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Inhibition of gamma-secretase activity impedes uterine serous carcinoma growth in a human xenograft model

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HIGHLIGHTS

- Uterine serous carcinoma (USC) exhibited increased Notch pathway expression compared to endometrioid carcinoma.
- Notch inhibition leads to *in vitro* and *in vivo* activities in the majority of USC cell lines and xenografts.
- Notch inhibition with paclitaxel and carboplatin leads to synergistic anti-tumor activity in subsets of USC patient derived xenografts (PDXs).

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ABSTRACT

Objective. Uterine serous carcinoma (USC) represents an aggressive subtype of endometrial cancer. We sought to understand Notch pathway activity in USC and determine if pathway inhibition has anti-tumor activity.

Methods. Patient USC tissue blocks were obtained and used to correlate clinical outcomes with Notch1 expression. Three established USC cell lines were treated with gamma-secretase inhibitor (GSI) *in vitro*. Mice harboring cell line derived or patient derived USC xenografts (PDXs) were treated with vehicle, GSI, paclitaxel and carboplatin (P/C), or combination GSI and P/C. Levels of cleaved Notch1 protein and *Hes1* mRNA were determined in GSI treated samples. Statistical analysis was performed using the Wilcoxon rank sum and Kaplan–Meier methods.

Results. High nuclear Notch1 protein expression was observed in 58% of USC samples with no correlation with overall survival. GSI induced dose-dependent reductions in cell number and decreased levels of cleaved Notch1 protein and *Hes1* mRNA *in vitro*. Treatment of mice with GSI led to decreased *Hes1* mRNA expression in USC xenografts. In addition, GSI impeded tumor growth of cell line xenografts as well as UT1 USC PDXs. When GSI and P/C were combined, synergistic anti-tumor activity was observed in UT1 xenografts.

Conclusions. Notch1 is expressed in a large subset of USC. GSI-mediated Notch pathway inhibition led to both reduced cell numbers *in vitro* and decreased tumor growth of USC some xenograft models. When combined with conventional chemotherapy, GSI augmented anti-tumor activity in one USC PDX line suggesting that targeting of the Notch signaling pathway is a potential therapeutic strategy for future investigation.

Introduction

Endometrial cancer is the most prevalent gynecologic malignancy and the fourth leading cancer among women in the United States. In 2014, approximately 52,000 women will be diagnosed with endometrial cancer and more than 8500 patients will not survive their disease [1]. Investigators have characterized endometrial cancers as either type I or type II [2]. Type I carcinomas account for the majority of endometrial cancer and present with early stage, low grade tumors of endometrioid histology. In contrast, type II endometrial cancers are aggressive, high grade subtypes that encompass a spectrum of histologies including carcinosarcoma, clear cell carcinoma and uterine serous carcinoma (USC) [3].

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Although USC represents the most common type II carcinoma, it accounts for only 10% of all endometrial cancers. USC frequently presents as advanced stage, metastatic disease resistant to conventional chemotherapy, and accounts for a disproportionate 40% of disease-related deaths [4]. Unlike type I endometrial cancers, USC can be primarily treated with cytoreductive surgery followed by platinum-based chemotherapy and radiation treatment [5]. Despite this multi-modality approach, recurrence and chemoresistant disease are common [6] highlighting the need for investigation of novel therapeutic options including targeted therapy.

The Notch signaling pathway plays an important role in multiple developmental and cellular processes, including regulation of cell proliferation, differentiation, apoptosis and stem cell self-renewal [7]. In humans, there are four Notch receptors (Notch1-4), each of which can be activated through binding with one of its ligands (Jagged and Delta-like) on neighboring cells [8]. This activation induces proteolytic cleavage of the Notch receptor by a disintegrin and metalloprotease (ADAM), followed by further cleavage by gamma-secretase and release of the Notch intracellular domain (NICD). Subsequent translocation of the NICD to the nucleus leads to transcriptional activation of target genes, such as members of the *Hes* and *Hey* families [8,9].

Deregulation of the Notch pathway has been demonstrated in a variety of malignancies with Notch1 and Notch3 most widely implicated in malignant transformation [10]. Both oncogenic and tumor-suppressive effects of the Notch pathway have been observed [11]. Interestingly, Notch was shown to be one of the most consistently activated signaling pathways in serous ovarian cancer, and scientific reports have supported that Notch signaling contributes to ovarian cancer pathology [12–14]. In contrast, there is limited evidence implicating Notch signaling in endometrial cancer pathogenesis. Some investigators have reported increased Notch1 protein expression in endometrioid endometrial cancers with one study also detecting a rise in Notch3 expression [15–17]. In addition, an elevated level of Notch1 or Notch3 protein in endometrioid endometrial carcinoma was significantly associated with worse overall survival [16]. In contrast, one recent study showed a decrease in mRNA levels of all four Notch receptors in endometrioid endometrial tumors, relative to expression in benign endometrium [18]. Although the role of Notch signaling in USC is currently unclear, the similar histology, clinical behavior and genomic profile of serous ovarian tumors and USC suggest that the analysis of Notch gene and protein expression in USC could reveal a new therapeutic target in the treatment of this tumor.

Given the role of Notch in human cancers, a variety of Notch pathway inhibitors have been developed [19]. Gamma-secretase inhibitors (GSIs) that block the cleavage of all four Notch paralogs are the most frequently utilized Notch-targeting agents [20]. In *in vitro* and *in vivo* models of serous ovarian cancer, Notch pathway inhibition led to decreased cell proliferation and xenograft growth, and increased sensitivity to chemotherapeutic agents [10,21]. No analyses of Notch pathway inhibition in serous endometrial cancer have been reported to date. More recently, *in vitro* GSI treatment of Ishikawa cells, which were originally derived from a well differentiated endometrial adenocarcinoma, resulted in decreased proliferation and increased apoptosis [22]. In another recent analysis, GSI treatment decreased the invasive potential of the KLE endometrial cancer cell line without inhibiting cell proliferation [16]. These data highlight a possible role for GSI in endometrial cancer therapy.

We initially aimed to confirm Notch1 protein expression in a spectrum of endometrioid endometrial cancers. More importantly, since Notch1 protein expression in USC had not been reported, we analyzed nuclear Notch1 expression levels in 45 specimens and correlated our findings with their respective clinical outcomes. In addition, we analyzed the functional significance of Notch signaling in USC biology by determining the effects of the pan-GSI MRK-003 [23–25] on cell proliferation in USC cell lines. GSI-mediated inhibition of the Notch pathway was confirmed by analyzing post-treatment levels of both the Notch1 nuclear intracellular domain (NICD1) and the Notch target gene *Hes1* mRNA. Finally, USC cell line derived xenografts as well as USC patient derived xenografts (PDXs) were used to evaluate the anti-tumor activity of MRK-003 as a single agent and in combination with platinum-based chemotherapy to determine if pan-Notch inhibition can synergize with an established therapy. This investigation suggested that nuclear Notch1 levels are increased in high grade endometrioid endometrial cancers and in USC. Gamma-secretase inhibition decreased USC cell proliferation *in vitro* in a dose dependent fashion and down-regulated levels of NICD1 and *Hes1*. Furthermore, GSI treatment impeded USC xenograft growth *in vivo* in a subset of cell line derived xenografts and PDXs. Administration of MRK-003 with paclitaxel and carboplatin (P/C) demonstrated synergistic anti-tumor activity in one of two primary USCs that had high nuclear Notch1 expression.

Methods

Tissue samples

An endometrial cancer tissue microarray consisting of 98 endometrioid endometrial cancers representing the spectrum of grade was obtained from US Biomax, Inc. Following Institutional Review Board (IRB) approval, we identified 45 patients with USC who underwent surgical staging at our institution from 2000 to 2012 and obtained formalin fixed, paraffin embedded (FFPE) tumor blocks of surgically removed USC tissue from each patient.

Notch1 immunohistochemistry

Following blocking of non-specific binding, sections of the endometrial cancer tissue microarray, primary human USC samples and USC xenograft samples were incubated with a mouse monoclonal anti-Notch1 antibody (Novus Biologicals) overnight at 4 °C. A biotinylated goat anti-mouse secondary antibody (Santa Cruz Biotechnology) was applied for 1 h at room temperature. VECTASTAIN ABC reagents (Vector Laboratories) and 3,3′-diaminobenzidine chromogen (Dako) were used for visualization of staining. Vector Laboratories' M.O.M. kit reagents were used according to the manufacturer's instructions when staining xenograft sections to prevent non-specific staining of mouse cells. All slides were reviewed and scored by a pathologist blinded to the nature of the samples. Notch1 nuclear expression was scored by a pathologist on a 0–3+ scale.

Cell lines and culture

The three established human USC cell lines ARK1, ARK2 and SPEC2 [26,27] were cultured in either RPMI 1640 medium supplemented with 10% fetal bovine serum (ARK1 and ARK2) or MEM medium containing Earle's salts and L-glutamine supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 2% MEM 100× Vitamin Solution and 1% MEM 100× Non-Essential Amino Acids (SPEC2). All three cell lines were incubated at 37 °C in 5% CO_2.

Drugs

MRK-003 was provided by Merck Research Laboratories. Paclitaxel was purchased from Sigma-Aldrich and carboplatin was obtained from the clinical pharmacy at our institution.

In vitro treatment of USC cell lines

For dose response experiments, ARK1, ARK2 and SPEC2 cells were plated on 6-well plates and incubated overnight in their complete growth medium with 1% fetal bovine serum (FBS). A stock of 10 mM MRK-003 in DMSO was diluted in medium containing 1% FBS and cells were treated in duplicate for 48 h with either medium only or increasing concentrations of MRK-003. Cells were collected and counted after