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Xanthohumol decreases Notch1 expression and cell growth by cell cycle arrest and induction of apoptosis in epithelial ovarian cancer cell lines

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ABSTRACT

Objective. Notch1 signaling is active in ovarian cancer and is a promising pathway for new therapies in ovarian cancer. We have previously detected high Notch1 expression in ovarian tumors. Xanthohumol has been shown to inhibit cancer cell growth and invasion, including Kaposi's sarcoma, which also highly expresses Notch1. We hypothesized that the Notch1 signaling pathway is targeted by xanthohumol leading to decreased ovarian cancer cell growth.

Methods. SKOV3 andOVCAR3 cells were utilized. MTT growth assays were conducted following treatment with xanthohumol. Quantitative RT-PCR and Western blot analyses were conducted to assess Notch1 down-regulation. Luciferase reporter assays were performed to assess functional down-regulation of Notch1. Cell cycle analysis was performed by flow cytometry.

Results. Significant growth inhibition and down-regulation of Notch1 transcription and protein expression were found following xanthohumol treatment. In addition, xanthohumol increased Hes6 transcription and decreased Hes1 transcription, known downstream targets of Notch 1. These observations were associated with cell cycle inhibition as demonstrated by an increase in p21 expression and S and G2/M cell cycle arrest confirmed by an increase in phosphorylated cdc2. Furthermore, an increase in the apoptotic markers, cleaved caspase-3 and cleaved PARP were observed.

Conclusion. Xanthohumol was a potent inhibitor of ovarian cancer cell growth, and our results suggest that xanthohumol may be influencing the Notch1 pathway. These findings suggest that xanthohumol could be useful as a therapeutic agent in ovarian cancer.

Introduction

Ovarian cancer is the fifth most common cancer in women in the United States and is the deadliest gynecologic malignancy. In 2009, there was an estimated 21,880 new cases diagnosed in the United States with 13,850 deaths [1]. Due to the difficulty of early detection, most cases of ovarian cancer are stage III or IV when discovered resulting in only a 15–20% cure rate [2]. Following surgical debulking and chemotherapy, 60–70% of ovarian cancer patients relapse and develop resistance to platinum-based chemotherapy [3]. Therefore, it is critical to identify alternative strategies to treat ovarian cancer.

Notch signaling is an evolutionarily conserved pathway throughout the animal kingdom [4,5]. Notch is a type 1 transmembrane receptor that functions as the main regulator of cell fate determination during development and is also involved in regulating cell proliferation and survival. Upon binding with one of its ligands, two proteolytic cleavages result. The first cleavage of the extracellular portion of Notch is catalyzed by an ADAM-family metalloprotease, TACE, followed by the second cleavage by γ-secretase that releases the Notch intracellular domain (NICD) from the inner cell membrane allowing it to translocate to the nucleus where it interacts with DNA-binding CSL along with its coactivator Mastermind and histone acetyltransferases, which convert CSL from a transcriptional repressor to a transcriptional activator [6]. Known transcription targets of Notch signaling include the hairy enhancer of split (Hes) genes, NF-κB, cyclin D1, and c-myc [7]. Conversely, in the absence of Notch signaling, CSL binds to the promoters of its target genes and recruits corepressors and histone deacetylases [6]. Notch upregulation has been observed in T-ALL, breast cancer, colon adenocarcinomas, and ovarian cancer [6,8–10]. While another group has investigated Notch1 expression in ovarian cancer, the focus was on full-length and the extracellular domain in ovarian tumors and cell lines [11]. In contrast, our focus is on the intracellular domain. Previous findings in our lab have found abundant NICD expression in 76% of tested ovarian cancer samples and that suppression of NICD by Notch 1 siRNA resulted in significant growth inhibition [12].

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The majority of antioxidants in our diet are derived from polyphenols [13]. Flavonoids are polyphenols that are found in fruits, vegetables and beverages, and chalcones are flavonoids that have an open C-ring in which the two aromatic rings are joined by a three carbon α, β-unsaturated carbonyl system [13,14]. Xanthohumol is the most abundant prenylated chalcone found in the dried hop cones produced by the female inflorescences of the hop plant (*Humulus lupulus L.*) [14,15]. The beer brewing industry accounts for 98% of the world use of hops and is used as a preservative, to provide bitterness, and to stabilize foam [16,17]. However, the average xanthohumol content found in beer, 0.96 mg/L or 1.95 μM, is not enough to produce a protective effect because xanthohumol is readily isomerized to isoxanthohumol [16,18,19]. In addition to being investigated as a chemotherapeutic agent, xanthohumol is an effective anti-inflammatory and anti-estrogenic agent [20]. Xanthohumol inhibits aromatase activity, which is a key enzyme in the conversion of estrogens from testosterone [21].

In this study, the effects of xanthohumol on ovarian cancer cells were analyzed. Our interest in pursuing the effect of xanthohumol on ovarian cancer stemmed from research of novel therapeutics in other Notch 1 expressing tumors. This led us to a previous publication of xanthohumol use in Kaposi sarcoma tumor cells, which have been found to overexpress Notch1 [22]. In an *in vivo* model, it was shown that xanthohumol treatment reduced Kaposi sarcoma tumor growth [16]. Based on the studies outlined above, we hypothesized that xanthohumol would inhibit ovarian cancer cell growth through down-regulation of the Notch1 pathway.

Materials and methods

Cell culture, xanthohumol treatment and cell proliferation assay

SKOV3and OVCAR3, cells were purchased from ATCC (Manassas, VA) and maintained in the previously published manner [12].Cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) rapid colorimetric assay [23]. Cells were plated at a density of 25,000 cells per well in quadruplicate in 24 well plates and incubated for 24 h. The cells were then treated with xanthohumol at doses ranging from 0 to 30 μmol/L and incubated for 6 days. Every 2 days the medium was removed and the cells were treated with xanthohumol. MTT assays were performed every 2 days by replacing the medium with 250 μl of RPMI-1640 containing MTT (0.5 mg/ml) and incubating at 37 °C for 4 h. After incubation, 750 μl of DMSO was added to each well and thoroughly mixed. The plates were then placed on an orbital shaker for 5 min and then measured at 540 nm using a spectrophotometer (μQuant, BioTek Instruments, Winooski, VT). Control and treated groups were measured in quadruplicate and repeated 3 separate times to ensure consistency. Data was collected using KCJunior software (BioTek).

Transient transfection and luciferase reporter assay

In an effort to clarify whether the Notch pathway was targeted by xanthohumol, we undertook a functional Notch 1 assay. By constructing a *CBF1* reporter assay, luciferase activity can be measured when functional Notch 1 binds to the *CBF1* transgene. A plasmid containing four copies of the *CBF1*-binding elements in pGL2pro (Promega) was obtained from Dr. Diane Hayward (The Johns Hopkins University). The cells were co-transfected using TransIT 2020 (Mirus Bio LLC, Madison, WI) with 2 μg of the *CBF1* plasmid and 0.5 μg of a plasmid containing the β-galactosidase gene driven by the CMV promoter, which as used as an internal control for transfection efficiency. The following day, the cells were treated with xanthohumol, incubated for 2 days, and then lysed with Reporter Lysis Buffer (Promega). Luciferase assays were performed using the Luciferase Assay System (Promega) and a luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI), and β-galactosidase enzyme assays (Promega) were performed on a plate reader (μQuant, BioTek Instruments). Luciferase activity was normalized to β-galactosidase and plotted as relative light units (RLU).

Western blot

Protein was isolated on days 2 and 4 of treatment with lysis buffer and total cellular protein concentration was determined using the 660 nm Protein Assay Reagent (Pierce, Rockford, IL). Cell extracts were loaded onto either 7% NuPAGE® Tris-Acetate gels or 10% NuPAGE® Bis-Tris gels (Invitrogen). Proteins were then transferred onto nitrocellulose membranes (BioRad, Hercules, CA). After transfer, the membranes were blocked in milk and incubated at 4 °C overnight with the following primary antibodies: cleaved Notch1 (Val1744) (Cell Signaling Technology, Danvers, MA), $p21^{WAF1/CIP1}$ (Cell Signaling Technology), phosphorylated cdc2 (Tyr15) (Cell Signaling Technology), cleaved caspase-3 (Cell Signaling Technology), cleaved PARP (Cell Signaling Technology), GAPDH (Trevigen, Gaithersburg, MD) and β-actin (Cell Signaling Technology). The membranes were then washed with wash buffer containing phosphate buffered saline and 0.01% Tween-20 (Biorad) and incubated with the appropriate horseradish peroxidase-conjugated rabbit or mouse secondary antibody. After incubation, the membranes were washed with wash buffer and developed with Immun-Star (Biorad) for β-actin and GAPDH and SuperSignal West Femto chemiluminescent substrate (Pierce) for Notch1, $p21^{WAF1/CIP1}$, cleaved caspase-3, and cleaved PARP and exposed to film. All Western blots were done in triplicate to ensure consistent results.

Quantitative RT-PCR

RNA was isolated on day 2 of treatment using an RNeasy Mini kit (QIAGEN, Valencia, CA) and quantified. cDNA was synthesized from 2 μg of RNA with the iScript™ cDNA Synthesis kit (BioRad) on a thermocycler (MJ Research, Waltham, MA). Quantitative RT-PCR for NICD was performed in a volume of 25 μl containing 400 ng cDNA, 200 nM forward primer, 200 nM reverse primer, and the iQ™ SYBR® Green Supermix (Biorad) in triplicate using an iCyler iQ Real-Time PCR Detection System (Biorad). Primers were obtained from Integrated DNA Technologies (Coralville, IA) and are listed in Table 1. The reactions were performed under the following conditions: 3 min at 95 °C, 35 cycles of 30 s at 95 °C, 25 s at 60 °C, and 30 s at 72 °C followed by 1 min at 95 °C and 1 min at 55 °C. Cycle numbers obtained at the log-linear phase of the reaction were plotted against a standard curve prepared with serially diluted control samples. Expression of NICD was normalized to s27 mRNA and plotted as the mean of triplicate wells. Quantitative RT-PCR reactions for Hes1 and Hes6 were performed in a volume of 25 μl containing 400 ng cDNA, 200 nM forward primer, 200 nM reverse primer, and the SsoFast™ EvaGreen® Supermix (BioRad) using the CFX Real-Time PCR Detection System and analysis software (BioRad). The reactions were performed under the following conditions: 30 s at 95 °C, 40 cycles of 5 s at 95 °C and 5 s at 60 °C. The expression of mRNA was normalized to the housekeeping gene, s27, and determined using the $2^{-\Delta\Delta Ct}$ method.

Table 1
Primers used for quantitative reverse-transcription PCR.

NICD	Forward	5′-GTCAACGCCGTAGATGACCT-3′
	Reverse	5′-TTGTTAGCCCCGTTCTTCAG-3′
Hes1	Forward	5′-TTGGAGGCTTCCAGGTGGTA-3′
	Reverse	5′-GGCCCCGTTGGGAATG-3′
Hes6	Forward	5′-AGCTCCTGAACCATCTGCTC-3′
	Reverse	5′-GACTCAGTTCAGCCTCAGGG-3′
s27	Forward	5′-TCTTTAGCCATGCACAAACG-3′
	Reverse	5′-TTTCAGTGCTGCTTCCTCCT-3′