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Research article

Curcumin-mediated regulation of Notch1/hairy and enhancer of split-1/survivin: molecular targeting in cholangiocarcinoma

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ABSTRACT

Background: Cholangiocarcinoma (CCA) is highly malignant and characterized by poor prognosis with chemotherapeutic resistance. Therefore, continued development of novel, effective approaches are needed. Notch expression is markedly upregulated in CCA, but the utility of Notch1 inhibition is not defined. Based on recent findings, we hypothesized that curcumin, a polyphenolic phytochemical, suppresses CCA growth *in vitro* via inhibition of Notch1 signaling.

Methods: Established CCA cell lines CCLP-1 and SG-231 were treated with varying concentrations of curcumin (0–20 μM). Viability was assessed through 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide and clonogenic assays. Evaluation of apoptosis was determined via Western analysis for apoptotic markers and Caspase-Glo 3/7 assay. Cell lysates were further analyzed via Western blotting for Notch1/HES-1/survivin pathway expression, cell cycle progression, and survival.

Results: Curcumin-treated CCA cells exhibited reduced viability compared with control treatment. Statistically significant reductions in cell viability were observed with curcumin treatment at concentrations of 7.5, 10, and 15 μM by approximately 10%, 48%, and 56% for CCLP-1 and 13%, 25%, and 50% for SG-231, respectively. On Western analysis, concentrations of ≥10 μM showed reductions in Notch1, HES-1, and survivin. Apoptosis was evidenced by an increase in expression of cleaved poly [ADP] ribose polymerase and an increase in caspase activity. Cyclin D1 (cell cycle progression) expression levels were also reduced with treatment.

Conclusions: Curcumin effectively induces CCA (CCLP-1 and SG-231) growth suppression and apoptosis at relatively low treatment concentrations when compared with the previous research. A concomitant reduction of Notch1, HES-1, and survivin expression in CCA cell lines provides novel evidence for a potential antitumorigenic mechanism-of-action. To our knowledge, this is the first report showing reduction in HES-1 expression via protein analysis after treatment with curcumin. Such findings merit further investigation of

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curcumin-mediated inhibition of Notch signaling in CCA either alone or in combination with chemotherapeutic agents.

1. Introduction

Cholangiocarcinoma (CCA) is a highly malignant adenocarcinoma that manifests among cholangiocytes of the intrahepatic, distal extrahepatic, and perihilar hepatobiliary tree epithelium [1,2]. Although rare, it is characterized as the second most common primary liver tumor behind hepatocellular carcinoma and as the most common primary malignancy of the biliary epithelium [1–3]. Potential causes of CCA include numerous viral and inflammatory etiologies. Many cases are associated with risk factors including primary sclerosing cholangitis, *Opisthorchis viverrini* (infection from liver fluke), hepatolithiasis, and biliary malformation (choledochal cysts, Caroli disease, and thorotrast) [1].

Rates of survival can vary depending on the anatomic location of the carcinoma and the extent of metastasis [1,2,4]. CCA generally displays poor prognosis because incipient stages of progression are marked by silent clinical findings [1,2,4–8]. It is documented that up to 50% of patients diagnosed with CCA present with nodal involvement [4]. Surgical intervention is cited as the only curative treatment for CCA but is significantly limited as the disease enters advanced metastasis [1,2,4–7]. Five-year survival rates for patients with a diagnoses of intrahepatic, distal extrahepatic, and hilar CCA receiving surgical intervention are 22%–44%, 27%–37%, and 11%–41%, respectively [9]. On resection, nodal involvement has been documented to display prognostic significance [10,11]. Relapse of cancer after surgical removal is typically local, but distant metastases do occur and are more frequent with perihilar involvement [9].

Broad resistance profiles have complicated the advancement of chemotherapeutic targeting in CCA. There is indication that chemotherapy improves survival rates to 11.7 mo with cisplatin plus gemcitabine combination therapy in patients without resectable tumors [12]. Evidence is currently lacking in support of adjuvant therapy in patients with resectable tumors [4]. Transarterial chemoembolization has been noted to further increase survival in unresectable patients despite increasing rates of toxicity [13,14]. Given dismal rates of survival after diagnosis and surgical intervention, there is an exceptional need for greater advancement in chemotherapeutic design.

Notch1, a transmembrane signaling protein associated with cellular proliferation and survival, is dysregulated in many cancers and is notably upregulated in CCA cell lines [15–20]. On binding its ligand on adjacent cells, the intracellular signaling domain of Notch1 is cleaved by γ-secretase [16]. Proteolytic cleavage releases the intracellular domain for translocation to the nucleus where it acts as a transcription factor. Transcriptional activation by Notch1 increases the expression of protein signaling components including hairy and enhancer of split-1 (HES-1), survivin, and cyclin D1. Given its documented association with cellular proliferation and survival, chemotherapeutic targeting of the Notch1 signaling pathway has garnered significant interest [16,21].

Curcumin, a polyphenolic phytochemical derived from the rhizomatous plant, turmeric (*Curcuma longa*), has been found to attenuate cancer cell growth through regulation of inflammatory and tumorigenic signaling pathways [21–29]. Turmeric has been used for its antibiotic, antiseptic, and anti-inflammatory properties long before its mechanism of action was interpreted [27–30]. Previous research has demonstrated that curcumin (when applied at concentrations up to 50 μM) serves to regulate growth of CCA cell lines *in vitro* via (1) inhibition of I kappa B kinase/Nuclear factor kappa B (NF-κB) phosphorylation, (2) inhibition of the STAT-3 pathway, (3) increased peroxisome proliferator-activated receptor expression, (4) suppression of the Akt activation pathway, and (5) attenuation of survival protein expression (including B-cell lymphoma 2, B-cell lymphoma-extra large, survivin, and others) with increased cell death [25]. Additional findings have shown that curcumin elicits similar inhibition of carcinogenic pathways in golden hamsters affected with CCA [26].

Studies approaching the therapeutic targeting of Notch1 in CCA are currently limited. Inhibition of γ-secretase has shown to reduce tumor growth and metastatic potential, but these findings were not supported by documented reductions in Notch1 signaling [31]. Endocannabinoid-mediated targeting of Notch1 and the structural isoform Notch2 has also resulted in CCA tumor reduction [18]. The mechanism of endocannabinoid targeting of this study was not elucidated, but the authors suggested a potential modification in γ-secretase activity.

Curcumin-mediated regulation of Notch1 signaling has been demonstrated previously in the treatment of pancreatic cancer cells [21], oral carcinoma [32], and esophageal cancer cells [33], but its therapeutic utility as a Notch1 inhibitor in CCA remains undefined. Based on our preliminary findings, we propose that curcumin reduces CCA growth *in vitro* via inhibition of Notch1 signaling.

After treating CCA cell lines with curcumin, we assessed (1) cellular viability, (2) expression levels of Notch1 signaling (including downstream targets HES-1, survivin, and cyclin D1), and (3) changes in apoptotic markers. Thereby, it was our intent to provide additional evidence toward the therapeutic potentials of curcumin in the treatment of CCA.

2. Methods

2.1. Cell culture and treatment

CCA cell lines CCLP-1 and SG-231 were derived from the human intrahepatic biliary epithelium and have kindly been provided by Dr Anthony J. Demetris, University of Pittsburgh (Pittsburgh, PA). CCLP-1 cells were maintained in Dulbecco Modified Eagle Medium (Sigma–Aldrich, St. Louis, MO) and supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA), 1% penicillin/streptomycin (P/S; Life Technologies), 1% 4-(2-

hydroxyethyl)-1-piperazineethanesulfonic acid (Life Technologies), and 1% nonessential amino acids (Life Technologies). SG-231 cells were maintained in minimum essential medium alpha ×1 (Life Technologies) and supplemented with 10% fetal bovine serum, 1% P/S, and 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic. Cells were stored in a 37°C humidified incubator containing a 5% CO_2 environment. Treatment solutions were established by dissolving curcumin (Sigma–Aldrich) into dimethyl sulfoxide (DMSO; Sigma–Aldrich).

2.2. Cellular viability assay

Viability of CCLP-1 and SG-231 cells lines was assessed through 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide colorimetric assay. Cells were plated into 48-well plates and were allowed to adhere overnight. Curcumin was added at various (0–30 μM) concentrations in quadruplicate. Control groups were established using DMSO. After incubating in treatment for 48 h, media were replaced by 125-μL Roswell Park Memorial Institute media (Life Technologies) containing 0.5 mg/mL 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide. After 3 h of incubation, 375 μL of DMSO was added to each well. Absorbance was then measured in each well at 540 nm (Infinite M200 PRO; Tecan, San Jose, CA). From the absorbance data, percent of cell viability was calculated. This value was obtained by dividing the absorbance of treatment group by their respective control.

2.3. Colony formation assay

To confirm growth inhibition by administration of curcumin, colony formation assay was performed on CCA cell lines. Cells were plated onto a 6-well plate and allowed to adhere overnight. Cells were then treated with varying concentrations of curcumin (0–20 μM) for 4 d. Media were replaced every 4 d for 2 wk. Then, the cells were fixed with crystal violet staining, and images were taken using the Molecular Imager ChemiDocXRS$^+$ imager with image lab software (Bio-Rad, Hercules, CA), as described [34].

2.4. Western analysis

Cell lysates were prepared using radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Waltham, MA), as described [34]. Protein content in cell lysates was quantified using bicinchoninic acid assay (Pierce, Rockford, IL). Thirty micrograms of quantified protein was loaded into sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (Bio-Rad Laboratories). After separation, protein was transferred to nitrocellulose membrane using a Trans-Blot Turbo (Bio-Rad). After transfer, membrane was blocked in milk solution (1 × phosphate-buffered saline, 5% dry milk, 0.05% Tween20) for 30–60 min. Nitrocellulose membranes containing protein were incubated in primary antibodies overnight. Primary antibodies used in this study included Notch1, cyclin D1, and survivin (1:500); livin and HES-1 (1:200); glyceraldehyde 3-phosphate dehydrogenase (1:4000; Santa Cruz Biotechnologies, Santa Cruz, CA); poly [ADP] ribose polymerase (PARP; 1:1000); and cleaved PARP (1:2000; Cell Signaling Technology, Beverly, MA). After primary antibody incubation, membranes were washed with 1 × phosphate-buffered saline, 0.05% Tween20 buffer and placed in milk solution containing anti-rabbit or anti-mouse antibody (Santa Cruz Biotechnology) at 1:10,000 dilution. After incubating for 1.5 h, membranes were washed again in 1 × phosphate-buffered saline, 0.05% Tween20 buffer and developed using SuperSignal West Dura, West Femto (Thermo Fisher Scientific) and Clarity (Bio-Rad) developing kits. Developed membranes were then analyzed and exposed under the Molecular Images ChemiDocXRS$^+$ imager to view bands representing the protein of interest. Further evaluation and quantitation of the protein bands were analyzed via ImageJ software (NIH, Bethesda, MD).

2.5. Functional apoptotic analysis

In addition to Western blot evaluation of apoptotic markers, apoptosis was further delineated through use of a functional assay. The activation of Caspase 3/7 was studied in both SG-231 and CCLP-1 cell lines after administration of curcumin. CCA cell lines were treated with varying concentrations of curcumin (0–20 μM) for 2 d or 4 d. Cell lysates were analyzed for Caspase 3/7 activity via the Caspase-Glo 3/7 assay kit (Promega, Madison, WI). Lysates were added into 96-well plates in which 25 μL of Caspase-Glo 3/7 reagent was added in quadruplicate. Luminescence was measured with Infinite M200 Pro (Tecan), and the resultant data were calculated as fold change compared with control.

2.6. Statistical analysis

Analysis of variance with Bonferroni *post hoc* testing was performed using a statistical analysis software package (IBM SPSS Statistics version 22, New York, NY). A P value of <0.05 was considered significant. Data were represented as ± standard error, and all experiments were performed in triplicate.

3. Results

3.1. Curcumin-treated CCA cells exhibited reduced viability and colony-forming ability

There was progressive reduction in cellular viability of SG-231 and CCLP-1 cell lines with increasing concentrations of curcumin after 48 h of treatment (Fig. 1A). Cellular viability was represented as a percent of the cells exposed to DMSO control treatment. Statistically significant reductions in growth were observed at concentrations of 7.5, 10, and 15 μM by approximately 13%, 25%, and 50% for SG-231 and 10%, 48%, and 56% for CCLP-1, respectively, after 48 h of treatment (P value <0.0001; Fig. 1A). Cellular viability was reduced at concentrations as little as 5 μM of treatment in each cell line (P value <0.05). To further confirm the reduction in cellular viability, colony-forming ability of the CCA cell lines was assessed. As shown in Figure 1B, the clonogenic ability of CCA cell lines was reduced after administration of curcumin treatment.

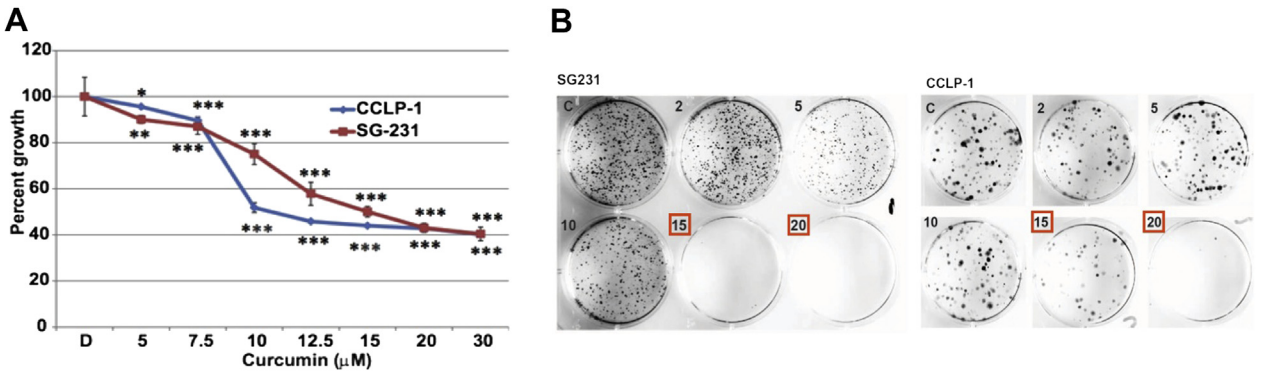

Fig. 1 – Curcumin treatment inhibited (A) cellular proliferation and (B) colony-forming ability in CCA cell lines. Statistically significant reductions in percent growth were observed at ≥5 μM of curcumin in both CCLP-1 and SG-231 cell lines at 48 h (*P* value < 0.05). Treatment doses causing greater than 50% growth reduction occurred at concentrations of curcumin over 10 and 15 μM for CCLP-1 and SG-231, respectively. *P value <0.05, **P value <0.001, ***P value <0.0001. (B) Significant reductions in colony-forming ability of SG-231 and CCLP-1 cell lines occurred at 15 and 20 μM of curcumin treatment. (Color version of figure is available online.)

3.2. *Increasing concentrations of curcumin treatment in SG-231 and CCLP-1 cell lines induced apoptosis and attenuated cell-cycle progression*

To further delineate the mechanism by which curcumin mitigates cellular proliferation, Western analysis was used. On Western analysis, both SG-231 (Fig. 2A) and CCLP-1 (Fig. 2B) cell lines showed evidence of apoptotic induction via reduction in the antiapoptotic protein livin. Additionally, proapoptotic markers were evident as the presence of cleaved PARP was exhibited at concentrations of ≥10 μM for SG-231 and ≥15 μM for CCLP-1. To confirm curcumin induces apoptosis, caspase 3/7 activity was evaluated (Fig. 2C). Similar to protein analysis, curcumin stimulated a marked increase in caspase activity starting at concentrations of 10 and 15 μM for SG-231 and CCLP-1, respectively. In addition to inducing apoptosis, administration of curcumin also had an effect on cell-cycle progression. Dose-dependent reductions of cyclin D1 in SG-231 and CCLP-1 cell lines with treatment were observed and indicate the presence of treatment-induced modulation of G1 to S phase cell-cycle progression.

3.3. *Curcumin suppressed Notch1/HES1/survivin pathway signaling in SG-231 and CCLP-1 cell lines*

To understand the mediation of cell viability inhibition and apoptotic induction, we investigated the role of curcumin in the Notch signaling pathway via Western analysis. At concentrations of ≥10 μM, curcumin effectively reduced expression of Notch1 in both SG-231 (Fig. 3A) and CCLP-1 (Fig. 3B) cell lines. Curcumin's effect on Notch signaling was further exemplified by concomitant reductions in downstream targets HES-1 and survivin.

4. Discussion

The high degree of severity in which CCA presents has resulted in significant challenges for therapeutic design. Currently, surgical intervention is the only curative option for affected patients. Such treatment; however, is limited to certain populations. Patient survival depends significantly on tumor stage, multiplicity of lesions, differentiation, and lymph node involvement [4,35–38]. A lack of currently effective adjuvant therapy and generalized chemotherapeutic resistance in unresectable patients warrants the continued development of novel molecular targeting.

The Notch1 pathway has been associated with tumorigenesis in many cancers given its role in cellular proliferation, survival, and metastasis [16]. The documented upregulation of Notch1 in CCA has stimulated interest for targeting Notch1 signaling components as a therapeutic strategy [16,18–20,31]. Therefore, we investigated the role of Notch1 modulation in two CCA cell lines, SG-231 and CCLP-1, and delineated its effect on cellular proliferation and growth.

Curcumin is a natural compound derived from *C longa* that has shown the ability to effectively modulate inflammatory and tumorigenic signaling associated with CCA carcinogenesis through *in vivo* and *in vitro* studies [25,26]. Furthermore, curcumin has demonstrated effectiveness toward Notch1 signaling pathway modulation in pancreatic cancer cells [21], oral carcinoma [32], and esophageal cancer cells [33]. Currently, there are limited data involving curcumin-mediated targeting of the Notch1 pathway and cross talk between pathways in CCA. Wang *et al.* [21] demonstrated that curcumin inhibits the NF-κB pathway and that this mitigation is partly through Notch1. Interestingly, further advancement has suggested that Notch1 is directly upstream of NF-κB, and the resultant inhibition of Notch1 directly correlates with NF-κB modulation and more importantly downstream target inhibition of key apoptotic and survival proteins such as survivin, cyclin D1, and cleaved PARP. We believe that the effect of curcumin, similar to that of Wang *et al.*, directly targets the Notch pathway and potentially other parallel pathways. For instance, there have been studies involving knockout of Notch1 in the presence of curcumin leading to a synergistic cellular growth inhibition [21]. Therefore, additional pathways maybe involved during the administration of curcumin and

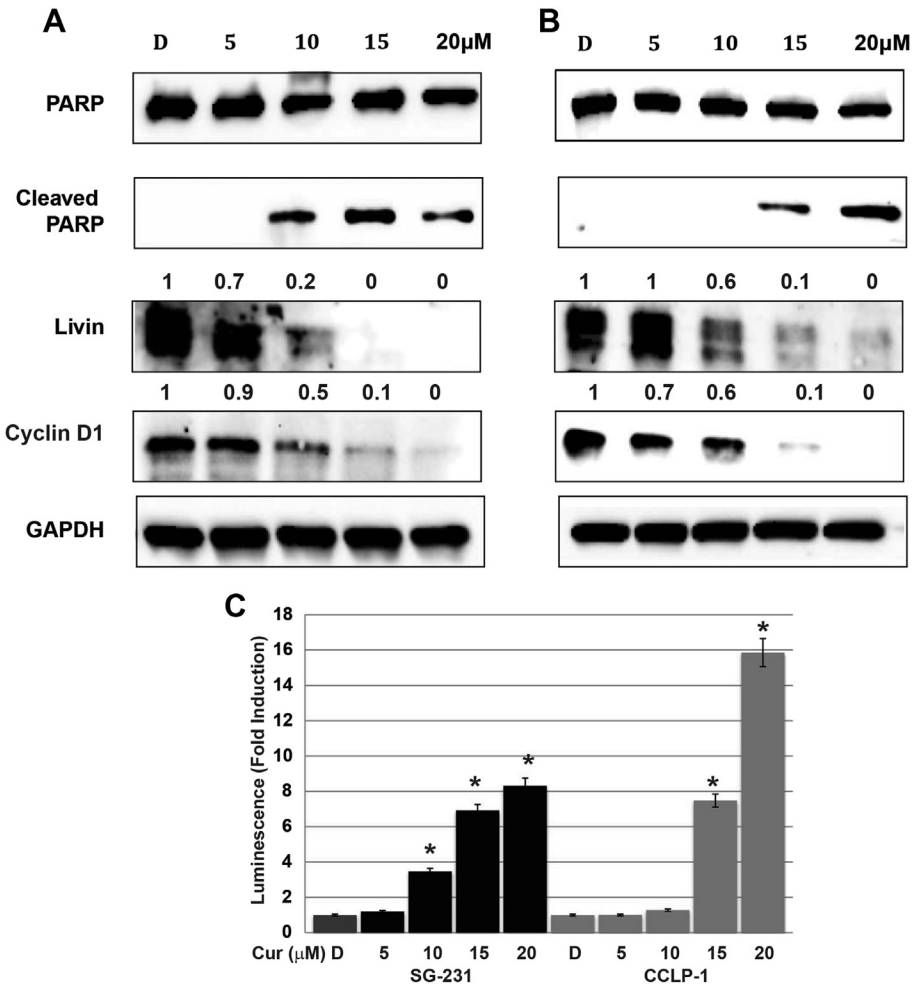

Fig. 2 – Treatment with curcumin induced apoptosis in SG-231 (A) and CCLP-1 (B) cell lines. Apoptosis was evidenced by reduced expression of livin with congruent elevations in cleaved PARP. Reduced advancement of the cell cycle was supported by progressive reductions in cyclin D1 expression with treatment. Glyceraldehyde 3-phosphate dehydrogenase was used as a loading control. Relative protein expression compared with control is listed above each sample. (C) Apoptotic induction was further evaluated through a caspase 3/7 assay. Increasing concentrations of curcumin (0–20 μM) stimulated caspase activity evidenced at 10 μM for SG-231 and 15 μM for CCLP-1, respectively. Caspase activity described as fold increase compared with control. *P value <0.05 when compared with SG-231 control. *P value <0.05 when compared with CCLP-1 control.

further strengthens its role as a potential therapy. Therefore, given this background, we investigated the effectiveness of curcumin on CCA growth and/or viability and characterized its ability to attenuate Notch1 signaling in CCA cell lines.

Cellular viability and colony-forming unit ability of SG-231 and CCLP-1 cell lines were significantly reduced after treatment with curcumin. This correlates with previous findings of curcumin on CCA growth potential [25,26]. Fifty percent reduction in SG-231 and CCLP-1 cellular viability occurred at 10 and 15 μM of treatment, respectively. Furthermore, colony-forming unit ability was reduced at 15 and 20 μM of treatment in each cell line. Impressively, the concentrations used in this study fall well below what are achievable within the human plasma limits, indicating a relatively low toxicity profile at the concentrations that are actively reducing proliferation [23].

It is worth noting that during preliminary examination of CCA cell lines, we found two interesting points not shown in the figures. First, screening of Notch isoforms in CCA revealed that Notch1 was highly expressed compared to the other three isoforms. Second, endogenous Notch1 expression is greater in SG-231 compared with that of the CCLP-1 cell line. Consequently, this will affect downstream targets so as Hes-1, and so forth; however, the effect is minimal and the proof of concept is maintained. Western blotting studies showed livin was reduced with increasing treatment concentration. Cleavage components of PARP that often present with apoptosis were observed at $\geq$10 μM of curcumin for SG-231 cell lines and at $\geq$15 μM for CCLP-1 cell lines. The induction of apoptosis was further confirmed through enhancement of caspase activity. Additionally, cyclin D1 was reduced in both cell lines with treatment indicating decreased G_1/S cell cycle interface progression. Furthermore, cyclin D1 and survivin are known targets of Notch1 and have been suggested that they may play a role in the development of resistance to

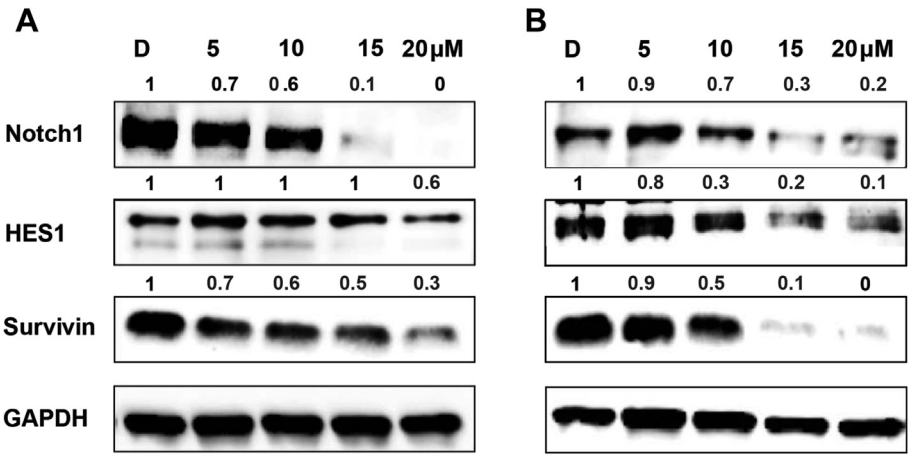

Fig. 3 – Expression levels of the Notch1 signaling pathway were reduced with increasing concentrations of curcumin treatment in both SG-231 (A) and CCLP-1 (B) cell lines. Curcumin treatment decreased Notch1, HES-1, and survivin expression. Glyceraldehyde 3-phosphate dehydrogenase was used as a loading control. Relative protein expression compared with control is listed above each sample.

chemotherapeutic agents [39–42]. Therefore, we believe that inhibition of Notch signaling pathway members may further sensitize CCA cell lines to chemotherapy.

The present findings of our study suggest a potential antitumorigenic mechanism-of-action for curcumin in the targeting of Notch1 signaling in CCA. To our knowledge, this is the first report showing reduction in Notch1/HES-1 expression in CCA via protein analysis after treatment with curcumin. The effectiveness of curcumin in suppressing CCA tumor viability and growth *in vitro* is supported in this study through various mechanisms and has been supported by previous research [25,26]. Furthermore, phase I and II clinical trials have shown the effectiveness of curcumin with use in colorectal [43] and pancreatic [44] cancers. Curcumin supplementation has also seen few negative side effects in patients receiving high doses of treatment in combination with other chemotherapy including gemcitabine [44–46].

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Disclosure

The authors reported no proprietary or commercial interest in any product mentioned or concept discussed in the article.

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