Corp., St. Louis, MO).

Proliferation assays. Cells (10,000 per well) in six-well plates were seeded. Cells were counted with trypan blue dye exclusion at days 1, 3, and 5. Counts were reported as means of three to six wells. Each cell growth assay was repeated at least once, at least in triplicate. For assessment of the percentage of cells in the cell cycle using Ki67 staining (DAKO Corp., Carpinteria, CA), immunocytochemistry was done on 5- to 8-µm sections of formalin-fixed, paraffin-embedded cell blocks of cultured cells. Sections were developed using the ABC method (Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin. Three randomly selected ×160 fields per slide were analyzed visually by a blinded observer, and percentage of stained cells was quantified and analyzed.

Detection of apoptotic cell death. Cell lines were plated in chambered slides in duplicate in at least two separate experiments. Once the cells reached 70% confluency, they were fixed with 40 g/L PBS buffered 4% formaldehyde for 20 min, after which they were stained with Hoechst 33258 (10 mg/L). Slides were then examined using fluorescence microscopy. Apoptotic cells were defined based on condensed nuclear morphology. Six random fields at ×1000 magnification were counted per slide by a blinded observer, and the number of apoptotic cells per total nuclei was recorded.

Dose-response curves for TRAIL- and Fas ligand–mediated growth inhibition. For CaOV3 and SNU251 transfectants, 5,000 cells were plated in each well of a 96-well plate and treated with varying concentrations of full-length recombinant soluble TRAIL (R&D Systems, Minneapolis, MN), Fas ligand (FasL; Alexis-Axxora, San Diego, CA), or media + DMSO for 40 h. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay (Promega, Madison, WI). For SKOV3 cells in which Six1 was knocked down, 1,000 cells per well were plated as above 48 h after electroporation. The rest of the assay was done as outlined for CaOV3 and SNU251 cells. Means of five–six wells are reported and experiments were repeated at least once in quintuplet or sextuplet. Representative dose-response curves are shown.

Patient specimens. Fresh tissue specimens were obtained under an approved Institutional Review Board protocol immediately after evaluation by a pathologist and stored in RNAlater stabilization buffer (Qiagen). Staging was done by one of two authors (K.B. or S.D.). A portion of each specimen was mounted on ornithine carbamyl transferase compound, snap-frozen, and stored at −80°C, and another portion was fixed in formalin and paraffin embedded. Sections (5–8 µm) were cut and verified using H&E staining.

Statistical analysis. Results were compared using Student’s t test and one-way ANOVA for normally distributed variables and χ², Fischer’s exact test, and Mann-Whitney U test for nonparametric variables. Survival data were analyzed using the Kaplan-Meier method with analysis of significance via the log-rank test. The SPSS (SPSS, Inc., Chicago, IL) data analysis software was used.

Results

Six1 is expressed in epithelial ovarian carcinoma cell lines but not in cultured normal ovarian surface epithelial cells. The Six1 homeobox gene is overexpressed in a number of cancers, including breast (22) and hepatocellular (33) carcinoma, rhabdomyosarcoma (26, 34), and Wilms’ tumor (35). Because numerous tumorigenic mechanisms are shared between breast and ovarian carcinoma (36, 37), we set out to determine whether Six1 is overexpressed in ovarian carcinoma. To this end, we examined the expression of Six1 in a number of OCC and cultured normal OSE cells. We found that Six1 mRNA, as measured by both Northern blot analysis (Supplementary Fig. S1) and quantitative RT-PCR (Fig. 1A), is increased in OCC (CaOV3, SKOV3, and SNU251) compared with OSE. Figure 1B shows that SIX1 protein expression follows the same pattern with highest levels observed in the
Six1 overexpression promotes a proliferative and antiapoptotic phenotype in ovarian carcinoma cells. To determine whether Six1 overexpression contributes to the tumorigenic phenotype and, more specifically, to resistance to TRAIL-mediated apoptosis, we next overexpressed Six1 in both CaOV3 and SNU251 cells, both of which express Six1 endogenously but at lower levels than the SKOV3 cell line. Stable Six1 and control chloramphenicol acetyl transferase (CAT) transfectants were generated and examined for Six1 mRNA and protein as shown in Fig. 1A and D and in Supplementary Fig. S2A. Two stable Six1-overexpressing clones and two CAT control clones from each line (CaOV3 and SNU251) were chosen for further analyses. The levels of over-expression of Six1 (fg/ng rRNA) achieved in these CaOV3 and SNU251 clones were less than or equivalent to the level of expression of endogenous Six1 mRNA in SKOV3 cells as determined by quantitative RT-PCR (average level of Six1 expression in SNU 251-Six1 clones = 262 fg/ng rRNA, CaOV3-Six1 clones = 395 fg/ng rRNA, and SKOV3 = 437 fg/ng rRNA), showing that we overexpressed the gene at the physiologic levels expressed in the TRAIL-resistant SKOV3 cells.

Six1 promotes the proliferation of precursor cells in normal development (15–20) and contributes to breast cancer (22, 23) and rhabdomyosarcoma (26) cell proliferation. Before examining the effect of Six1 on TRAIL sensitivity, we first wished to determine if Six1 influences the proliferative potential of OCC, as it does in breast cancer and rhabdomyosarcoma. To this end, we compared the growth of CaOV3-Six1 and SNU251-Six1 cells with CaOV3-CAT and SNU251-CAT control cells. The Six1-overexpressing cells increased in number at a faster rate than CAT control cells in both cell lines (Fig. 2A; Supplementary Fig. S2B). To determine whether Six1 overexpression influences proliferation per se in OCC as opposed to simply decreasing apoptosis, we measured the proliferative index of the CaOV3 transfectants using Ki-67 staining. In CaOV3-Six1 clones, 56 ± 16% of the cells (average of two clones) were positive for Ki-67, compared with 41 ± 8% of the CaOV3-CAT cells (average of two clones); this increase is statistically significant (P = 0.04; Fig. 2B). Consistent with previous results in breast cancer cells where Six1-dependent increases in proliferation are mediated by cyclin A1 (23), we found that Six1 overexpression in OCC led to an up-regulation of cyclin A1 (Fig. 2C).

Organs in Six1 knockout mice show decreased proliferation and increased apoptosis (17–20), and high levels of Six1 overexpression are observed in the TRAIL-resistant SKOV3 cells. Together, these data suggest that the Six1 protein may confer both proliferative and survival advantages. However, the role of Six1 in cancer cell survival has not been established. Hoechst staining for nuclear chromatin condensation revealed at least a 3-fold decrease in basal apoptosis in Six1-overexpressing cells compared with CAT controls (Fig. 3A; P = 0.02), suggesting that Six1 overexpression in OCC increases net cell numbers by decreasing apoptosis in addition to stimulating proliferation.

TRAIL resistance in cell lines overexpressing Six1. SKOV3 cells, which express significantly higher levels of Six1 mRNA and protein than CaOV3 cells (Fig. 1), are reported to be more resistant to TRAIL-mediated apoptosis (38). We thus asked whether Six1 overexpression in CaOV3 and SNU251 cells, at levels similar to endogenous expression in SKOV3 cells, could confer resistance to TRAIL. As Fig. 3B shows, Six1 overexpression in CaOV3 cells decreased TRAIL sensitivity from an IC50 of ~3 ng/mL TRAIL in CaOV3-CAT clones to an IC50 of ~11 ng/mL TRAIL in CaOV3-Six1 clones. Similar results were obtained in SNU251 cells where the IC50 was ~7 ng/mL TRAIL in SNU251-CAT clones and ~15 ng/mL TRAIL in SNU251-Six1. In contrast, Six1 overexpression did not confer resistance to the FasL (Supplementary Fig. S3).

To determine whether the high levels of Six1 in SKOV3 cells are responsible for their resistance to TRAIL-mediated apoptosis, we knocked down Six1 in SKOV3 cells using targeted siRNA. Two
pSUPER-based siRNA constructs targeting Six1 were transfected into SKOV3 cells (Six1C and Six1F). In addition, a pSUPER construct targeting luciferase (Luc), which engages the siRNA machinery but does not affect Six1 levels, was transfected into SKOV3 cells as control. When examined at the onset of the TRAIL assay, Six1 protein was almost completely absent in SKOV3 cells transfected with the pSUPER Six1C construct and was reduced by 50% (as determined by densitometric scanning of a Six1 Western blot) in cells transfected with the pSUPER Six1F construct (Fig. 4A). As expected, the pSUPER Luc siRNA had no effect on Six1 levels (Fig. 4A). SKOV3 cells transfected with the pSUPER Six1C, in which Six1 was effectively knocked down, were dramatically sensitized to TRAIL (IC₅₀ 15 ng/mL TRAIL) compared with SKOV3 cells transfected with the pSUPER Luc control (IC₅₀ not reached at highest TRAIL concentration used, 1,000 ng/mL; Fig. 4B). In addition, SKOV3 cells transfected with the Six1F construct, in which Six1 was reduced by 50%, were partially sensitized to TRAIL (Fig. 4B). Because the extent of Six1 knockdown by each of the siRNAs correlated with the sensitivity to TRAIL, these data, together with Six1 overexpression studies, conclusively show that Six1 expression modulates sensitivity to TRAIL. In contrast, complete Six1 knockdown in SKOV3 cells (using Six1C) resulted in only a minimal (<2-fold) sensitization to FasL (Supplementary Fig. S4). Therefore, Six1 selectively and significantly inhibits TRAIL-mediated apoptosis.

Six1 is overexpressed in ovarian carcinomas. Because TRAIL resistance is potentially linked to the metastatic progression of ovarian cancers, the discovery that Six1 modulates the response of ovarian cancer cells to TRAIL could have significant implications both for ovarian cancer prognosis as well as for therapeutic interventions. For this reason, it is important to know whether Six1 overexpression is prevalent in ovarian carcinomas, and whether its expression is related to cancer survival. To address these issues, we isolated RNA from normal ovaries, cystadenomas, and early-stage (stage I) and late-stage (stages II, III, and IV) ovarian carcinomas and did quantitative RT-PCR to examine Six1 mRNA levels. It should be noted that because normal ovary and cystadenoma samples may contain a larger proportion of non-epithelial cells than either early-stage or late-stage tumors, an effort was made to selectively separate and analyze the surface epithelium of normal ovaries and cystadenomas as opposed to the underlying stroma at the time of excision. Using a cutoff of 100 fg Six1/ng RNA, 0 of 5 (0%) normal postmenopausal ovaries, 4 of 13 (30%) cystadenomas, 3 of 6 (50%) early-stage cancers (stage I), and 17 of 27 (63%) late-stage cancers with loco-regional peritoneal dissemination (stages II–IV) were shown to overexpress Six1 (Fig. 5A). Six1 mRNA is elevated in early-stage ovarian tumors compared with normal ovary (mean relative expression, 117 ± 34 fg/ng RNA versus 32 ± 16 fg/ng RNA; ~ 50% (as determined by densitometric scanning of a Six1 Western blot) in cells transfected with the pSUPER Six1F construct (Fig. 4A). As expected, the pSUPER Luc siRNA had no effect on Six1 levels (Fig. 4A). SKOV3 cells transfected with pSUPER Six1C, in which Six1 was effectively knocked down, were dramatically sensitized to TRAIL (IC₅₀ ~ 15 ng/mL TRAIL) compared with SKOV3 cells transfected with the pSUPER Luc control (IC₅₀ not reached at highest TRAIL concentration used, 1,000 ng/mL; Fig. 4B). In addition, SKOV3 cells transfected with the Six1F construct, in which Six1 was reduced by 50%, were partially sensitized to TRAIL (Fig. 4B). Because the extent of Six1 knockdown by each of the siRNAs correlated with the sensitivity to TRAIL, these data, together with Six1 overexpression studies, conclusively show that Six1 expression modulates sensitivity to TRAIL. In contrast, complete Six1 knockdown in SKOV3 cells (using Six1C) resulted in only a minimal (<2-fold) sensitization to FasL (Supplementary Fig. S4). Therefore, Six1 selectively and significantly inhibits TRAIL-mediated apoptosis.

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Figure 2. Six1 increases OCC proliferation concomitant with cyclin A1 induction. A, cell numbers in CaOV3-Six1 cells and CAT-transfected controls. Higher cell numbers are observed at 3 and 5 d after plating in the CaOV3 cells (P < 0.001). Solid black line, Six1-A; dashed line, Six1-B; dotted line, CAT-A; gray line, CAT-B. B, Ki-67–positive cells in CaOV3-Six1 clones compared with CAT controls. Percentage of positive cells in Six1-A and Six1-B combined is 56 ± 16% compared with CAT-A and CAT-B combined, which is 41 ± 8% (P = 0.04). C, Western blot analysis of cyclin A1 (CycA1) protein expression in CaOV3-Six1 transfectants and CaOV3-CAT controls.

Figure 3. Six1 overexpression leads to a decrease in basal apoptosis and to resistance to TRAIL. A, percentage of cells with condensed nuclei in CaOV3-Six1 clones compared with CaOV3-CAT cells (P = 0.02 CAT versus Six1 clones). B, effect of 40 h of TRAIL on Six1-overexpressing CaOV3 and CAT control transfectants. Percentage survival was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay.
Six1 expression cannot be attributed merely to its presence in an epithelial cell population. These data show that Six1 mRNA is overexpressed in ovarian carcinoma and suggest that Six1 expression contributes to a more malignant phenotype.

To examine whether Six1 overexpression may contribute to a more malignant phenotype, we determined the relationship between levels of Six1 mRNA in patients with late-stage ovarian cancer and survival. In support of a role for Six1 in tumor progression, late-stage ovarian cancer patients expressing high levels of Six1 (>300 fg/ng 18S rRNA) exhibited significantly shortened survival at an overall median follow-up of 11 months than late-stage ovarian cancer patients with lower levels of Six1 overexpression (see Supplementary Table S1 for a summary of clinical-pathologic details of all ovarian tumors used in the Kaplan-Meier analysis). Of seven ovarian cancer patients with Six1 >300 fg/ng rRNA, four have died with a median survival of 17 months; two are alive with disease; and one is disease-free at 36 months of follow-up. In contrast, the 20 ovarian cancer patients with late-stage disease that exhibited Six1 levels below 300 fg/ng rRNA are all alive with a median follow-up of 12 months, and 8 of 20 patients are disease-free. Survival effects observed are independent of stage, histology, extent of debulking, or chemosensitivity. Thus, high levels of Six1 overexpression are significantly associated with poor clinical outcome (P = 0.0015; Fig. 5B). As many, but not all, late-stage ovarian carcinomas are of the serous subtype (see Supplementary Table S1), we further restricted the Kaplan-Meier analysis specifically to serous carcinomas to remove any differences in Six1 expression that may be due to tumor histology. Supplementary Figure S5 shows that in serous ovarian carcinomas, high levels of Six1 expression (>300 fg/ng 18S rRNA) also correlate with significantly shortened survival (P = 0.015). This shows that Six1 overexpression is significantly associated with worsened survival within a histologic ovarian tumor subtype (serous) as well as within all late-stage ovarian carcinomas regardless of histologic subtype.

Discussion

In this study, we first show that Six1 overexpression in OCC leads to increased proliferation that is associated with increased A-type

Figure 4. Six1 knockdown sensitizes OCC to TRAIL-mediated apoptosis. SKOV3 cells were transiently transfected with plasmids expressing siRNA sequences against Six1 and luciferase (Luc) as outlined in the methods. A, SIX1 Western blot analysis showing efficiency of knockdown with siRNA constructs. In the experiment shown, the Six1C construct completely knocked down SIX1 expression, whereas the Six1F construct only partially knocked down Six1. Confirmatory Western blots were done for each experiment (three experiments done) and showed similar results, with the Six1C construct always giving a near complete knockdown, and the Six1F construct resulting in partial or no knockdown. B, effect of TRAIL on cells transfected with a control (Luc, dotted line) construct, an efficient Six1-targeting construct Six1C (solid black line), and a less efficient (see Western blot) Six1-targeting construct Six1F (dashed line). Experiment was done thrice (each condition in quintuplet), and the level of Six1 knockdown correlated with TRAIL sensitivity in each experiment.

Figure 5. Six1 mRNA is overexpressed in ovarian carcinomas compared with normal ovary and is associated with poor prognosis in late-stage cancers. A, quantitative RT-PCR for Six1 in normal ovary (n = 5), cystadenoma (n = 13), early-stage (stage I) ovarian carcinomas (n = 6), and late-stage metastatic (stages II–IV) ovarian carcinomas (n = 27). Dotted line runs across graph at 100 fg Six1/ng 18S rRNA. B, survival curves for patients with tumor Six1 levels >300 fg/ng rRNA (solid line) compared with Six1 levels <300 fg/ng rRNA (dashed line). Median follow-up of 11 months (log-rank, P = 0.0015).
cyclicity. These findings suggest that the mechanism by which Six1 stimulates cell cycle progression is similar in ovarian and breast cancer (23) as well as in rhabdomyosarcomas (34). Most importantly, we further show that Six1 overexpression leads to decreased apoptosis in OCC. Although many oncogenes that increase proliferation concomitantly increase apoptosis (thus requiring a second "hit" to inhibit apoptosis before tumors can form; ref. 39), an oncogene that simultaneously increases proliferation and decreases apoptosis would be expected to have profound effects in the cell, leading to rapid tumor growth.

We then show that Six1 specifically influences the TRAIL apoptotic pathway. Six1 overexpression causes increased resistance to TRAIL, and knockdown of Six 1 in ovarian carcinoma cells dramatically sensitizes cells to TRAIL, while having only a minimal effect on FasL. sensitization. A recent report suggested that Six1 expression leads to phosphorylation of Akt (34), a known survival factor whose activity would be expected to result in some protection against both TRAIL-mediated (40) and FasL-mediated (41) apoptosis. This effect perhaps explains the minimal sensitization to FasL observed with Six1 knockdown. However, because Six1 overexpression (which increases OCC proliferation) actually decreases TRAIL but not FasL sensitivity, and because Six1 knockdown dramatically sensitizes SKOV3 cells to TRAIL (>60-fold with complete Six1 knockdown), whereas an equivalent Six1 knockdown only sensitizes cells 2-fold to FasL, we suggest that Six1 plays a specific role in conferring resistance to TRAIL. Both TRAIL- and FasL-mediated apoptosis are extrinsic pathways of apoptosis that use the same intracellular signaling pathways (6, 9). Therefore, the greatly increased inhibition of TRAIL-mediated apoptosis by Six1 suggests that Six1 is specifically modulating events that influence TRAIL receptor activation at the cell surface. The mechanism by which Six1 confers resistance to TRAIL remains to be determined.

The ability of Six1 to confer resistance to TRAIL-mediated apoptosis in cancer cells has several important consequences. Expression of TRAIL on natural killer cells has been shown to play an important role in immunosurveillance against tumor cells and is responsible for suppression of metastasis (42). TRAIL-deficient mice are susceptible to both tumor initiation and metastasis, further underscoring the role of TRAIL as a tumor suppressor (11). Mutations of TRAIL receptors are associated with metastatic breast cancer (14), and expression of TRAIL in breast and ovarian cancers is associated with better prognosis (43, 44). Finally, TRAIL therapies decrease metastases in a number of mouse models (45, 46). Together, these data suggest that tumors expressing molecules that confer resistance to TRAIL are likely to be more biologically aggressive. Indeed, in this study, we have shown that Six1 mRNA overexpression is associated with late-stage ovarian carcinoma and more specifically confers a worse prognosis in late-stage disease. It should be noted that most early-stage ovarian carcinoma samples examined in this study were histologically mucinous or endometrioid, whereas the majority of late-stage ovarian carcinomas were histologically serous. Although it is formally possible that the difference in Six1 expression correlates with histologic subtype, this does not negate the finding that high Six1 expression correlates with poor prognosis, as this is true even within a specific histologic subtype (serous only; Supplementary Fig. S5). Given the few late-stage mucinous or endometrioid tumors, we are unable to definitively study the trivariate relationship among Six1 expression, tumor stage, and histology.

Survival analysis done on late-stage ovarian carcinomas (stages II–IV) showed that patients whose tumors had levels of Six1 >300 fg/ng rRNA had a shortened survival. Poor prognosis associated with high levels of Six1 was independent of presenting CA125 level, grade, histology, and extent of debulking. These observations suggest that Six1 expression may be an important independent prognostic indicator of poor survival for ovarian cancer patients. In support of this concept, a recent study showed that Eya2, a Six1 coactivator, is amplified and up-regulated in ovarian cancer, and its overexpression was significantly associated with shortened survival in advanced disease (47). We conclude that the Six1-Eya regulatory network, known to play an important role in the development of numerous organs (17, 48, 49), may also play a role in ovarian carcinoma.

A second important consequence of Six1 overexpression in ovarian carcinoma is that it may alter tumor response to TRAIL-related therapies. Recombinant TRAIL and/or agonistic monoclonal antibodies are currently in phase I clinical trials against advanced solid malignancies, including ovarian cancer (9). Because Six1 mRNA is overexpressed in 63% of late-stage ovarian carcinomas, and because TRAIL resistance is conferred by Six1 overexpression, it is highly likely that Six1 levels in patient tumors will influence the outcome of these clinical trials. Hence, the status of Six1 expression in ovarian tumors may serve not only as a prognostic indicator of outcome but also as a functional screening strategy to predict whether treatment targeting TRAIL-mediated apoptosis may be of benefit.

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