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Original research article

The effect of *Atractylodes lancea* (Thunb.) DC. on the Notch signaling pathway

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ABSTRACT

Cholangiocarcinoma (CCA) is a cancer of the bile ducts and liver with a low survival rate. Development and progression of the disease involve many changes to gene expression including to genes involved in Notch signaling. The Notch signaling pathway is a highly conserved pathway that regulates differentiation, proliferation, and apoptosis among other things, mediated through direct cell-to-cell contact, and is implicated in many kinds of cancer including CCA. *Atractylodes lancea* (Thunb.) DC. (AL) is a plant used in traditional Thai medicine that has been shown to have anti-CCA activity. In this study, Notch pathway gene expression was analyzed from venous blood samples taken from Thai patients with advanced-stage CCA participating in a phase II clinical trial. Patients were given either standard palliative treatment (control) or oral capsule pharmaceutical formulation of standardized crude ethanolic AL extract with a daily dose of 1,000-1,500 mg. In total, 15 patients were included in the study. Twenty-four genes were analyzed for differential gene expression using nCounter® Analysis System, NanoString Technology and data was analyzed by performing fold change analysis. Patients' gene expression on day 28 of treatment was compared against day 1 of treatment (baseline) for each treatment group. Data analysis revealed 20 genes had a significant fold change in expression from day 1 to day 28. Of these 20 genes, 7 genes had a significant fold change in expression in two treatment groups (DLL4, DTX4, HES1, HES5, HDAC1, APH1B, and LFNG), and 2 genes had a significant fold change in all three treatment groups (JAG1 and CREBBP). In conclusion, oral administration of standardized crude ethanolic AL extract affects expression of Notch signaling pathway-related genes in CCA patients; most notably, JAG1 expression was down-regulated in response to AL treatment, in a dose-dependent manner.

Keywords: cholangiocarcinoma, *Atractylodes lancea*, Notch signaling, differential gene expression

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1. Introduction

Cholangiocarcinoma (CCA) is a cancer of the epithelial cells of bile ducts and hepatic parenchyma.[1,2] Metastasis and recurrence remain major issues for CCA, diagnosis often comes in late stages, and the overall survival is less than 10% .[2,3] Concerningly, both the incidence and mortality rate of CCA have been on the rise for the past few decades.[4-12] Existing treatments are suboptimal and the need for novel therapies is urgent, especially ones that are affective against late stage CCA and can prevent or reduce metastasis and recurrence.

Several signaling pathways are involved with CCA development and might be the target for anti-CCA development. The Notch signaling pathway is an important pathway involved in differentiation, proliferation, and apoptosis. When a Notch ligand from one cell binds to a Notch receptor from another cell, the Notch receptor changes conformation and goes through a series of cleavages. This results in the Notch intracellular domain (NICD) of the Notch receptor being cut loose. The NICD is transported to the nucleus where it interacts with transcription factors, ultimately leading to transcription of Notch-target genes.[13] There are many other proteins involved in this signaling cascade that affect various points in the signaling pathway.

Notch signaling has been implicated in various cancers including CCA.[13,14] Increased levels of Notch1 and its associated NICD are pro-tumorigenic in CCA.[15] Several Notch genes (Notch1, Notch4, JAG1, *etc.*) have been reported to be up-regulated in intrahepatic cholangiocarcinoma (iCCA), with correlating to tumor size and survival rates.[16,17] Increased expression of signaling molecules upstream of Notch such as PI3K and AKT; also induces CCA progression.[15] Gamma secretase inhibitors have also shown anti-CCA activity *in vitro*, further evidence of the pro-CCA role that Notch signaling plays.[14]

Atractylodes lancea (Thunb.) DC. (AL) extract was found to be the most potent and most selective against CCA cells *in vitro* (CL-6 cell line).[18] The dried rhizomes of this plant contain many compounds including sesquiterpenoids, monoterpenes, polyacetylenes, phenolic acids, and steroids.[1] The two most abundant compounds within are atractylodin and β-eudesmol,[1] these two compounds are both bioactive, have demonstrated anti-CCA activity, and can be used as marker compounds for standardization and quality control of the AL extract.[1,19] AL has been shown to have anticancer activity, and particularly anti-CCA activity, in both *in vitro* and *in vivo* studies. In fact, AL has been found to possess a number of biological activities against CCA cells. A series of *in vitro* and *in vivo* studies showed promising activity of *A. lancea* for the treatment and control of CCA, and its potential for use in humans.[18,20-23] In the first ever clinical phase I study of AL, the pharmacokinetics and safety of capsule formulation (CMC) of the standardized AL extract were evaluated in healthy Thai participants. The report showed the safety and tolerability of the AL formulation in Thai participants.[22] Clinical efficacy and safety of the CMC capsule formulation of AL formulation in advanced-stage CCA patients was then subsequently studied (Na-Bangchang, unpublished data). The present study aimed to investigate the effect of AL formation on the Notch signaling pathway in CCA patients.

2. Materials and Methods

2.1 Development of pharmaceutical AL formulation

The extraction process for the standardized crude ethanolic AL extract used in this study was developed in a previous study.[19] AL rhizomes were washed, cut, oven dried at 50° C, then ground into a fine powder. This powder was double macerated with 95% ethanol at room temperature with agitation for 24 h. After, the solvent was filtered then put

in a rotary evaporator under reduced pressure to concentrate the solution. Finally, the concentrated solution was dried using a freeze dryer. The resulting extract was stored at 4°C until further use.

Quality control for the AL extract was done using one of the main bioactive components, atractylodin, as a marker compound for HPLC testing.[19]

The safety and toxicity of AL have been tested in both clinical and non-clinical trials.[20-22] No mortality, serious toxicity or histopathological abnormalities in any organ have been observed. In animal models, the only symptoms observed were stomach irritation and general central nervous system (CNS) depression with all symptoms clearing within 2 hours. In a phase I clinical trial of AL, no adverse events were reported nor were any heart arrythmias or other serious clinical symptoms reported.

2.2 Study design

This study was part of the study: "Phase IIa Clinical Trial for Dose-Finding", which was conducted at Sakol Nakorn Hospital, Sakol Nakorn Provice, Thailand (Na-Bangchang, unpublished data). The approval of the study protocol was obtained from The Human Research Ethics Committee of Thammasat University (Medicine), Thammasat University (MTU-EC-OO-4-203/63). Written informed consent was obtained from all research participants. Patients were diagnosed with CCA by CT scan, clinical examination, and/or tissue biopsy. The patients included in this study were those that remained in the clinical trial until at least day 28. Those that withdrew or passed away were not included in this study.

The clinical trial consisted of three groups: two experimental and one control:

1. AL 1,000 mg of per day, in conjunction with standard supportive therapy for the entire 90-day trial.

2. AL 1,500 mg: the dose ramp-up period for this group was conducted as follows:

Day 1-14: 1,000 mg of AL per day

Day 15-28: 1,500 mg of AL per day

3. Control group with standard palliative treatment.

2.3 Blood sample collection

Blood samples (3 mL each) from days 1 and 28 of the aforementioned study were used for gene expression analysis. Blood samples were collected from venous blood in EDTA tubes then centrifuged at 1,500xg for 15 min. The whole blood was kept at -80°C until further analysis. All samples were transported to Thammasat University under temperature control to prevent RNA degradation.

2.4 RNA extraction

The RNA extraction was performed using TRIzol (Invitrogen™, Waltham, USA). Two hundred µl of whole blood samples taken from study participants on days 1 and 28 were mixed with TRIzol (2.5 ml) and then chloroform (0.5 ml). The mixture was then centrifuged for 30 min at 12,000xg, 4°C. The supernatant was then collected and incubated with isopropanol overnight. After incubation, the tubes were centrifuged again for 30 min at 12,000xg, 4°C. The pellet was then washed with 75% ethanol, then centrifuged for 5 min at 12,000xg, 4°C. The supernatant was then discarded and the tubes were allowed to air dry, leaving only the RNA pellet. The pellet was then resuspended in DEPC-treated water and stored at -80°C until further use.

2.5 Gene expression analysis

The RNA samples were analyzed using nCounter PanCancer Pathways Panel by NanoString (NanoString Technologies, Seattle, WA, USA). The genes involved in the Notch signaling pathway were set according to information provided by NanoString Technologies. Differential gene expression was analyzed via fold change analysis. The threshold of significance for differential gene expression was set at 2-fold up-/down-regulation.

3. Results

3.1 Study population

Treatment group 1 consisted of 6 patients, treatment group 2 consisted of 5 patients, and treatment group 3 (control) consisted of 4 patients. All patients gave blood samples on days 1 and 28. The demographic data was shown in Table 1. All patients had stage 4 intrahepatic CCA (iCCA).

3.2 Comparison of Notch pathway gene expression to baseline

The fold change in expression level from day 1 to day 28 was compared for each treatment group. In total, there were 20 genes that had significant differential gene expression in at least one treatment group (Table 2).

In the control group receiving 0 mg AL per day, the expression of 10 genes was significantly up-regulated (HDAC1, APH1B, JAG1, LFNG, HDAC2, DTX4, MFNG, NOTCH2, HES1, and DLL3) while the expression of 3 genes was significantly down-regulated (DTX3, CREBBP, and NOTCH3).

In the group receiving 1000 mg AL per day, the expression of 5 genes was significantly up-regulated (APH1B, CREBBP, JAG2, JAG1, and HES5) while the expression of 4 genes was significantly down-regulated (PTCRA, HES1, LFNG, and DLL4).

In the high-dose group receiving 1500 mg AL per day, the expression of 7 genes was significantly up-regulated (CREBBP, DLL4, DTX4, HES5, HDAC1, NOTCH1, and KAT2B) while the expression of 2 genes was significantly down-regulated (DTX1 and JAG1).

3.3 Comparison of Notch pathway gene expression among study groups

Comparing the fold change values among the three groups, there were 2 genes for which the fold change in expression was significant for all three groups (Table 2) . First, JAG1 expression decreased in a dose-dependent manner (Figure 1A) ; second, CREBBP expression increased in a dose-dependent manner (Figure 1B).

Relative to the control group, the treatment group receiving 1,000 mg AL per day showed decreased expression of APH1B, LFNG, and HES1 (Figure 2A-C) in addition to the previously mentioned changes seen for JAG1 and CREBBP. The gene expression pattern of patients who received 1,500 mg AL per day relative to control was slightly different from the differences in the 1,000 mg AL treatment group (Figure 3).

The differential gene expressions between the two dosages of AL are shown in Figure 4. DLL4 and HES5 were upregulated when AL dosage was increased in addition to the previously mentioned changes seen for JAG1 and CREBBP.

Table 1. The demographic data.

	Control	Group I	Group II
N	4	6	5
Age (years)	68.5 (62-75)	65 (59-83)	76 (57-84)
Sex	Male: 1 (25%) Female: 3 (75%)	Male: 3 (50%) Female: 3 (50%)	Male: 4 (80%) Female: 1 (20%)
CCA stage	IV: 4 (100%)	IV: 6 (100%)	IV: 5 (100%)

Table 2. The genes with significant fold change in expression (>2 or <0.5) in at least one group. Fold change calculated as Day 28/Day 1.

Gene name	Control	AL 1000 mg	AL 1500 mg
JAG1	7.64	2.34	0.26
CREBBP	0.47	2.39	5.20
HDAC1	11.18	NS	2.45
APH1B	10.53	2.45	NS
LFNG	7.63	0.33	NS
HDAC2	5.99	NS	NS
DTX4	4.97	NS	2.65
MFNG	4.65	NS	NS
NOTCH2	3.78	NS	NS
HES1	3.55	0.41	NS
DLL3	2.11	NS	NS
DTX3	0.48	NS	NS
NOTCH3	0.37	NS	NS
DLL4	NS	0.33	3.01
HES5	NS	2.15	2.56
NOTCH1	NS	NS	2.35
KAT2B	NS	NS	2.12
DTX1	NS	NS	0.45
JAG2	NS	2.36	NS
PTCRA	NS	0.44	NS

NS: not significant

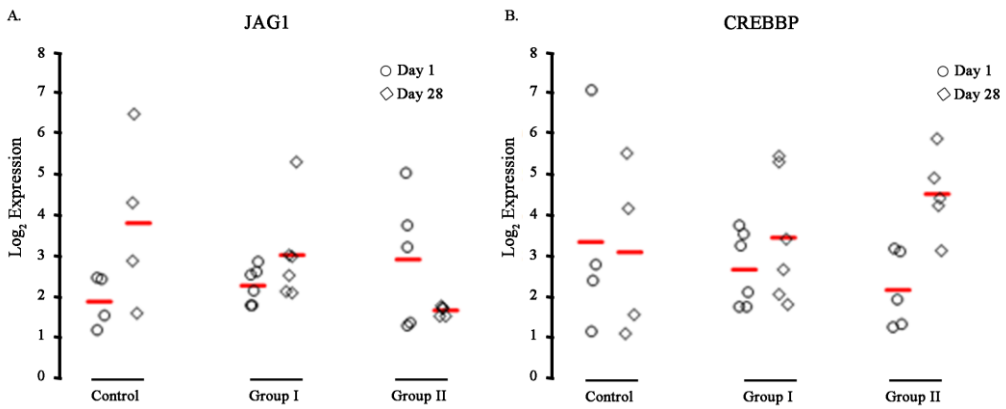

Fig. 1. The gene expression among three groups after 28 days of treatment for JAG1 (A.) and CREBBP (B.). Data is presented as Log_2 expression level in individual patients.

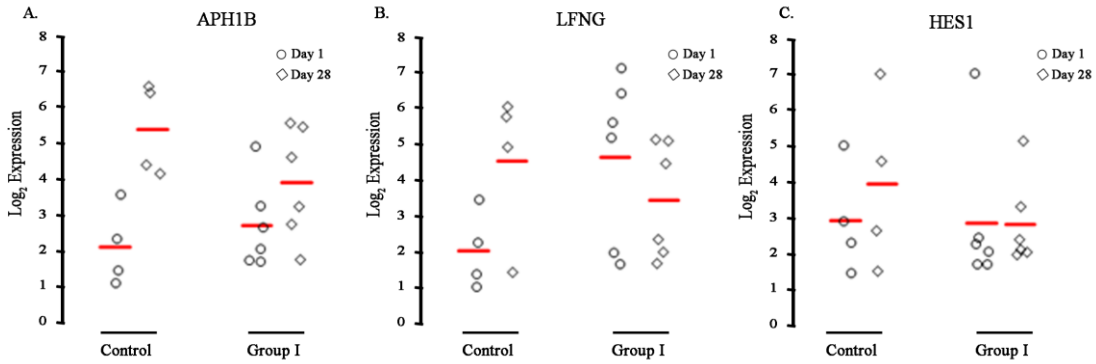

Fig. 2. The pattern of gene expression after 28 days in patients receiving 1,000 mg AL per day (group 1) compared to control (no AL treatment) for APH1B (A.), LFNG (B.), and HES1 (C.). Data is presented as Log_2 expression level in individual patients.

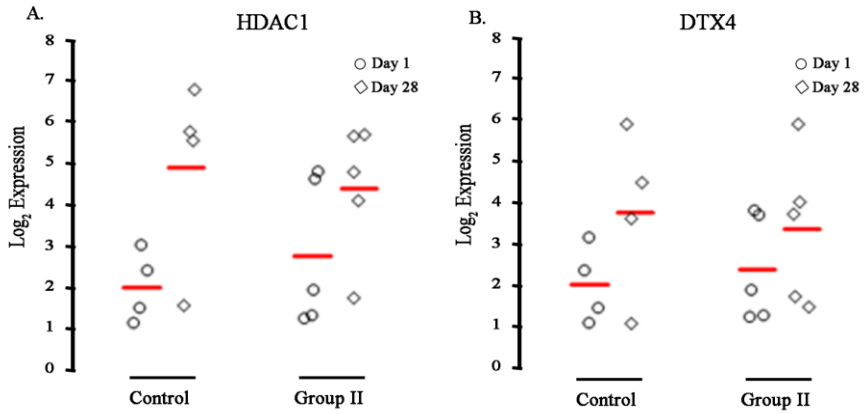

Fig. 3. The pattern of gene expression after 28 days in patients receiving 1,500 mg AL per day (group 2) compared to control (no AL treatment) for HDAC1 (A.) and DTX4 (B.). Data is presented as Log_2 expression level in individual patients.

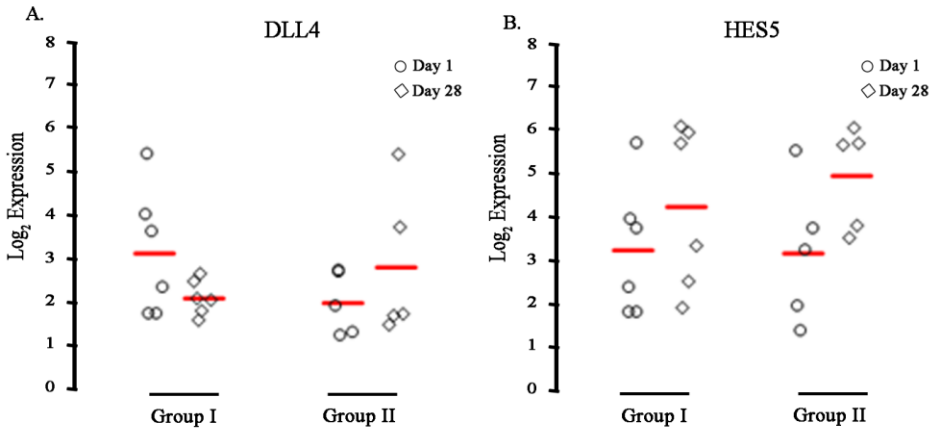

Fig. 4. The pattern of gene expression after 28 days in patients receiving 1,000 mg AL per day (group 1) compared to 1,500 mg AL per day (group 2) for DLL4 (A.) and HES5 (B.). Data is presented as Log_2 expression level in individual patients.

4. Discussion

In this study, blood samples were used to monitor patient response to AL treatment. Blood sample is a useful tool for cancer treatment which can be used for monitoring reaction to therapy, it is minimally invasive, poses less risk/harm to patients, is cost-efficient, and can be performed repeatedly which enables monitoring of a patient as they react to therapy in real-time and over the course of treatment.[24] Quite interestingly, it was shown that mRNA expression profiles from blood serum in CCA patients matched that of CCA tumor tissue.[25] Whether this translates to whole blood is unknown but this represents a compelling area for future research.

The recent results confirm the effect of the AL formulation on Notch signaling, as reported in previous studies.[26] The Notch signaling pathway is an important pathway that has been linked with several types of cancer, including CCA.[13,14] CCA development has been observed in instances of over-activation of the Notch signaling pathway.[15] There have been a number of studies done on Notch signaling genes and how they relate to CCA development and progression. Notch1, NICD1, Notch2, Notch3 atypical receptor, JAG1, Notch4, AKT, mTOR, and Hes-1 have all been found to be upregulated in CCA, either *in vitro* or *in vivo*.[14,15] The aberrant expression of these genes has been associated with cell transformation, cell proliferation, cell survival, DNA damage, metastasis, and ultimately larger tumor sizes and lower survival rates.[14,15]

In the control group with no AL treatment, 3 Notch signaling pathway-related genes were significantly differentially expressed. Interestingly, Notch1 was not significantly changed but Notch2 expression was highly up-regulated while Notch3 expression was down-regulated. In iCCA, Notch1 and Notch4 were shown to be up-regulated while Notch2 and Notch3 were down-regulated in patient tissue samples; additionally, Notch1 was upregulated in patients with tumor sizes larger than 5 cm.[16] However, in another study also using resected tissue, it was found that while Notch1 and NICD1 expression was consistently up-regulated in iCCA, Notch2, Notch3, and Notch4 were not differentially expressed, underlining the heterogeneity of CCA.[27] This same study concluded that Notch1 overexpression induces cells to undergo the epithelial-to-mesenchymal transition (EMT) and encourages metastasis. In mice, knocking out Notch2 was shown to delay tumor development and all tumors that did develop were hepatocellular carcinoma-like lesions and hepatocellular adenomas, none were iCCA, demonstrating that Notch2 is required for iCCA development.[28] This could relate to the findings of this study which found that Notch2 mRNA was significantly up-regulated in whole blood of the control group. The role Notch3 plays in CCA is still not completely understood and further research will need to be done in order to draw conclusions on its potential as a target for therapy.[15]

JAG1 is a ligand for Notch receptors; JAG1 has been found to be pro-tumorigenic in a long list of cancers including CCA and hepatocellular carcinoma.[16,26,29] Furthermore, JAG1 expression has been associated with tumor angiogenesis, neoplastic cell growth, cancer stem cell maintenance, and metastasis in cancers.[26] Without any treatment, increased JAG1 expression led to increased CCA progression. Levels of both JAG1 protein and mRNA have been found to be elevated in iCCA patient tissue samples[17] Suppressed JAG1 expression has reduced proliferation and increased apoptosis of CCA *in vitro*. The resulting down-regulation of whole-blood JAG1 expression in CCA patients receiving AL treatment is in agreement with report from Vanaroj et al.,[26] both key bioactive components of AL, atractylodin and β-eudesmol down-regulated JAG1 mRNA and protein levels. These results are encouraging as there is much evidence suggesting that

reduced JAG1 signaling will improve outcomes for CCA patients. However this translates to clinical outcomes is a target of research.

Two isoforms of histone deacetylases (HDAC1 and 2) were highly expressed in control group. HDACs can promote carcinogenesis by repressing tumor-suppressor genes, and they are overexpressed in numerous types of tumors including CCA. [30-32] HDACs are indirectly involved in Notch signaling via this transcription regulation.[33,34] Two Deltex isoforms- 3 and 4 (DTX3/4) were found up- and down- regulated, respectively.

Interestingly, CREBBP expression was up-regulated in response to AL treatment; this is an important gene that provides instructions for making CREB binding protein, which affects the activity of a wide range of genes by regulating transcription via histone acetylation.[35] In this way, CREBBP is indirectly involved in various signaling pathways including Notch. CREBBP protein plays an essential role in controlling cell growth and division and prompting cells to mature and differentiate.[36] CREBBP mutations have been reported in various cancers including B cell lymphomas, follicular lymphoma, skin squamous cell carcinoma, and salivary gland carcinoma among others though its role in CCA is not yet well studied.[37] Mutations in CREBBP have been found in female CCA patients from China.[38] The current study is to our knowledge the first to report the down-regulation of CREBBP in CCA patients. Regarding the role of CREBBP specifically in Notch signaling, CREBBP activity has been linked to the expression of FBXW7, a key Notch signaling inhibitor. [35] How exactly the up-regulation of CREBBP translates to clinical outcomes in CCA will need further research.

All these genes together were showed the over-activation of Notch signaling pathway with might be induced CCA progression.

The results from AL treatment were different among the two dosages. Only two genes showed similar results; the fold change of JAG1 gene expression was reduced from 7.64 to 2.33 and 0.26 with control, AL1000 mg, and AL 1500 mg, respectively. While CREBBP gene was increased from 0.47 to 2.39 and 5.20, respectively. The changes of these two genes were dose dependent. The reduction of JAG1 was also observed in *in vitro* studies from Vanaroj *et al*.[26] which showed that atractylodin and β-eudesmol, which are major bioactive compounds in AL, suppressed JAG1 gene and protein expression.

CREBBP expression was decreased in the control group and increased in both AL treatment groups in a dose-dependent manner. However, there is little information available on CREBBP in the context of CCA so the impact it has is still inconclusive. CREBBP is inherently a more difficult gene to comprehensively understand as it is so wide reaching. It is able to act as a cofactor for many different transcription factors, regulating thousands of genes, in areas such as proliferation, cell cycle, differentiation, and DNA damage repair, in almost all cell types, sometimes even acting in opposing pathways simultaneously.[37]

The fold change in expression of HDAC1 was markedly reduced by the 1500 mg AL treatment as compared to the control group (2.45 vs. 11.18, respectively). HDAC1 represents a promising target for CCA therapy, so the affect AL had on its expression is notable. HDAC1 regulates the expression of other genes through chromatin remodeling via histone deacetylation. In a recent study, HDAC1 was shown to be upregulated in both clinical tissue samples as well as multiple CCA cell lines. Furthermore, the authors showed, both *in vitro* and *in vivo*, that inhibiting HDAC1 expression reduced CCA cell proliferation, tumorigenicity, migration, invasion, and metastasis.[30]

5. Conclusion

In conclusion, this study demonstrated that AL treatment affects the expression of Notch signaling pathway-related genes in CCA patients based on whole-blood mRNA profiling. Most notably, AL down-regulated JAG1 expression and up-regulated CREBBP expression in a dose-dependent manner. It is possible these changes may also be reflected in CCA tumor tissue, though that is yet to be determined. Future research should investigate if the observed effect of AL extends to the CCA tumor tissue in the clinical setting.

There were some limitations to this study. First, the sample size was small which limited the statistical power to find significant changes in gene expression. This is the first time AL has ever been studied in CCA patients so the pool of potential participants is small. Additionally, the drop-out rate of study participants posed a challenge as only those remaining in the study until at least the second blood sampling (day 28) could be included. The sample size should be taken into consideration when drawing conclusions from the results presented. Second, other studies that have looked at differential gene expression in cancer patients have done so using tissue samples whereas this study used blood samples. Both are valid ways of analyzing gene expression but the results of the two different methods may not be exactly one to one. Tissue samples are able to show gene expression in tumor tissue whereas blood samples show gene expression that is not exclusively from tumor tissue cells; however, blood samples enable easy repeat sampling. Third, this study did not analyze clinical outcomes. Lastly, this study only observed changes in Notch signaling gene expression, it did not observe any other pathways. Future research should use a larger study population, follow up to include clinical outcomes and analyze data covering more pathways to discover other potential targets for AL treatment.

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Conflicts of Interest

The authors declare no conflict of interest.

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