Upregulation of AGR2vH facilitates cholangiocarcinoma cell survival under endoplasmic reticulum stress via the activation of the unfolded protein response pathway

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Received June 13, 2019; Accepted November 28, 2019

DOI: 10.3892/ijmm.2019.4432

Abstract. Cholangiocarcinoma (CCA) is an epithelial cell malignancy arising within the biliary tree in the liver. CCA is usually diagnosed at an advanced stage, subsequent to developing with metastasis. Recently, anterior gradient-2 (AGR2) was characterized as one of the most highly upregulated genes among all metastasis-associated genes in highly metastatic CCA cell lines. Previous reports have demonstrated that AGR2 is required for triggering the unfolded protein response (UPR) pathway to support cancer cell survival, particularly under endoplasmic reticulum (ER) stress conditions. A previous study identified an AGR2 short isoform generated by aberrant splicing, AGR2vH, which contributed to the metastatic phenotype of CCA cells. The aim of the present study was to determine the function of AGR2vH in UPR pathway activation to support cancer cell survivability and apoptosis evasion. Subsequent to experimentally inducing ER stress in AGR2vH-overexpressing CCA cells using tunicamycin, the UPR pathway was activated by the upregulation of UPR marker genes (activating transcription factor 6, eukaryotic initiation factor 2a and spliced X-box binding protein 1), UPR proteins [binding immunoglobulin protein/glucose-regulated protein (GRP)78 kDa and phosphorylated eukaryotic translation initiation factor 2a] and UPR downstream targets (GRP94). In addition, the results were verified by AGR2vH knockdown using specific small interfering RNAs. Under ER stress conditions, the overexpression of AGR2vH reduced the number of apoptotic cells by decreasing caspase-3/7 activity and downregulating c/EBP homologous protein mRNA and B-cell lymphoma-2 (Bcl-2)-associated X protein expression, whereas the Bcl-2 protein was upregulated, resulting in a higher number of viable cells. The results of the present study support the previous data that indicate that an oncogenic AGR2vH isoform may not only promote metastasis-associated phenotypes, but also CCA cell survival and apoptosis evasion, thereby favoring cancer progression.

Introduction

Cholangiocarcinoma (CCA), or bile duct cancer, arises from the epithelium in the biliary tract. CCA development has been associated with infection by carcinogenic liver flukes, Opisthorchis viverrini, therefore, this type of cancer exhibits the highest incidence and mortality rates in Southeast Asia, particularly in Thailand (1). Diagnosis and treatment of CCA is difficult in a majority of cases, as the cancer is often detected when the patients are at an advanced stage, with metastases to the liver, lungs, lymph nodes or other secondary organs (2,3).

A previous model was established for studying the in vitro metastasis of CCA using a pair of human CCA cell lines without and with high metastatic activity, namely KKU-213 and KKU-213L5, respectively. KKU-213 is the parental cell line and KKU-213L5 was selected in vivo through the fifth serial passage of tissues from pulmonary metastases formed following the tail vein injection in NOD/scid/Janus kinase 3 mice. KKU-213L5 cells were revealed to exhibit the most prominent metastatic phenotype compared with the parental KKU-213 cells. The mRNA expression profiles of 77
metastasis-associated genes were determined using a quantitative (q)PCR array, which revealed that anterior gradient-2 (AGR2) was the most highly upregulated among 77 genes that were predominantly upregulated in KKU-213L5 cells compared with parental KKU-213 cells (4).

AGR2 is a protein that localizes in the anterior border of the embryonic ectoderm and is crucial in cementing gland development in the early embryonic development in *Xenopus laevis* (5). Human AGR2 is classified as an enzyme in the protein disulfide isomerases (PDIs) family. The 13,304 base pair (bp) of the AGR2 gene on chromosome 7 encodes for a 996-bp 8-exon mRNA, which translates into a 175-amino acid protein (6). AGR2 is typically localized in the endoplasmic reticulum (ER) and is involved in the production of cysteine-rich proteins, including the mucin family proteins, which cannot fold correctly, and AGR2 is a key enzyme that directly functions as the isomerase enzyme for the folding of newly synthesized proteins and prevents the accumulation of unfolded or misfolded proteins (14). Activated PERK phosphorylates a downstream target, eukaryotic initiation factor 2 (eIF2), and phosphorylated (p-)eIF2α promotes the expression of transcription factor ATF4, which regulates numerous UPR pathway target genes involved in ER stress-mediated apoptosis, including C/EBP homologous protein (CHOP) (15).

In 2014, the first evidence of AGR2 splicing was reported in prostate cancer, including 6 spliced variant transcripts, including AGR2vB, AGR2vC, AGR2vE, AGR2vF, AGR2vG and AGR2vH (16). A previous study reported the aberrant splicing of AGR2 in CCA cells, which characterized the highly upregulated AGR2vH transcript and its function in promoting the metastasis-associated phenotypes of CCA cells, including migration, invasion and adhesion capacities. Of note, only AGR2vH was predictably translatable into a protein isoform that consists of 67 amino acids, which were truncated from 175 amino acids in AGR2 (17,18). The oncogenic properties of AGR2vH, which enhance the metastatic ability of CCA cells, were recently demonstrated (17). It was previously reported that the suppression of AGR2vH in highly metastatic KKU-213L5 cells reduced their migration and invasion abilities, whereas the overexpression of AGR2vH in parental KKU-213 cells promoted cancer cell migration, invasion, adhesion and proliferation (17).

Regarding the characterization of AGR2, it is a gene that is highly and specifically upregulated in the metastatic CCA cell line (4) and the upregulation of this gene coincides with the aberrant splicing of AGR2 mRNA, and AGR2vH are specific to metastatic CCA cells (17). Previous results have demonstrated that the AGR2vH isoform enables metastasis-associated phenotypes in CCA cells. Additionally, another important function of AGR2vH was proposed (17). Taking into account experiments in other cancer types that proved that the AGR2 wild-type is required for interaction with BiP/GRP78, which is the UPR pathway activator as mentioned above (10), the present study attempted to investigate the functional ability of AGR2vH in the activation of the UPR pathway in a model of CCA.

Prospectively, AGR2vH may serve as an alternative partner molecule contributing to the survival of CCA cells. The aim of the present study was to determine the effect of AGR2vH on UPR pathway response and cell viability/apoptosis following the overexpression and knockdown of AGR2vH in CCA cells, particularly when experimentally inducing ER stress in the cancer cells. The activation of the UPR pathway was investigated by the expression of UPR-sensitive markers and UPR pathway activation proteins. In addition, the number of dead cells and the activity of caspase enzymes in the apoptosis pathway and the survival of CCA cells were investigated.

### Materials and methods

**Cell lines and cell culture.** The two CCA cell lines used in the present study included KKU-213, which was obtained from the Japanese Collection of Research Bioresources Cell Bank, and KKU-213L5, a highly metastatic CCA cell line derived from the parental KKU-213 cell, which was established in a previous study (4). Cells were provided from the Cholangiocarcinoma Research Institute, Faculty of Medicine, Khon Kaen University (Khon Kaen, Thailand). The cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% v/v fetal bovine serum with 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Waltham, MA). The cell lines were grown in a humidified incubator at 37°C with 5% CO2 in air.
previously described (17). Briefly, AGR2vH-overexpressing cells were plated in a 6-well plate at 2.5x10^4 cells per well for the induction of unglycosylated-proteins in the ER. Tunicamycin was used to block the activity of glycosylase, which resulted in the accumulation and culture. Geneticin (Sigma-Aldrich; Merck KGaA) was dissolved in dimethyl sulfoxide and the single clones were selected using 2 mg/ml Geneticin (Sigma-Aldrich; Merck KGaA) in Opti-MEM I reduced-serum medium (Gibco; Thermo Fisher Scientific, Inc.) and maintained at 37˚C in a humidified incubator in a 5% CO2 atmosphere.

**Transfection and overexpression of AGR2vH in CCA cells.** AGR2vH-overexpressing KKU-213 cells were established as previously described (17). Briefly, AGR2vH mRNA was amplified by specific primers, as listed in Table I, with the relevant restriction sites to clone into the pCR® 2.1-TOPO® cloning vector (Invitrogen; Thermo Fisher Scientific, Inc.). The AGR2vH nucleotide sequences were analyzed and confirmed before being sub-cloned into the p3XFLAG-CMV-14 expression vector (Sigma-Aldrich; Merck KGaA). Either pCMV14-AGR2vH or pCMV14-Empty vector (5 μg/μl) were transfected into KKU-213 cells by Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) and cultured for 48 h at 37˚C, and the single clones were selected using 2 mg/ml Genetin G418 (Thermo Fisher Scientific, Inc.) and subjected to expansion and culture.

**Experimental induction of ER stress.** Tunicamycin was used to block the activity of glycosylase, which resulted in the accumulation and culture. 2% agarose gel electrophoresis was performed to analyze the migration of unglycosylated proteins in the ER. The thermocycling conditions were as follows: 95˚C for 5 min for a pre-denaturation, followed by 30 cycles of 95˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec, and final extension at 72˚C for 5 min. The PCR products were mixed with 6X non-mutagenic fluorescent (SYBR-Green) DNA staining reagent (Nanolite, GeneDireX, Inc.) and analyzed by 2% agarose gels detected by ImageQuant™ LAS 500 (GE Healthcare Life Sciences) and quantitated using ImageQuant TL 7.0 software (GE Healthcare Life Sciences).

**Preparation of RNA and RT-PCR.** Total RNA was isolated from the cells using an E.Z.N.A® Total RNA kit I (Omega Bio-Tek, Inc.). The concentrations of RNA samples were measured, and 1 μg total RNA was used to synthesize the complementary DNA using the HisenScriptTM RH [-] RT PreMix kit (Intron Biotechnology, Inc.) according to the manufacturer's protocol. All cDNA samples were stored at -80˚C until use. For the determination of gene expression by the amplification of synthesized cDNA, PCR was performed under optimized conditions. The reaction mixture contained 0.2 μg cDNA template, 0.4 μM each of the forward and reverse primers with a total volume of 20 μl of 1X MyTaqTM HS Red Mix (Bioline Reagents Limited). The house-keeping gene β-actin was used as an internal control for semi-quantitative normalization. The primers for the target genes were based on previous studies including AGR2vH (17), XBPl (20), BiP/GRP78 (11), ATF6, CHOP (21), eIF2α and GRP94 (22) and are listed in Table I. The thermocycling conditions were as follows: 95˚C for 5 min for a pre-denaturation, followed by 30 cycles of 95˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec, and final extension at 72˚C for 5 min. The PCR products were mixed with 6X non-mutagenic fluorescent (SYBR-Green) DNA staining reagent (Nanolite, GeneDireX, Inc.) and analyzed by 2% agarose gel electrophoresis at 90 volts for 30 min and visualized under ultraviolet illumination of agarose gels detected by ImageQuant™ LAS 500 (GE Healthcare Life Sciences) and quantitated using ImageQuant TL 7.0 software (GE Healthcare Life Sciences).

**Protein extraction and western blot analysis.** Cells were lysed with lysis buffer containing 7M urea, 2M thiourea, 4% -[3-cholamidopropyl)dimethylaminonio]-1-propanesulfonate, and protease and phosphatase inhibitors (Roche Diagnostics). The protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Inc.). Equal amounts of protein (30 μg/lane) were separated by 12% SDS-PAGE and western blot analysis were performed as previously described (17). Briefly, membranes were blocked with 5% non-fat milk in TBST for 2 h at room temperature. Subsequently, membranes were incubated with primary antibodies overnight at 4˚C and incubated with secondary antibodies for 1 h at room temperature. Antibodies against BiP/GRP78 (cat no. E-AB-31742; 1:2,000), horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG) (cat no. AP182P; 1:5,000) and HRP-conjugated anti-mouse IgG (cat no. E-AB-20864; 1:2,000), B-cell lymphoma-2 (Bcl-2; cat no. E-AB-15522; 1:650) and Bcl-2-associated X (BAX; cat no. E-AB-30629; 1:1,000) were purchased from Elabscience (Houston, TX, USA) whereas antibodies for β-actin (cat no. A5441; 1:1,000), BiP/GRP78 (cat no. E-AB-31742; 1:2,000), B-cell lymphoma-2 (Bcl-2; cat no. E-AB-15522; 1:650) and Bcl-2-associated X (BAX; cat no. E-AB-30629; 1:1,000) were purchased from Sigma-Aldrich; Merck KGaA. Protein bands were detected with an enhanced chemiluminescence system (Bio-Rad Laboratories, Inc.) and visualised with ImageQuant™ LAS 500 (GE Healthcare Life Sciences).
Flow cytometry. Apoptosis was assessed by Annexin V-Phycoerythrin/7-Amino-Actinomycin staining using a Muse™ Annexin V and dead cell assay kit (Merck KGaA). Cells were plated in a 6-well plate at 2.5x10^5 cells per well for 24 h prior to tunicamycin treatment. After 24 h treatment at 37˚C, 100 µl Muse™ Annexin V & dead cell reagent and an equal volume of 4x10^5 cells from each of the groups were mixed. Subsequent to incubating for 20 min at room temperature, the numbers of live, dead and apoptotic cells were analyzed using Muse® cell Analyzer and Muse 1.5.0.0 Analysis software (Merck KGaA) (25).

Caspase 3/7 activity assay. Cells were plated in a 96-well black plate at 2x10^4 cells per well for 24 h prior to tunicamycin treatment. After 24 h treatment, caspase 3/7 activities were analyzed using an Apo-ONE® Homogeneous caspase 3/7 assay (Promega Corporation), based on the cleavage of the non-fluorescent caspase substrate Z-dEVd-R110 by caspase-3/7 to create fluorescent Rhodamine 110 (26), according to the manufacturer's protocol. The fluorescence signal of each well was measured by a fluorescence microplate reader (EnSpire Multimode Plate reader; PerkinElmer, Inc.). With regards to the measurement of the fluorescence intensities, the assay suggested that the excitation wavelength be set at 499 nm, and the emission wavelength at 521 nm (27,28).

Cell viability assay. Cells were plated in a 96-well plate at 3x10^3 cells per well for 24 h prior to tunicamycin treatment. After 24 h treatment, 10 µl Cell Counting Kit-8 (CCK-8) reagent (Sigma-Aldrich; Merck KGaA) was added to each
well. Cells were incubated for 4 h at 37°C, and the absorbance at 450 nm was measured using a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Inc.).

**Statistical analysis.** Experiments were performed in biological triplicate. Data were calculated and presented as the mean ± standard deviation. Statistical significance between two groups was determined using a Student’s t-test (two tailed). One-way analysis of variance with a Least Significant Difference post-hoc test was performed to compare multiple groups using SPSS 17 (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Expression of AGR2vH on AGR2vH-overexpressing cells.** Semi-quantitative RT-PCR was performed to evaluate the expression of AGR2vH once the cells were transfected with pCMV14-Empty and pCMV14-AGR2vH vector. The expression of AGR2vH was substantially increased in AGR2vH-overexpressing cells when compared with the untransfected control cells, but the expression level of AGR2vH in AGR2vH-overexpressing cells was still lower compared with KU-213L5 cells (Fig. 1A). Furthermore, RT-qPCR confirmed the results of RT-PCR (P<0.01; Fig. 1B). In addition, the cell viability following AGR2vH transfection into KU-213 cells was not significantly altered at 48 h post-transfection compared with KU-213 cells (untransfected control) (Fig. 1C).

**Experimentally induced ER stress.** To optimize the different concentrations of tunicamycin, the expression of ER stress markers (XBP1s and BiP/GRP78) was determined after 24 h treatment using <4 µg/ml tunicamycin. XBP1s was significantly upregulated at 2 µg/ml when compared with untreated cells, as demonstrated using RT-PCR and confirmed by qPCR (P<0.05; Fig. 2A). Similar results were obtained for BiP/GRP78 by RT-PCR and qPCR (P<0.05; Fig. 2B). Therefore, 2 µg/ml tunicamycin was selected for ER stress induction.

**Activation of the UPR pathway and UPR downstream.** The present study sought to investigate UPR response following AGR2vH overexpression and knockdown in CCA cells and UPR response under ER stress-inducing conditions. On determination of the UPR marker gene expression, the notable upregulation of XBP1s, ATF6 and eIF2α was observed when the AGR2vH-overexpressing cells were under ER stress conditions (Fig. 3A). Similar results were observed in AGR2vH-depleted cells, with the downregulation of these markers under ER stress conditions, particularly ATF6 and eIF2α (Fig. 3B). The expression of GRP94 mRNA, an ER chaperone downstream of the UPR pathway, was upregulated in AGR2vH-overexpressing cells (Fig. 3C) and downregulated in AGR2vH-depleted cells, particularly under ER stress conditions (Fig. 3D). In addition, the activation of the UPR pathway was confirmed by determination of BiP/GRP78 and p-eIF2α protein expression under ER stress-inducing conditions. It was revealed that BiP/GRP78 and p-eIF2α protein expression was upregulated in AGR2vH-overexpressing cells (Fig. 3E), whereas they were downregulated in AGR2vH-depleted cells (Fig. 3F).

**Effects of AGR2vH on cell apoptosis.** The apoptosis of CCA cells was determined using flow cytometry. Reduction of the apoptotic cell population, particularly cells in late apop-
tosis, was observed in AGR2vH-overexpressing cells under ER stress-inducing conditions when compared with empty vector-transfected cells (Fig. 4A and B). The results were also confirmed by caspase 3/7 activities, which were significantly decreased in AGR2vH-overexpressing cells under ER stress conditions when compared with empty vector-transfected cells (P<0.001; Fig. 4C). In addition, the present study observed the downregulation of c HOP mRNA, which is the ER stress-induced apoptosis gene (Fig. 4D), and upregulation of the anti-apoptotic Bcl-2 protein and downregulation of the apoptotic BAX protein (Fig. 4E), particularly when the AGR2vH-overexpressing cell were under ER stress.

**Effects of AGR2vH on cell survival.** The mRNA expression of AGR2vH was upregulated in AGR2vH-overexpressing cells under ER stress-inducing conditions, and downregulated in AGR2vH-overexpressing cells with AGR2vH depletion (Fig. 5A). The expression of AGR2vH was directly associated with the survival of cCA cells, which was determined using the CCK-8 assay. Cell survival was significantly increased with AGR2vH overexpression in cells under ER stress-inducing condition when compared with empty vector-transfected cells (P<0.01), while the cell viability of AGR2vH-overexpressing cells with AGR2vH depletion under ER stress-inducing conditions was significantly decreased when compared with AGR2vH-overexpressing cells (P<0.001; Fig. 5B).

**Discussion**

AGR2vH, produced from the aberrant splicing of AGR2, promotes the metastatic phenotype of cCA cells. Of note, only AGR2vH is predicted to be translatable into a 67-amino acid protein isoform (17). In addition, AGR2vH was revealed to contribute to the migration and invasion of cCA cells (17,18). The dimerization of AGR2 is required to activate the UPR pathway by interaction with BiP/GRP78 for the recovery of cellular ER stress and the increase of cancer cell survival (10). Prospectively, AGR2vH may serve as an alternative partner molecule, which may readily interact with BiP/GRP78 to activate the UPR pathway when ER stress occurs in cancer cells.

For the verification of ER stress, the BiP/GRP78 protein chaperone, which activates the UPR pathway, was upregulated. In addition, XBP1 was spliced, which removes a 26-nucleotide intron from XBP1 mRNA, into XBP1s, resulting in the increased expression of the chaperon proteins (29). In the present study, BiP/GRP78 and XBP1s were upregulated. In addition, AGR2 was demonstrated to be upregulated under ER stress conditions to facilitate protein
folding in the cells (30). In the present study, AGR2vH expression was induced. The activation of the UPR pathway under ER stress conditions is based on three ER transmembrane receptors, namely IRE1, ATF6 and PERK (31). The present study investigated the expression of unspliced (XBP1u)/XBP1s downstream of IRE1. AGR2vH-overexpressing cells exhibited XBP1u downregulation and XBP1s upregulation, which induces the expression of genes involved in restoring protein folding, including BiP/GRP78 and PDI5 (12). A previous study demonstrated that the expression of eIF2α, a downstream target of PERK, was upregulated following tunicamycin treatment (22). The present study reported that eIF2α mRNA and activated p-eIF2α protein were upregulated under conditions of ER stress by 2 µg/ml tunicamycin, and were upregulated in AGR2vH-overexpressing cells. Furthermore, in the present study, the expression of GPR94, a downstream target of ATF6 (32), was upregulated in association with the increased expression of ATF6.

In addition, the expression of cHOP, a molecule involved in ER stress-induced apoptosis, is low under non-stress conditions but increases under ER stress conditions (33). In the present study, the expression of cHOP in cCA cells was upregulated under ER stress-inducing conditions, but was downregulated in AGR2vH-overexpressing cells. Of note, the present study also demonstrated changes in the expression of anti-apoptotic and apoptotic proteins, and revealed that the expression of AGR2vH decreased cancer cell apoptosis under ER stress-inducing conditions. (A) Comparative determination of apoptotic cells among AGR2vH-overexpressing cells. (B) Quantitative analysis of the total apoptotic cells among AGR2vH-overexpressing cells. (C) Detection of caspase 3/7 activity in AGR2vH-overexpressing cells. (D) mRNA expression of CHOP, an ER stress-induced apoptosis gene. (E) Expression of anti-apoptotic protein Bcl-2 and apoptotic protein BAX. The data in B and C are presented as the mean ± standard deviation. **P<0.01 and ***P<0.001 with comparisons shown by lines. AGR2vH, Anterior gradient-2 spliced variant H; ER, endoplasmic reticulum; CHOP, C/EBP homologous protein; Bcl-2, B-cell lymphoma 2; BAX, Bcl-2-associated X protein; bp, base pair; NC, negative control.

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In addition, the expression of CHOP, a molecule involved in ER stress-induced apoptosis, is low under non-stress conditions but increases under ER stress conditions (33). In the present study, the expression of CHOP in cCA cells was upregulated under ER stress-inducing conditions, but was downregulated in AGR2vH-overexpressing cells. Of note, the present study also demonstrated changes in the expression of anti-apoptotic and apoptotic proteins, and revealed that the expression of AGR2vH expression was induced.

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Bcl-2 and BAX were associated with the survivability of CCA cells with AGR2vH overexpression and knockdown.

In conclusion, the upregulation of AGR2vH activated the UPR pathway and the expression of UPR downstream markers, decreasing cell apoptosis via decreased caspase-3/7 activity and contributing to the survival of CCA cells, particularly under ER stress-inducing conditions. These results support the potential application of this molecule as an alternative therapeutic target for CCA.

Acknowledgements

Not applicable.

Funding

The present study was supported by The Naresuan University Research Scholarship for Graduate Students, the Thailand Research Fund; grant no. TRF-MRG6080014) and the Thailand Research Fund and Office of the Higher Education Commission (grant no. TRF-MRG6080014).

Availability of data and materials

The datasets used and/or analyze during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WK and SP designed the experiments. GS and JY performed the experiments. GS and WK contributed to data acquisition and wrote the manuscript. SW contributed to project design and edited the manuscript. SJ made substantial contributions to conception and design, and revised the manuscript for important intellectual content. All authors have read and approved the manuscript, and agree to be accountable for all aspects of the research in ensuring that the accuracy and integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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