

# Overexpression of oncogenic spliced-AGR2vH potentiates cholangiocarcinoma cell tumorigenicity with proteomic alterations

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## Research Article

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## Abstract

Upregulated expression of Anterior gradient 2 (AGR2) has been observed in cells, highly metastatic mouse models, nras-mutant zebrafish and cholangiocarcinoma (CCA) specimens derived from patients. Our previous study reported that AGR2 splicing into AGR2vH can promote CCA cell metastasis and survivability. This present study aimed to investigate the molecular mechanisms underlying AGR2vH tumorigenicity *in vitro* and *in vivo*. AGR2vH was determined in patient tissues and presented the upregulation in CCA tumor tissues compared with matched-normal adjacent tissues. During the *in vitro* studies, AGR2vH was ectopically overexpressed in KKU-213A cells. Established AGR2vH-overexpressing CCA cells were found to exhibit increased proliferative and clonogenic ability. For *in vivo* tumorigenicity, a higher tumorigenic potential was identified in AGR2vH-overexpressing cells xenograft mice. Moreover, liquid chromatography-mass spectrometry with protein bioinformatics was used to examine the proteomic alteration. The CCA cell proteome was altered, and it was indicated that AGR2vH may be associated with CCA cell proliferation via the activation of Wnt/β-catenin signaling pathway, which was verified via the comparative immunoblotting of β-catenin in cytoplasmic and nuclear fractionated proteins. These present results provided evidence that the upregulation of AGR2vH promotes the tumorigenicity of CCA cells, which was associated with an alteration of the CCA cell proteome.

## Introduction

Cholangiocarcinoma (CCA) is a malignant tumor type arising in the epithelium of the bile ducts, which is recorded as the fourth most common cause of cancer-related mortality in Thailand and is commonly associated with poor prognosis [1, 2]. Moreover, the worldwide incidence of CCA was estimated to be 85 per 100,000 individuals in the northeast region of Thailand [3]. Current food consumption behaviors and fluke infection result in an increased CCA incidence, which accounts for 3% of gastrointestinal cancers and represents ~2% of all cancer-related fatalities annually [4]. Common risk factors of CCA in southeast Asian countries, particularly in Thailand, are associated with chronic infection from liver flukes (*Opisthorchis viverrini*) as a result of consuming uncooked fish. One of the major problems associated with CCA are the non-specific incipient symptoms, which make CCA management difficult. A recent study investigated the factors associated with CCA initiation, and Tg (fabp10: nras<sup>61K</sup>) transgenic zebrafish were generated for monitoring CCA induction. Gross morphological and ICC marker analyses were used to successfully examine the histopathological features of intrahepatic CCA. Anterior gradient 2 (AGR2) was identified as the most upregulated gene in Tg (fabp10: nras<sup>61K</sup>) transgenic zebrafish, and was associated with the morphological liver changes and CCA initiation [5].

AGR2 is a member of the protein disulfide isomerase (PDI) gene family. Accumulating evidence has shown that AGR2 serves an important role in a variety of cellular functions and acts as the proto-oncogenic protein [6]. Previous studies have also observed the upregulation of AGR2 in various malignant tumors, including breast, lung and prostate cancer. Moreover, the upregulation of AGR2 was a poor prognostic indicator in breast and lung cancer [7-9]. Another previous study revealed that high expression of AGR2 was associated with tumor grade and metastasis of CCA [10]. Alternative splicing is another

hallmark of cancer, and also occurs in CCA, as mentioned in our previous review article [11]. Of note, aberrant alternative splicing of AGR2 was first characterized in prostate cancer, which marked the expression AGR2vH, and aberrant splicing transcript from AGR2 mRNA is a more favorable indicator for prostate cancer compared with prostate-specific antigen [12].

In CCA, the present study found that upregulation of AGR2 in metastatic CCA cells coincided with an aberrant splicing of AGR2 mRNA, and isoforms of AGR2, such as AGR2vE, AGR2vF and AGR2vH, are specific to the metastatic cells. Sequence analysis identified that only the AGR2vH transcript retained the start codon, which was predictably translated into protein. For the isoform AGR2vH, the AGR2 protein was altered at the dimerization motifs; three important domains were missing, including PDI domain, peptide binding domain and KTEL-ER retention domain, but the protein retained an ER-signal sequence at 1-20 amino acid residues and the adhesion domain at 21-40 amino acid residues. The adhesion domain of AGR2 was previously demonstrated to be involved in cell migration [13]. It was found that AGR2vH was upregulated in non-metastatic and highly metastatic CCA cells when compared with cholangiocytes. In addition, the ectopic expression of AGR2vH in non-metastatic KKU-213A cells can promote the cell migratory, invasive and adhesive abilities [13]. Moreover, AGR2vH can support CCA cell survivability under ER stress-inducing conditions [14].

The present study aimed to examine the expression of AGR2vH in human CCA tissues and identify the molecular mechanisms involved in the effects of AGR2vH overexpression on CCA cell proliferation *in vitro* and *in vivo*. The molecular networks between individual molecules were also analyzed via *in silico* analysis, and signaling pathways that are associated with cell proliferation were investigated using proteomics analysis of AGR2vH-overexpressing cells.

## Materials And Methods

### Sample collection

Human tissues were provided by the Cholangiocarcinoma Research Institute, Faculty of Medicine, Khon Kaen University. Paired tumor and adjacent non-tumor tissues were obtained from 5 patients with CCA (1 male and 4 females with 53-70 years old) who underwent surgery at Department of Surgery at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University. Written informed consent was obtained from subjects (HE521209). Experimental design and procedures were approved by Naresuan University Institutional Review Board for Human Ethic (approval no. NU-IRB0895/61). For the CCA samples, five tissues were selected from clearly differentiated tumor and non-tumor areas, as selected from the tissues as in a previous study [15]. These tissues included paraffin-embedded tissues of patients with intrahepatic CCA who underwent liver resection. Diagnosis was evaluated using clinical data, imaging analysis, tumor markers and pathology. Immunohistochemical studies for pathological diagnosis included antibodies against cytokeratin-7 (CK7), cancer antigen or carbohydrate antigen 19-9 (CA19-9), hepatocyte paraffin 1 (HepPar1) and  $\alpha$ -fetoprotein. The tumor tissues were verified as CCA based on the following criteria when either CK7 $^+$  or HepPar1 $^+$ , with or without CA19-9 $^+$ , were found. The study protocol

(previous sample collection) was approved by The Human Research Ethics Committee, Khon Kaen University, Thailand (approval no. HE551407) before the study was conducted.

### **Establishment of stable AGR2vH-overexpressing cells**

The procedure of AGR2vH-overexpressing cell establishment was conducted as described in our previous studies [13, 14]. The AGR2vH amplicon was amplified, sequenced and digested with *Hind*III and *Eco*RI restriction enzymes (Thermo Fisher Scientific, Inc.), before being inserted into p3XFLAG-CMV-14 expression vector (Sigma-Aldrich; Merck KGaA). The pCMV14-AGR2vH and pCMV14-empty vectors were transfected into KKU-213A cells using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) and the single clone was selected using Geneticin G418 (Thermo Fisher Scientific, Inc.). The successful establishment of AGR2vH overexpressing cells was confirmed using reverse transcription (RT)-PCR and RT-quantitative (q)PCR.

### **Cell line and cell culture**

The human CCA cell lines, KKU-213A [16] and KKU-213L5 [10], were provided by the Cholangiocarcinoma Research Institute, Faculty of Medicine, Khon Kaen University. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% v/v FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO<sub>2</sub>.

### **RNA extraction, RT-PCR and RT-qPCR**

Total RNA was extracted from cells or tissues using RiboZol™ reagent (Amresco LLC) following the manufacturer's instructions. cDNA synthesis was performed using a HiSenScript™ RH(-) cDNA Synthesis kit (Intron Biotechnology, Inc.).

The PCR reaction was performed using 1X MyTaqTM HS Red mix (Bioline; Meridian Bioscience) under optimized conditions using AGR2vH specific primers, which are described in previous studies [13, 14]. β-actin was used as an internal control for semi-quantitative normalization. The amplification procedure consisted of the following steps: Initial denaturation at 5 min at 94°C, followed by 28 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at the primers specific annealing temperature, extension for 30 sec at 72°C, and a final extension for 7 min at 72°C. PCR products were analyzed via 1% agarose gel electrophoresis, detected using ImageQuant™ LAS 500 (Cytiva) and quantitated using ImageQuant TL 7.0 software (GE Healthcare). RT-qPCR was performed for relative quantification of gene expression using 1X LightCycler® 480 SYBR Green I Master (Roche Applied Science) under optimized conditions (13,14), and analysis was conducted using the LightCycler® 480 system (Roche Applied Science). The expression levels of the target genes were normalized with β-actin using the relative quantification formula of 2<sup>-ΔΔCq</sup> [17].

### **Cell proliferation assay**

Cell proliferation was examined using a Cell Counting Kit-8 (CCK-8) kit (Dojindo Molecular Technologies, Inc.) using water soluble tetrazolium substrate. Cells were plated at 3,000 cells per well in a 96-well plate. After incubation for 24, 48 and 72 h, 10 µl CCK-8 reagent was added to each well and incubated at 37°C for 2 h, and the absorbance was measured at 450 nm. The average absorbance at 450 nm of the determinate cells at 24 h in each group was normalized as 1.00 for calculation of relative of cell proliferation. The experiment was performed independently in biological triplicate.

### **Colony formation assays**

Anchorage-dependent growth was evaluated using a clonogenic assay in 6-well plates. pCMV14-empty and pCMV14-AGR2vH cells were seeded at 500 cells per well and incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 14 days. Colonies were fixed in 4% paraformaldehyde, stained with crystal violet solution and counted under a light microscope with 4X magnification.

### **Animal experiments**

All animal studies adhered to protocols approved by the Naresuan University Institutional Animal Care and Use Committee (Project no. NU-AE610623; Approval no. 6101025). Twelve Male BALB/CAJcl-Nu/Nu mice (age, 4-6 weeks) were obtained from Nomura Siam International Co., Ltd., housed under specific pathogen-free conditions and cared for according to the institutional guidelines for animal care.

In total, six mice were used in each group to establish the subcutaneous xenograft model. Then, 2.5x10<sup>6</sup> pCMV14-AGR2vH4 transfected KKU-213A cells or pCMV14-Empty transfected KKU-213A cells in 150 µl PBS were subcutaneously injected into the right flanks of nude mice. The body weight of the mice and the diameters of tumors were monitored every 2 days for 3 weeks, and tumor volume (mm<sup>3</sup>) was calculated using the following equation: Tumor length (mm) x tumor width (mm)<sup>2</sup>/2. All animals were sacrificed on day 21 after xenograft transplantation using an overdose of sodium pentobarbital ( $\geq$ 100 mg/kg) via intraperitoneal injection. Tumors were excised and tumor weight was measured.

### **Liquid chromatography-tandem mass spectrometry (LC-MS/MS) and protein bioinformatics**

Total protein was extracted from pCMV14-AGR2vH4 transfected KKU-213A cells or pCMV14-Empty transfected KKU-213A cells, using RIPA buffer (0.05 M Tris-HCl pH 7.4, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.15 M sodium chloride, 0.002 M EDTA and 0.05 M sodium fluoride) and measured using a Bradford assay. The concentration of each protein sample was adjusted to 10 µg/µl and subjected to a phosphoprotein enrichment procedure (Pierce; Thermo Fisher Scientific, Inc.). Then, 50 µg protein was subjected to in-solution digestion, mixed with 50 ng/µl trypsin (Promega Corporation) and incubated at 37°C overnight. The digested samples were dried and protonated with 0.1% formic acid before injection into LC-MS/MS. Tryptic peptide samples were prepared for injection into an Ultimate3000 Nano/Capillary LC system (Thermo Fisher Scientific, Inc.) coupled to a Hybrid quadrupole Q-ToF impact II™ (Bruker Daltonics) equipped with a Nano-captive spray ion source. Electrospray ionization was carried out using CaptiveSpray. Mass spectra were obtained in the positive-ion mode over the range (m/z) 150-

2,200 (Compass 1.9 software; Bruker Daltonics). MaxQuant 1.6.5.0 was used to quantify the proteins using an Andromeda search engine to correlate MS/MS spectra to the Uniprot *Homo sapiens* database [18].

Only peptides with a minimum of seven amino acids and  $\geq 1$  unique peptide were required for protein identification. Only proteins with  $\geq 2$  peptides and  $\geq 1$  unique peptide were considered as being identified and used for further analysis. As a search FASTA file, 954 proteins present in the *Homo sapiens* proteome downloaded from Uniprot. The proteome data obtained were further analyzed via UniProt, Gene Ontology (GO), Vocalno plot, Heat map and Panther analyses.

### **Protein extraction and western blotting**

For whole cell protein lysate, cells were lysed with RIPA lysis buffer containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate and protease and phosphatase inhibitors. For cytoplasmic and nuclear protein preparation, cells were washed with cold PBS, and harvested in cell lysis buffer (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl and 0.5 mM DTT), and the supernatants were first collected as the cytoplasmic fraction. Then, the nuclear pellets were collected, washed twice with ice cold PBS and lysed with nuclear lysis buffer (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA and 0.5 mM DTT) [19]. Phosphatase inhibitors and protease inhibitors (Roche Diagnostics GmbH) were added at each step.

The protein concentration was determined using the Bradford assay. The 40, 20 and 10 µg whole cell proteins, cytoplasmic protein and nuclear protein, respectively, were separated via 12% SDS-PAGE and transferred to a PVDF membrane (Bio-Rad Laboratories, Inc.). After blocking with 5% skimmed milk, the membrane was incubated with primary antibodies overnight at 4°C. The primary antibodies were used at a dilution of 1:500 for b-catenin (Elabscience, Inc.), AKT (Elabscience, Inc.) and phosphorylated (p)-AKT (Elabscience, Inc.), and 1:5,000 for GAPDH (Sigma-Aldrich; Merck KGaA). After incubation with specific secondary antibodies (1:2,000), the expression of protein was observed using an ECL solution (Bio-Rad Laboratories, Inc.).

### **Statistical analysis**

Experiments were performed in triplicate. Data are presented as the mean  $\pm$  SD. An unpaired Student's t-test (two tailed) was used for comparison between each group using SigmaPlot software (SigmaPlot 11.0; Systat Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

## **Results**

### **Determination of AGR2vH mRNA upregulation in CCA tissues**

The expression level of AGR2vH was analyzed in a series of matched CCA and adjacent normal bile duct epithelium tissue samples from five CCA cases. A significant increase of AGR2vH mRNA expression was observed in CCA tissues compared with adjacent non-tumor tissues (Fig. 1a and 1b).

Then, the association of AGR2vH expression in tumors of these five cases was examined. Case 1 and case 2 had slightly increased AGR2vH expression in tumors, and were recorded as stage I and II, respectively. On the other hand, cases 3 and 5 were recorded as stage II with metastasis and stage IVA, respectively. In addition, while no staging was documented for case 4, perineural and lymph node invasions were mentioned in the clinical report. Therefore, AGR2vH expression trends in tumor tissues were associated with the stage and aggressiveness of CCA.

### **AGR2 overexpression in CCA cells promotes cell proliferation and anchorage-dependent growth**

In *in vitro* experiments, the effects of AGR2vH in CCA tumorigenicity were investigated using established AGR2vH overexpressing cells in a non-metastatic KKU-213A cell line from our previous studies [13, 14]. The expression of AGR2vH in pCMV14-AGR2vH cells and pCMV14-empty cells was assessed using RT-PCR and RT-qPCR techniques. AGR2vH expression was verified in two selected clones (AGR2vH4 and AGR2vH8), and it was confirmed that both established cell lines stably contained pCMV14-AGR2vH (Fig. 2a and 2b).

Cell proliferation was monitored at 24, 48, 72 and 96 h using a CCK-8 assay. The relative proliferative ability of KKU-213A, pCMV14-Empty, pCMV14-AGR2vH4, pCMV14-AGR2vH8 and KKU-213AL5 cells was examined by normalizing to the proliferation indicated at 24 h. Ectopic expression of AGR2vH in non-metastatic KKU-213A cells slightly increased CCA cell proliferation at 24 and 48 h, and significantly promoted the cell proliferation at 72 and 96 h (Fig. 2c).

Previous studies have reported that AGR2 impacts cell proliferation *in vitro* in esophageal, pancreatic and breast cancer cells. Therefore, the role of AGR2vH overexpression in anchorage-dependent growth was examined. Overexpression of AGR2vH markedly enhanced anchorage-dependent proliferation in both pCMV14-AGR2vH4 and pCMV14-AGR2vH8 cells when compared with KKU-213A and pCMV14-Empty transfected cells (Fig. 2d and e). Therefore, AGR2vH can promote CCA cell proliferation and the formation of cancer cell colonies under anchorage-dependent culture conditions.

### **AGR2vH promotes the *in vivo* tumorigenesis of CCA cells in a xenograft tumor model**

The effect of AGR2vH on cancer tumorigenesis was investigated in the *in vivo* model. The AGR2vH4-overexpressing cell xenograft tumors derived from a subcutaneous injection of pCMV14-AGR2vH4 transfected CCA cells grew more rapidly, as indicated by increased tumor size in the representative mice (Fig. 3a) and a higher tumor volume (Fig. 3b), compared with the control group. Moreover, xenograft tumors are presented (Fig. 3c), and an increase in tumor size and weight was observed in the AGR2vH-overexpressing xenograft when compared with control group. The maximum xenograft tumor size obtained in the study was 324.6 mm<sup>3</sup> (464.8 mg tumor weight) (Fig. 3d). In addition, the health condition of these experimental nude mice was observed by measuring the body weight and the results indicated that all of treatments were non-toxic to the mice (Fig. 3e).

### **Proteomics analysis of AGR2vH-overexpressing CCA cells**

Proteomics analysis of AGR2vH-overexpressing cells (pCMV14-AGR2vH4 transfected CCA cells) was examined using LC-MS/MS and analyzed via bioinformatics tools, which was compared with control cells. The data mining and analysis identified a total of 954 differentially expressed proteins (DEPs) in AGR2vH-overexpressing cells. To gain insight into the biological roles of these DEPs, GO category enrichment analysis was conducted. The GO categories include three groups: Biological process, molecular function and cellular component (Fig. 4a). Using a false discovery rate <0.05 threshold, it was identified that GO terms for biological processes were significantly enriched in 'cellular process', 'metabolic process' and 'biological regulation'. The molecular function mainly included 'binding', 'catalytic activity' and 'molecular function regulator'. For the cellular component, the enriched GO terms were 'organelle', 'cell' and 'protein-containing complex'.

Next, the DEPs were analyzed using the volcano plot (cut off <1-fold) and 55 downregulated proteins and 59 upregulated proteins were identified in AGR2vH-overexpressing cells. Heat map analysis of these upregulated and downregulated proteins (cut off < 2-fold) was performed with the list of accession numbers (Fig. 4b and table 1). Moreover, a pathway analysis using Panther was conducted for DEPs in AGR2vH-overexpressing cells. The results demonstrated that the overrepresented DEPs in AGR2vH-overexpressing cells were mainly associated with the Wnt signaling pathway and TGF- $\beta$  signaling pathway (Fig. 4c).

The association between AGR2vH and the molecular signaling that promoted CCA cell proliferation was then assessed. It was hypothesized that the ectopic expression of AGR2vH may promote CCA progression via an activation of the Wnt/b-catenin signaling pathway. To evaluate this hypothesis, Wnt/b-catenin signaling molecules (b-catenin, AKT and p-AKT) were detected using western blot analysis. The results demonstrated that b-catenin and p-AKT protein expression levels were significantly increased in AGR2vH4-overexpressing cells (Fig. 4d). The determination of b-catenin activation was performed via both western blot analysis of cytoplasmic/nuclear proteins, AGR2vH4-overexpressing cells presented a higher intensity of nuclear b-catenin, and a decreased cytoplasmic b-catenin expression.

## Discussion

The present study examined other molecular functions of AGR2vH, a spliced mRNA transcript that was generated from aberrant alternative splicing of AGR2 pre-mRNA, which was previously reported to be involved in CCA development and aggressiveness [13, 14]. With regards to the possible application of AGR2vH in prostate cancer, a high expression level of AGR2vH was found in the urine exosome and it may serve as a non-invasion detection biomarker [12]. Therefore, this present study examined AGR2vH expression in CCA and found that it was upregulated CCA cells, especially in highly metastatic CCA cells. Then, the contribution of AGR2vH to CCA metastasis was determined via the evaluation of CCA cell migratory and invasive abilities after AGR2vH overexpression and knockdown [13]. Moreover, it was found that AGR2vH could promote CCA cell survivability under ER stress conditions[14].

The present study examined AGR2vH expression in CCA patient tissues and identified that AGR2vH was upregulated when compared with healthy bile duct tissues from the same case. The ectopic expression of AGR2vH significantly increased the proliferative capacity of non-metastatic KKU-213A cells. In addition, AGR2vH promoted the clonogenicity of KKU-213A cells by promoting the anchorage-independent growth by increasing the colony number and size. Previous studies in various types of cancer have reported that only wild-type AGR2 was upregulated in cancerous tissues and was involved in the proliferation of ovarian cancer cells, as well as the survivability of papillary thyroid carcinoma and pancreatic cancer cells [20-22]. Of note, the molecular mechanism underlying the role of AGR2 in cell proliferation was identified in breast cancer cells, as it was suggested that transient suppression of AGR2 can inhibit cell proliferation and cell cycle progression via the reduction of cyclin D1 [23]. In addition, depletion of AGR2 significantly decreased cell viability via accumulation in the G<sub>0</sub>/G<sub>1</sub> phase and the induction of cellular senescence in prostate cancer [24].

The present study identified that DEPs in AGR2vH-overexpressing CCA cells may be associated with Wnt signaling activation. It has been reported that the Wnt/b-catenin signaling pathway is associated with tumorigenesis, metastasis and cancer cell stemness [25, 26]. In addition, CCA-related differentially methylated regions have been reflected in signaling pathway alterations, which may be affected by aberrant DNA methylation in CCA, and it was found that Wnt/b-catenin and TGF- $\beta$  are significantly enriched pathways in CCA [27]. Therefore, the present study examined the expression levels of b-catenin, AKT and p-AKT, which are the downstream molecules of the Wnt/b-catenin signaling pathway. The results demonstrated upregulation of b-catenin and activation of AKT phosphorylation in AGR2vH-overexpressing cells. Moreover, nuclear b-catenin expression and nuclear localization was increased in AGR2vH-overexpressing cells.

In human CCA, a previous study has identified that the expression level of b-catenin was upregulated in CCA and was correlated with a greater size of CCA tumors [28]. Furthermore, the expression of Wnt ligands was significantly increased in CCA tissues compared with non-cancerous tissues. It has also been demonstrated that the canonical Wnt signaling pathway is progressively activated in CCA and that the inhibition of the canonical Wnt signaling pathway reduces the tumor nodules in animal xenografts [29].

In conclusion, the present study revealed upregulation of AGR2vH in human CCA tissues. The overexpression of AGR2vH significantly increased CCA cell proliferation in the non-metastatic parental KKU-213A cells and xenograft animal model. The proteomics analysis revealed that Wnt/b-catenin signaling is a significantly enriched cancer-related pathway in CCA, and that the ectopic expression of AGR2vH increased the expression of Wnt downstream molecules. These results support the conclusion that AGR2vH may promote CCA cell proliferation by activating the canonical Wnt/b-catenin signaling pathway.

## Abbreviations

AGR2; Anterior gradient 2, AGR2vH; Anterior gradient 2 spliced variant H, AKT; Protein kinase B, CCA; cholangiocarcinoma, DEPs; Differential expressed proteins, GAPDH; Glyceraldehyde 3-phosphate dehydrogenase, LC-MS/MS; Liquid chromatography-tandem mass spectrometry, PCR; Polymerase chain reaction, PDI; Protein disulfide isomerase.

## Declarations

### Ethics approval and consent to participate

For CCA patients' tissues, the experimental design and procedures were approved by Naresuan University Institutional Review Board for Human Ethic (approval no. NU-IRB0895/61). Of notes, human tissues were provided by the Cholangiocarcinoma Research Institute, Faculty of Medicine, Khon Kaen University, therefore, the study protocol (sample collection) was approved by The Human Research Ethics Committee, Khon Kaen University, Thailand (approval no. HE551407) before the study was conducted.

For animal study, all animal studies adhered to protocols approved by the Naresuan University Institutional Animal Care and Use Committee (Project no. NU-AE610623; Approval no. 6101025).

### Author contributions

JY and CI performed most of the experiments, statistical analysis and drafted the manuscript. SS, NH and SR performed some of the experiments. DS, SP, SW and WK conceived the idea and designed some of the experiments. SJ and WK wrote and corrected the manuscripts.

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### Declarations

Competing interests: The authors declare that they have no competing interests.

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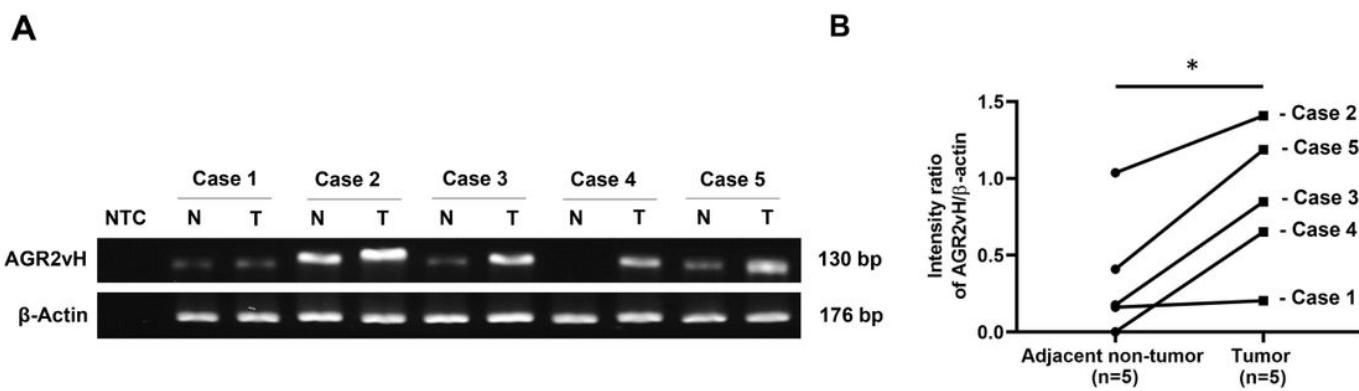
## Table 1

**Table 1.** List of 23 proteins that are differentially expressed in AGR2vH-overexpressing cells (pCMV14-AGR2vH transfected KKU-213A) compared with control cells (pCMV14-Empty transfected KKU-213A), as identified via LC-MS/MS.

Accession number	Symbol	Protein name	Function	Log2 ratio	p-value
Q8TF46.2	DIS3L	DIS3-like exonuclease 1	Molecular function	2.30	1.40E-12
Q9UPV0.3	CEP164	Centrosomal protein of 164 kDa	Cell division	2.30	1.40E-12
Q96BR6.2	ZNF669	Zinc finger protein 669	Transcriptional regulation	-2.01	5.16E-09
Q8N9V7.3	TOPAZ1	Testis- and ovary-specific PAZ domain-containing protein 1	Biological process	-2.02	4.70E-06
P28289.1	TMOD1	Tropomodulin-1	Actin-binding	-2.08	7.62E-09
A8MXY4.3	ZNF99	Zinc finger protein 99	Transcriptional regulation	-2.16	8.85E-06
Q9P291.1	ALEX1	Armadillo repeat-containing X-linked protein 1	Mitochondrial transport	-2.25	3.71E-04
Q8N1I0.3	Dock4	Dedicator of cytokinesis protein 4	Molecular function	-2.36	7.35E-07
Q13670.1	PMS2P11	Putative postmeiotic segregation increased 2-like protein 11	Molecular function	-2.47	3.35E-06
Q9P2F5.2	STOX2	Storkhead-box protein 2	Biological process	-2.66	7.16E-07
P47972.2	NPTX2	Neuronal pentraxin-2	Cellular process	-2.75	2.13E-10
Q9P255.2	ZNF492	Zinc finger protein 492	Transcriptional regulation	-2.76	1.55E-12
Q9BQS8.3	FYCO1	FYVE and coiled-coil domain-containing protein 1	Metal ion binding	-2.94	5.72E-08
Q9UQB9.1	AURKC	Aurora kinase C	Cell division	-2.97	9.80E-08
Q96NY8.1	NECTIN4	Nectin-4	Biological adhesion	-3.09	5.27E-06
Q99102.4	MUC4	Mucin-4	Cell adhesion	-3.10	6.29E-06
Q9P1U1.1	ACTR3B	Actin-related protein 3B	Actin-binding	-3.10	6.29E-06
Q9P202.3	WHRN	Whirlin	Signal transduction	-3.57	3.43E-06
Q5VUJ5.1	AGAP7P	Putative Arf-GAP with GTPase-ANK repeat and PH domain-containing protein 7	Molecular function	-3.57	3.43E-06
Q9UIW2.3	PLXNA1	Plexin-A1	Biological adhesion	-3.67	1.99E-11

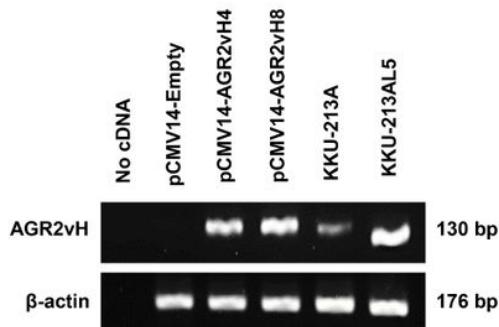
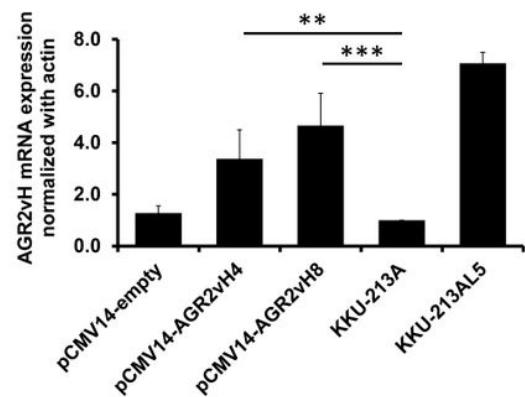
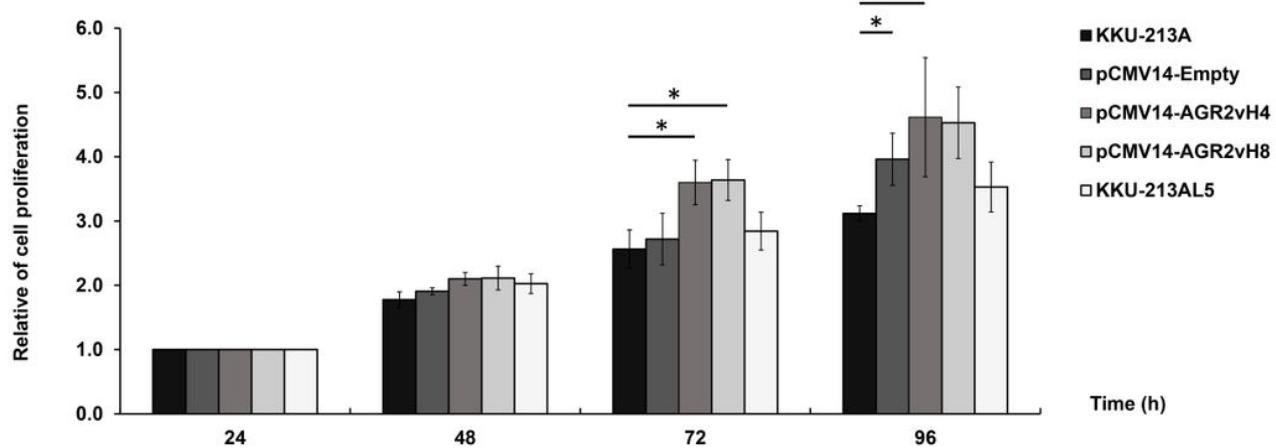
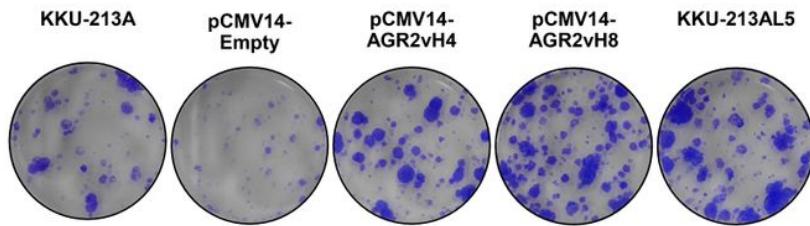
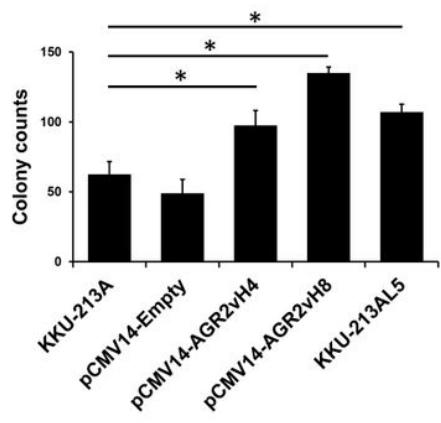
Q9GZV1.3	ANKRD2	Ankyrin repeat domain-containing protein 2	Biological regulation	-4.05	5.73E-10
Q96MC4.1	CEP295NL	CEP295 N-terminal-like protein	Biological regulation	-5.18	8.73E-12
Q15858.3	SCN9A	Sodium channel protein type 9 subunit alpha	Biological regulation	-5.62	2.53E-13

## Figures



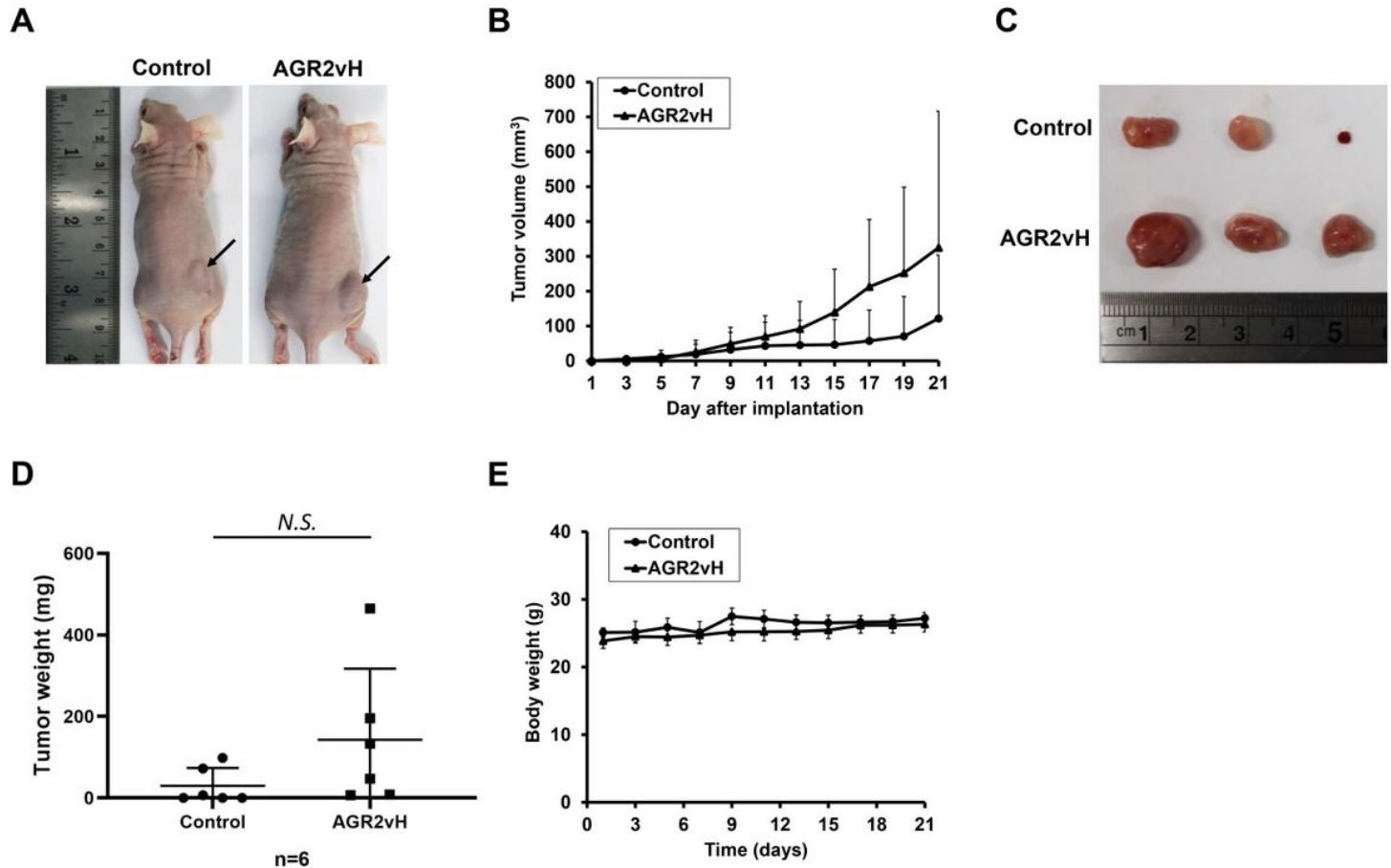
**Figure 1**

AGR2vH mRNA expression in match-paired CCA and adjacent non-tumor tissues. a AGR2vH mRNA expression in tumor area compared with adjacent non-tumor tissue area in five CCA cases as detected via RT-PCR. b Semi-quantitative of AGR2vH band intensities from RT-PCR results in five CCA cases. T, tumor area; N, non-tumor tissue area. \*P<0.05.

**A****B****C****D****E****Figure 2**

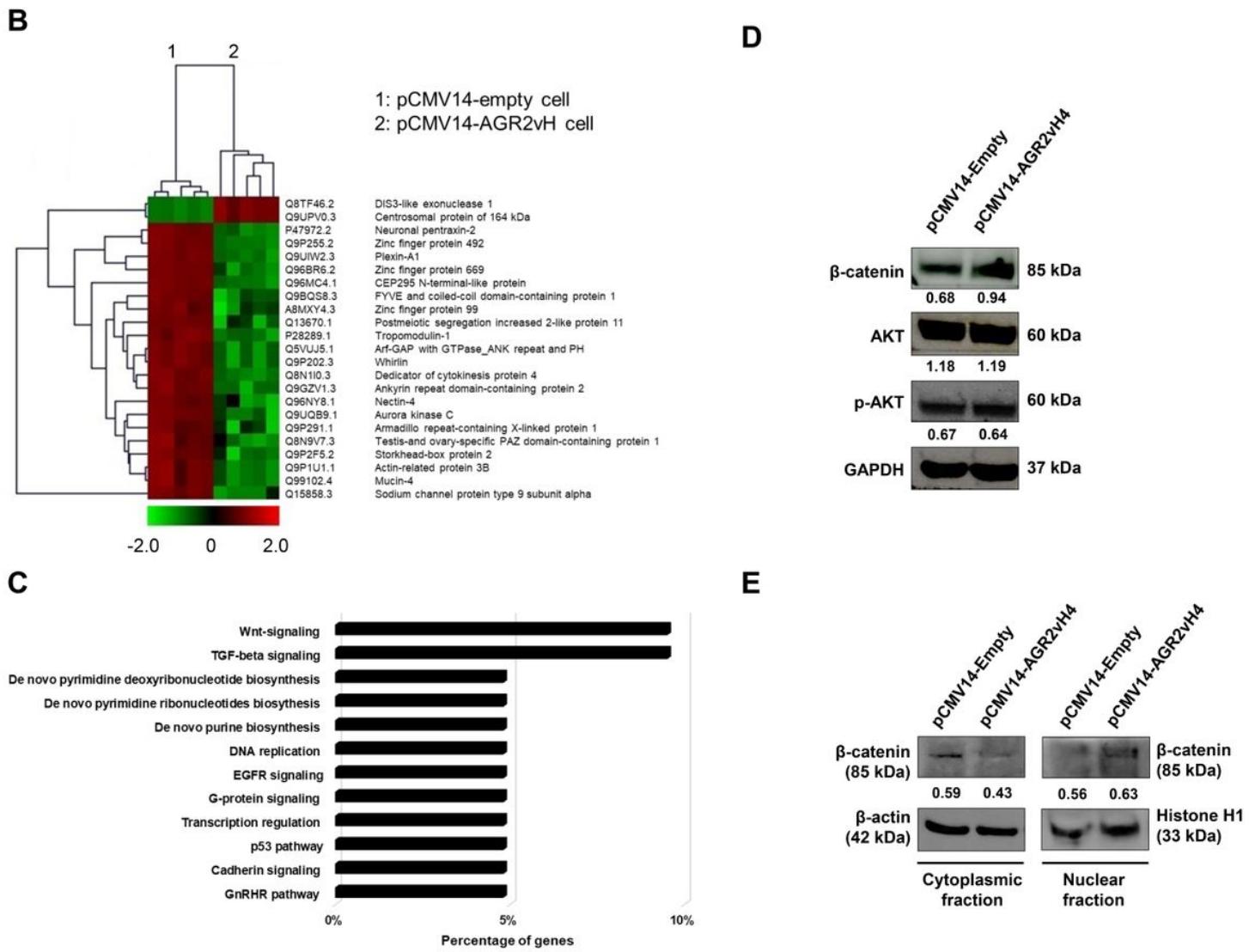
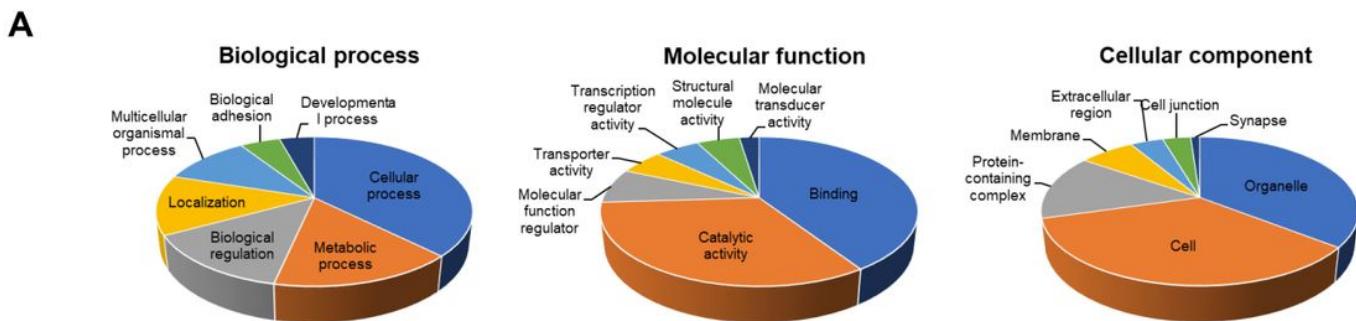
AGR2vH mRNA expression in AGR2vH-overexpressing cells, and effects of AGR2vH on CCA cell proliferation and anchorage-dependent growth. a RT-PCR validation of ectopic overexpression of AGR2vH in pCMV14AGR2vH-transfected cells. b Quantitative analysis of AGR2vH upregulation in pCMV14AGR2vH-transfected cells. c Proliferative rate of AGR2vH-overexpressing cells at 24, 48, 72 and 96 h as detected using a Cell Counting Kit-8 assay. d Cancer cell colony formation (Anchorage-dependent

growth ability) of AGR2vH-overexpressing cells from whole-well of clonogenic assay. e Colony count as detected by the clonogenic assay. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001.



**Figure 3**

Effect of AGR2vH on CCA growth in the BALB/CAJcl-Nu/Nu mice xenograft model. a Representative subcutaneous tumor formation of AGR2vH4-overexpressing cells in the xenograft animal model (n=6 for each group). b Volume of AGR2vH4-overexpressing cell xenograft tumor. c Size (21-days) of AGR2vH4-overexpressing cell xenograft tumor. d Weight of AGR2vH4-overexpressing cell xenograft tumor. e Comparative body weight of the mice in the AGR2vH4-overexpressing cell xenograft and control groups. AGR2vH, Anterior gradient 2; CCA, cholangiocarcinoma. N.S.=Non-statistically Significant.



**Figure 4**

Comparative proteomics analysis of AGR2vH4-overexpressing cells and control cells. a Annotation of 954 DEPs in AGR2vH4-overexpressing cells using GO analysis, and these were categorized into biological process, molecular function and cellular component. b Heat map analysis of DEPs of both upregulated and downregulated proteins (cut off <2.0-folds change) in AGR2vH4-overexpressing cells. c Pathway analysis using Panther for DEPs in AGR2vH4-overexpressing cells. d Western blot analysis identified the upregulation of b-catenin and p-AKT in AGR2vH-overexpressing cells. e Western blot analysis verified the

activation of b-catenin via the detection nuclear protein fractions compared with cytoplasmic protein fractions in AGR2vH4-overexpressing cells.