Inhibition of Notch Signaling in Combination with Paclitaxel Reduces Platinum-Resistant Ovarian Tumor Growth

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Accessibility
Inhibition of Notch signaling in combination with paclitaxel reduces platinum-resistant ovarian tumor growth

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INTRODUCTION

Ovarian cancer (OvCa) represents the second most common, and most lethal gynecologic malignancy in the United States. By the end of 2013, it is estimated that over 22,000 women will have been diagnosed with OvCa, and more than 15,000 patients will have succumbed to the disease (1). The high death rate is due in part to the fact that the majority of patients present with advanced stage disease possibly due to a lack of screening strategies that effectively detect the cancer in early stages. The most common subtype is high-grade serous carcinoma (70%), followed by endometrioid and clear cell variants (2). Despite the histologic variation, the current treatment regimen includes cytoreductive surgery as well as six cycles of taxane and platinum-based chemotherapy delivered IV or IP (3, 4). While 70–80% of patients respond to this first-line chemotherapy, recurrences occur frequently and a significant majority of those patients develop chemoresistant disease (5, 6). The overall prognosis of patients diagnosed with OvCa therefore remains poor, with a 5-year survival rate of approximately 50% (4). Insights into key molecular pathways that are overexpressed in OvCa and contribute to tumor progression, recurrence, and chemoresistance may lead to novel therapies that can improve the treatment strategies for these women.

The Notch signaling cascade has been implicated in numerous malignancies (7–11) and is one of the most altered pathways in serous OvCa (2). Notch signaling is involved in the regulation of proliferation, differentiation, cell fate, and survival and plays an important role in embryonic development as well as in self-renewal of adult stem/progenitor cells (12–14). Activation of the Notch signaling cascade has been implicated in numerous malignancies (7–11) and is one of the most altered pathways in serous OvCa (2). Notch signaling is involved in the regulation of proliferation, differentiation, cell fate, and survival and plays an important role in embryonic development as well as in self-renewal of adult stem/progenitor cells (12–14). Activation of the Notch pathway possibly due to a lack of screening strategies that effectively detect the cancer in early stages. The most common subtype is high-grade serous carcinoma (70%), followed by endometrioid and clear cell variants (2). Despite the histologic variation, the current treatment regimen includes cytoreductive surgery as well as six cycles of taxane and platinum-based chemotherapy delivered IV or IP (3, 4). While 70–80% of patients respond to this first-line chemotherapy, recurrences occur frequently and a significant majority of those patients develop chemoresistant disease (5, 6). The overall prognosis of patients diagnosed with OvCa therefore remains poor, with a 5-year survival rate of approximately 50% (4). Insights into key molecular pathways that are overexpressed in OvCa and contribute to tumor progression, recurrence, and chemoresistance may lead to novel therapies that can improve the treatment strategies for these women.

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signaling cascade occurs through binding of one of four Notch receptors (Notch1–4) with one of its ligands (Jagged and Delta-like) on a neighboring cell. Sequential proteolytic cleavage of the Notch receptor by a disintegrin and metalloprotease (ADAM) and γ-secretase leads to the release of the Notch intracellular domain (NICD). This active fragment subsequently translocates to the nucleus, where it activates transcription of target genes including members of the HES and HEY families (12, 15).

Recent studies have demonstrated expression of the Notch pathway in many malignancies including breast, intestinal, pancreatic, brain, and OvCa (10, 16–21). Genomic analyses of ovarian carcinomas as part of The Cancer Genome Atlas (TCGA) Project showed alteration of Notch signaling in 22% of analyzed tumors (2). In addition, Notch1 and Notch3 RNA transcript and protein are highly expressed in ovarian carcinomas (22–27) and elevated expression correlates with resistance to chemotherapy and decreased survival (28–30). Investigators have hypothesized that Notch signaling may promote the activity of a tumor-initiating cell population that can sustain the growth of ovarian tumors despite cytotoxic therapies that halt the progression of actively proliferating cancer cells (31–33). While this hypothesis is controversial, it would provide rationale for combining Notch pathway inhibition with conventional cytotoxic therapy.

Targeting of the Notch pathway is currently being investigated in a variety of cancers, with γ-secretase inhibitors (GSIs) being the most widely used Notch inhibitors (34, 35). Few phase I and II clinical trials have reported GSI anti-tumor activity though these studies have included only a few women with OvCa. Previous pre-clinical studies have shown that inhibition of Notch signaling blocks the growth of both OvCa cell lines in vitro and cell line-derived xenografts in vivo (36–38). In addition to inhibition of cell proliferation and induction of cell death, treatment with GSI is associated with an increased sensitivity of OvCa cell lines to platinum therapy (39) supporting the concept that treatment with Notch antagonists may be relevant in a recurrent disease setting (28).

In the current study, we analyzed the contribution of the Notch pathway to the pathobiology of serous OvCa. We assessed the expression of specific members of the Notch family and tested the anti-tumor activity of the GSI MRK-003 against patient-derived xenografts (PDXs) generated from serous carcinomas of the ovary or peritoneum. The pre-clinical efficacy of MRK-003 has been determined in several human cancers (8, 9, 34, 40–43), and we wanted to assess the effectiveness of this GSI as a single agent and in combination with standard cytotoxic chemotherapy in a cohort of clinically platinum-sensitive and resistant ovarian tumors. Treatment of mice harboring the ovarian tumor xenografts with MRK-003 resulted in reduced tumor growth in a subset of experiments. Combination cytotoxic chemotherapy and GSI demonstrated synergistic activity only in those platinum-resistant tumors suggesting Notch pathway inhibition may be more effective in the recurrent and refractory setting.

**MATERIALS AND METHODS**

**GENERATION AND PROPAGATION OF OVARIAN CANCER XENOGRAFTS**

Our PDX model utilizing primary human ovarian tumors has been described previously (44). Briefly, excess serous OvCa tissue or ascites was obtained at the time of surgery from patients who had given informed consent to participate in an Institutional Review Board approved tissue collection protocol. Following enzymatic processing, tumor-derived single-cell suspensions were depleted of hematopoietic and endothelial cells, resuspended in PBS, mixed with Matrigel (1:1), and injected subcutaneously (s.c.) in female NOD/SCID mice. The range of cells injected was 7.5 × 10^5 to 1.5 × 10^6 in each animal. Tumor formation in the injected animals was regularly monitored, and tumors were harvested from euthanized animals when they reached a diameter of 1–2 cm. The harvested xenografts were then enzymatically processed, depleted of H-2Kd positive mouse cells, and injected s.c. into NOD/SCID mice as described above. Using this transplantation system, cohorts of 15–40 mice injected with OvCa cells derived from a single patient were generated and subsequently used to perform treatment experiments.

**IMMUNOHISTOCHEMICAL ANALYSIS**

Notch1 and Notch3 protein expression in formaldehyde fixed and paraffin embedded sections of primary and xenograft ovarian tumors were analyzed by immunohistochemistry (IHC) using a mouse monoclonal anti-Notch1 antibody (Novus Biologicals) or a rabbit polyclonal anti-Notch3 antibody (Abgent). Non-specific binding of antibody was blocked by either the Vector Laboratories M.O.M. kit (in the case of Notch1) or 5% normal goat serum in PBS with 0.1% Triton X (Notch3) followed by incubation with the relevant primary antibody and the appropriate biotinylated secondary antibody. Subsequent treatment with Vectastain ABC reagents (Vector Laboratories), visualization with 3,3′-diaminobenzidine chromogen (DAB, Dako), and counterstaining with hematoxylin were performed.

**IMMUNOBLOTTING**

Whole cell lysates were prepared from frozen xenograft samples or cell lines using Mammalian Protein Extraction Reagent (Thermo Scientific) lysis buffer supplemented with inhibitors of endogenous protease, kinase, and phosphatase activity (all obtained from Sigma-Aldrich). Twenty micrograms of protein from each sample were separated on 7.5 or 10% polyacrylamide gels and transferred to a PVDF membrane. Following transfer, membranes were blocked with 5% milk in 1 × TBS, 0.1% Tween-20 (TBST) and incubated in diluted (1:1000) primary antibody overnight, according to the manufacturer’s recommendations. Primary antibodies used were a rabbit monoclonal anti-Notch1 antibody, a rabbit monoclonal anti-cleaved Notch1 (Val1744) antibody, and a rabbit monoclonal anti-Notch3 antibody (Cell Signaling). Membranes were then incubated with a horseradish peroxidase (HRP) conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology) and developed using a chemiluminescent detection reagent obtained from GE Healthcare Life Sciences. Equivalent protein loading was verified by stripping the blots and re-probing with a mouse anti-Pan-Actin antibody (NeoMarkers).

**CELL CULTURE AND IN VITRO TREATMENT WITH MRK-003**

The OVCAR3 and SKOV3 human OvCa cell lines were purchased from ATCC. For MRK-003 dose response experiments, equal numbers of OVCAR3 or SKOV3 cells were plated and serum-starved...
Table 1 | Clinical characteristics of patients whose tumors were analyzed in this study.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at diagnosis (years)</th>
<th>Stage</th>
<th>Grade</th>
<th>Progression free survival (months)</th>
<th>Overall survival (months)</th>
<th>Current status</th>
</tr>
</thead>
<tbody>
<tr>
<td>OV1</td>
<td>38.3</td>
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<td>3</td>
<td>34.1</td>
<td>57.9</td>
<td>Alive</td>
</tr>
<tr>
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<td>3</td>
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<td>37.6</td>
<td>Deceased</td>
</tr>
<tr>
<td>OV3</td>
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<td>IIIC</td>
<td>3</td>
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<td>Deceased</td>
</tr>
<tr>
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<td>22.4</td>
<td>28.9</td>
<td>Alive</td>
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<tr>
<td>OV5</td>
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<td>IIIC</td>
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<td>Deceased</td>
</tr>
<tr>
<td>OV6</td>
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<td>7.1</td>
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</tr>
<tr>
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<td>ND</td>
<td>4.9</td>
<td>6.5</td>
<td>Deceased</td>
</tr>
</tbody>
</table>

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FIGURE 1 | Variable expression of Notch1 and Notch3 in ovarian cancer specimens. (A,C) Immunohistochemical analyses of Notch1 and Notch3 expression was carried out on xenograft tumors generated from all seven tumors that were subsequently used for treatment studies (OV1–OV7), as well as on paired primary tissue samples when available (OV1–OV5). Representative images of Notch1 (A) and Notch3 (C) immunostaining are shown. (B,D) Western blotting was performed to analyze expression of Notch1 (B) and Notch3 (D) in xenografts derived from primary human serous ovarian cancers (OV1–OV7). Uncleaved, inactivated Notch1 and Notch3 as well as cleaved activated Notch1 and Notch3 are shown. An anti-Pan-Actin antibody was used to confirm equivalent loading of samples. The HCC1187 and SKOV3 cell lines were used as a negative control (−Cx) for Notch1 and Notch3, respectively. The MCF-7 cell line was used as a positive control (+Cx) for both Notch1 and Notch3.

reduction in OVCAR3 and SKOV3 cell counts was found compared to cells incubated with medium only (see Figure 2A). Subsequently, OVCAR3 and SKOV3 cells were treated with either MRK-003 at the relevant IC$_{50}$ (5 µM for OVCAR3, 10 µM for SKOV3) or vehicle control for 6 h and then harvested. The impact of MRK-003 on Notch signaling was assessed by immunoblotting analysis of γ-secretase-cleaved Notch1 levels and qPCR analysis of target gene Hes1 expression. We observed both a strong reduction in cleaved Notch1 protein levels as well as a marked decrease in Hes1 gene expression relative to the vehicle treated control in both cell lines following treatment with MRK-003 (see Figures 2B,C).

IMPACT OF MRK-003 IN VIVO TREATMENT ON Hes1 EXPRESSION
Our in vitro data suggest that MRK-003 specifically targets the Notch pathway in OvCa cell lines. To extend these findings, we assessed the effect of MRK-003 on Notch signaling in PDXs. OvCa xenografts were harvested from mice 6, 24, or 48 h after administration of a single dose of MRK-003 or vehicle control and expression of the Notch target gene Hes1 was analyzed by qPCR. In all analyzed xenografts, a 30–70% decrease in Hes1 mRNA levels, relative to levels in vehicle treated controls, was observed after exposure to MRK-003. Figure 3 is a representative example of these analyses and illustrates the
The γ-secretase inhibitor MRK-003 inhibits OVCAR3 and SKOV3 cell proliferation and decreases NICD1 and Hes1. (A) In vitro treatment of OVCAR3 and SKOV3 cells with MRK-003 leads to a dose-dependent reduction of cell proliferation. Cells were incubated with increasing concentrations (0–10 µM) of MRK-003 for 48 h in medium containing 1% FBS. OVCAR3 cells were treated in triplicate, and SKOV3 cells were treated in quadruplicate. Average relative changes in cell numbers are shown, and error bars represent the standard error of the mean. (B) Western blotting analysis of OVCAR3 and SKOV3 cells incubated with 5 µM (OVCAR3) or 10 µM (SKOV3) MRK-003 for 6 h reveals a marked decrease in expression of cleaved Notch1 (Val17/44). An anti-Pan-Actin antibody was used to confirm equivalent loading of samples. Control = cells incubated with medium only. (C) qPCR analysis of Hes1 gene expression in MRK-003 treated OVCAR3 and SKOV3 cells, harvested 6 h post treatment, showed significantly decreased Hes1 transcript levels compared with cells incubated with medium only. Relative changes in Hes1 mRNA expression are shown, normalized to expression of housekeeping gene β-actin.

Relative transcript levels of Hes1 in OV5 xenografts collected 6 h after treatment with MRK-003 or vehicle. OV5 was selected as a representative example as this was a platinum-resistant tumor that demonstrated a potent synergy with paclitaxel in our in vivo experiments.

Assessment of the impact of MRK-003 as a single agent or in combination with cytotoxic chemotherapeutics on ovarian cancer xenografts

The clinical characteristics of the seven patients whose tumors were used to generate primary xenografts are summarized in
MRK-003 monotherapy led to significant tumor growth reduction of OV5 xenografts, compared with vehicle treatment ($p = 0.04$, see Figure 5A). Again, no significant weight loss or other signs of toxicity were observed in mice treated with MRK-003 (data not shown).

**DISCUSSION**

In the current study, we confirm that members of the Notch signaling pathway are expressed in serous OvCa, and GSI-mediated targeting of this pathway leads to anti-proliferative effects *in vitro* and anti-tumor activity *in vivo*. While single agent GSI was tumorstatic in half of the analyzed PDXs, combination therapy with cytotoxic chemotherapy led to significant synergistic anti-tumor effects only in those xenografts originally derived from platinum-resistant OvCa patients. Collectively, these pilot data support the role of Notch signaling in OvCa growth particularly in the chemoresistant setting.

Various studies have detected Notch1 and Notch3 expression in ovarian tumors by IHC, real-time polymerase chain reaction, and/or immunoblotting. The results for Notch1 expression via detection by IHC have been variable and difficult to interpret. In one study of relative Notch1 expression in benign ovarian tissue and a spectrum of low- to high-grade ovarian carcinomas, Notch1 was rarely detected in benign tissue and its expression level correlated with increasing grade and clinical stage of disease (23). Interestingly, Notch1 was primarily confined to the cell membrane and the cytoplasm. The direct functional or clinical significance of increased cytoplasmic expression in OvCa is unclear given that cleaved Notch1 migrates to the nucleus and is considered the active form. In similar immunohistochemical analyses, no nuclear Notch1 expression was detected suggesting little to no role for Notch1 in OvCa (46). In contrast, Notch1 nuclear expression was evident in our cohort of samples but was highly variable among and within samples. Despite the variation between samples, however, the intensity of nuclear Notch1 expression in each primary tumor and its correlate xenografts was stable reinforcing that Notch1 expression remains consistent across subsequent xenograft generations. These data suggest that protein expression of Notch1 or 3 does not correlate with response to GSI suggesting many other molecular inputs must be involved with modulating response in the platinum-resistant setting.

While Notch1 IHC studies have generated conflicting results, immunoblotting analyses that have largely focused on Notch1 NICD expression in primary samples and established human OvCa cell lines are much less controversial. Rose et al. (22) found abundant levels of cleaved Notch1 in the OVCAR3, SKOV3, and CaOV3 cell lines as well as in approximately 75% of the primary OvCa samples analyzed. Consistent with published work (22, 36), we similarly detected cleaved Notch1 in OvCa cell lines and primary ovarian tumor samples by immunoblotting. Thus, in contrast to the collective IHC results, the data to date suggest that cleaved Notch1 is present in some samples thereby providing indirect evidence that Notch1 signaling is active in OvCa.

This hypothesis is further bolstered by functional studies that suggest Notch1 promotes OvCa cell proliferation. In one investigation, stable transfection of A2780 OvCa cells with the Notch1 NICD increased both cell proliferation and the rate of colony

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**Table 1.** Briefly, all samples were obtained from women with primarily advanced stage disease, three of whom manifested platinum-sensitive disease and markedly improved survival compared to the platinum-resistant patients. In three independent experiments, mice harboring xenografts derived from a platinum-sensitive tumor were randomized to receive either MRK-003 alone, P/C alone, the combination of MRK-003 and P/C, or vehicle alone. We observed a significant reduction in OV3 ($p = 0.02$) and OV4 ($p < 0.01$) PDX growth following administration of MRK-003 alone, compared with vehicle treated control tumors (see Figure 4). Treatment with P/C alone significantly decreased tumor volumes of all three analyzed platinum-sensitive PDXs, and combination therapy with MRK-003 and P/C was not more effective than treatment with P/C alone (see Figure 4). In Figure 4A, the combined P/C and MRK-003 arm was truncated early so that tumor could be harvested for post treatment investigation. No signs of toxicity were observed in mice receiving MRK-003 and animal weights remained stable during the course of treatment (data not shown).

The three primary human platinum-resistant serous ovarian tumors were also established as PDXs in immunocompromised mice. In three separate experiments, mice harboring the tumors were subjected to therapy with either MRK-003 alone, paclitaxel alone, MRK-003 in combination with paclitaxel, or vehicle alone. In all analyzed tumors, enhanced anti-tumor activity was found in mice treated with the combination of MRK-003 and paclitaxel, compared with paclitaxel alone and MRK-003 alone ($p < 0.03$, see Figure 5). Administration of paclitaxel as a single agent led to a significant decrease of xenograft growth in only one of three tumors (OV7, $p = 0.01$, see Figure 5C). In addition,
FIGURE 4 | MRK-003 treatment in vivo inhibits tumor growth in the majority of analyzed platinum-sensitive ovarian cancers. Three independent cohorts of mice bearing xenografts derived from clinically platinum-sensitive serous ovarian cancer were treated with vehicle, MRK-003 alone, paclitaxel/carboplatin (P/C) alone or P/C + MRK-003. A significant anti-tumor effect of treatment with MRK-003 alone was observed in OV3 (B) and OV4 (C) xenografts (p < 0.05). P/C alone and P/C + MRK-003 were equally effective in significantly reducing tumor growth of OV2 (A), OV3, and OV4 xenografts (p < 0.01). Tumor volumes were measured twice weekly. Error bars represent the standard error of the mean.

FIGURE 5 | MRK-003 and paclitaxel synergize to inhibit growth of platinum-resistant ovarian tumors. Three independent cohorts of mice bearing xenografts derived from clinically platinum-resistant serous ovarian cancer were randomly divided into four groups. Each group received vehicle, paclitaxel alone, MRK-003 alone or paclitaxel + MRK-003. Significantly heightened inhibition of tumor growth was observed upon combination treatment with paclitaxel + MRK-003, compared with treatment with either drug alone (p < 0.03). Tumors were measured twice weekly. Error bars represent the standard error of the mean. OV5 (A); OV6 (B); and OV7 (C).

formation (26). Others found that downregulation of γ-secretase activity reduced Notch1 and Hes1 mRNA and protein in the same A2780 OvCa cells and this decrease correlated with a decrease in cell proliferation (36). Since pan inhibition of γ-secretase likely inhibits activation of all Notch receptors, the observed effects may not be due to specific disruption of Notch1 signaling. Rose and colleagues (22) provided more direct evidence of Notch1 involvement in cell proliferation by siRNA-mediated reduction of Notch1 levels. The decrease in Notch1 in response to siRNA correlated with decreased cell proliferation. Thus, the in vitro data suggest that Notch1 signaling can influence the biology of OvCa.

Notch3 has also been implicated in the pathology of OvCa as its expression is evident in a significant number of analyzed OvCa samples. Unlike Notch1, however, investigators have focused on nuclear localization of Notch3 as a functional marker of pathway activation (25, 27, 29, 47). Like Notch1, Notch3 was also expressed at much lower levels in benign ovarian tissue when compared to malignant samples (25, 29), and higher expression levels were associated with either advanced stage (29) or recurrent disease (28, 48). At the genomic level, the Notch3 gene was amplified in a cohort of OvCa samples, as detected by fluorescence in situ hybridization, and this amplification positively correlated with increased Notch3 protein expression (24). This finding was supported by recent data from TCGA, which also showed that Notch3 was amplified in serous ovarian carcinoma (2). Our data support the finding that cleaved Notch3 is present in primary OvCa samples and likely contributes to the pathology of OvCa (24, 25, 39, 48). Blocking Notch3 signaling by a GSI or a Notch3 specific siRNA reduced cell number and led to an increase in apoptosis in OVCAR3 and A2780 OvCa lines (24). Interestingly, the Notch ligand Jagged-1 was abundantly
expressed in both OvCa cell lines and benign mesothelial cells derived from tumors (25). Both gene knockdown of Jagged-1 and transfection of the Notch3 intracellular domain (NICD3) suggest the existence of a juxtacrine loop that impacts ovarian cell-cell adhesion and tumor growth (25).

While Notch3 appears to play a role in OvCa cell proliferation, tumor growth, and metastasis, additional investigations have implicated Notch signaling in chemoresistance (28, 39, 48). Knockdown of Notch3 sensitizes OvCa3 cells to carboplatin (28). Additionally, Notch3 is overexpressed in cisplatin and cisplatin/paclitaxel-resistant OvCa cell lines and its inactivation by GSI or siRNA reverses this resistance (48). More recently, OVCA429 cells that were transduced with a retroviral vector expressing NICD3 showed an increase in expression of smooth muscle actin, Slug and Snail, consistent with an epithelial to mesenchymal transition. Interestingly, this shift was associated with an increase in resistance to carboplatin-induced apoptosis (49). McAuliffe and colleagues (39) suggested that tumorigenic OvCa stem cell (CSC) populations defined as CD44+ cells or the side population fraction [reviewed by Foster et al. (50)] were decreased with inhibition of γ-secretase activity. These data suggest that Notch signaling may support populations of CSCs in OvCa that are resistant to conventional cytotoxic therapies. Notch signaling can modulate other signaling pathways that may influence CSC function. Specifically, the Notch target Hes1 is expressed in both OvCa cell lines and benign mesothelial cells derived from tumors (25). Both gene knockdown of Jagged-1 and transfection of the Notch3 intracellular domain (NICD3) suggest the existence of a juxtacrine loop that impacts ovarian cell-cell adhesion and tumor growth (25).

In conclusion, this pilot investigation suggests specific Notch family members are variably expressed in OvCa and that MRK-003-mediated inhibition of Notch signaling reduces OvCa cell proliferation in vitro and inhibits tumor growth in vivo. While single agent anti-tumor activity was observed in many of the PDX analyses, GSI improved conventional cytotoxic therapy exclusively in combination with paclitaxel in those OvCa tumors known to be clinically platinum-resistant. Our results support the rationale for further investigation of the pre-clinical and clinical effectiveness of GSIs in OvCa, especially in the platinum-resistant setting.

AUTHOR CONTRIBUTIONS

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Notch inhibition in ovarian cancer

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28. Groeneweg et al. Notch inhibition in ovarian cancer


**Conflict of Interest Statement:** Sriram Sathyanarayan was an employee at Merck Oncology, Boston, MA, USA at the time this study was designed and initiated. All other authors reported no conflict of interest with the work presented in this manuscript.


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