รายงานวิจัยฉบับสมบูรณ์

โครงการ **ไอโซฟอร์มของโปรตีนแอนทีเรียกราเดียนท์ทูที่เกิดจากการสไปล์ซิ่งส่งเสริมกระบวนการเกิดมะเร็งและการดำเนินโรคของมะเร็งท่อน้ำดี** (MRG6080014)

โดย **ผู้ช่วยศาสตราจารย์ ดร.วรศักดิ์ แก้วก่อง**

มีนาคม 2562

สัญญาเลขที่ **MRG6080014**

รายงานวิจัยฉบับสมบูรณ์

โครงการ ไอโซฟอร์มของโปรตีนแอนทีเรียกราเดียนท์ทูที่เกิดจากการสไปล์ซิ่งส่งเสริมกระบวนการเกิดมะเร็งและการดำเนินโรคของมะเร็งท่อน้ำดี

ผู้ช่วยศาสตราจารย์ ดร.วรศักดิ์ แก้วก่อง
คณะวิทยาศาสตร์การแพทย์ มหาวิทยาลัยนเรศวร

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและต้นสังกัด

บทคัดย่อ

รหัสโครงการ: MRG6080014

ชื่อโครงการ: ไอโซฟอร์มของโปรตีนแอนทีเรียกราเดียนท์ทูที่เกิดจากการสไปล์ซิ่งส่งเสริมกระบวนการเกิดมะเร็งและการดำเนินโรคของมะเร็งท่อน้ำดี

ชื่อนักวิจัย: ผู้ช่วยศาสตราจารย์ ดร.วรศักดิ์ แก้วก่อง

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ระยะเวลาโครงการ: 2 ปี (4 เมษายน 2560 - 3 มีนาคม 2562)

มะเร็งท่อน้ำดีเป็นมะเร็งที่มีอุบัติการณ์และอัตราการตายสูงในประเทศไทย สาเหตุที่นำไปสู่ความรุนแรง คือ การแพร่กระจายของเซลล์มะเร็งไปยังอวัยวะต่างๆ จากที่มีการศึกษายีนที่สัมพันธ์กับการแพร่กระจายของมะเร็ง 77 ยีน พบว่า AGR2 มีการแสดงออกสูงสุดในเซลล์มะเร็งท่อน้ำดี ผู้วิจัยพบ splicing isoform ของ AGR2 ในรูปแบบของ AGR2vH แล้วนำมาศึกษาบทบาทต่อการแพร่กระจายของมะเร็งท่อน้ำดี รวมถึงกลไกต่อความรุนแรงของเซลล์มะเร็ง โดยการยับยั้ง (siRNA) และเพิ่ม (Overexpression) การแสดงออกของ AGR2vH ในเซลล์มะเร็ง แล้วติดตามคุณสมบัติการเจริญแบ่งตัว การเคลื่อนที่ การบุกรุกเนื้อเยื่อ การยึดเกาะ และการเปลี่ยนแปลงรูปร่างของเซลล์ พบว่าคุณสมบัติเหล่านี้แปรผันตามการแสดงออกของ AGR2vH ที่ทำการทดลอง (***Yosudjai J, et al. 2018 in Biomedicine & Pharmacotherapy***) และยังพบว่า เมื่อเหนี่ยวนำให้เซลล์มะเร็งเข้าสู่สภาวะเครียดของเอนโดพลาสมิกเรติคูลัม การเพิ่มการแสดงออกของ AGR2vH เข้าไป ทำให้เซลล์มะเร็งมีการกระตุ้น Unfolded Protein Response Pathway ทำให้มีอัตราการรอดชีวิตสูงขึ้นและเข้าสู่กระบวนการตายน้อยลง (***Submitted manuscript to Cell Stress & Chaperone, January 28, 2019***) ระหว่างดำเนินงานวิจัย ผู้วิจัยได้ประมวลองค์ความรู้เป็นบทความทบททวนวรรณกรรม เรื่อง ความผิดปกติที่เกิดขึ้นหลังการถอดรหัสของยีนสามารถสังเคราะห์โปรตีนไอโซฟอร์มที่มีเกี่ยวข้องกับการพัฒนาของมะเร็งท่อน้ำดี (***Yosudjai J, et al. 2019 in Biomedical Reports***) ทั้งนี้ ระยะเวลาของโครงการวิจัยได้สิ้นสุดลง แต่ผู้วิจัยยังค้นคว้าบทบาทหน้าที่ของโมเลกุลนี้ต่อไป ภายใต้การสนับสนุนของที่ปรึกษาโครงการวิจัย ดร.ศิวนนท์ จิรวัฒโนทัย (คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล) โดยความร่วมมือกับ ดร.สิทธิรักษ์ รอยตระกูล (สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยี (สวทช.)) และ Professor Dr. Jeeyun Lee (Samsung Medical Center, Samsung Hospital, South Korea)

คำหลัก: แอนทีเรียกราเดียนท์ทู สไปล์ซิ่ง มะเร็งท่อน้ำดี

Abstract

Project Code: MRG6080014

Project Title: Alternative splicing isoform of anterior gradient 2 promotes tumorigenesis and contributes to cholangiocarcinoma progression

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Project Period: 2 years (4 April 2017 – 3 March 2019)

Cholangiocarcinoma (CCA) is a cancer of bile duct, considered to be an incurable and lethal cancer. High mortality rate of CCA patients is underlined by cancer metastasis, an ability of the cancer cells that spread to secondary organs. Here, we found that upregulation of AGR2 in metastatic CCA cells coincides with an aberrant splicing of AGR2 mRNA, and that isoforms of AGR2 RNA, such as AGR2vH are specific to the metastatic cells. We demonstrated that the AGR2vH isoform enables metastatic-associated phenotypes in CCA cells. Depletion of AGR2vH by siRNA in metastatic KKU-213L5 cell results in significant reduction of cancer cell migration and invasion whereas overexpression of AGRvH in non-metastatic KKU-213 cells promotes cancer cell migration, invasion, adhesion, and proliferation (***Yosudjai J, et al. 2018 in Biomedicine & Pharmacotherapy***). In addition, we aimed to determine the roles of AGR2vH on UPR pathway activation to support cancer cell survivability and to evade apoptosis. After experimentally induced ER stress into AGR2vH-overexpressing CCA cell, UPR pathway was activated and can reduced the number of apoptotic cells, by decreased caspase-3/7 activity and resulting in higher number of viable cells. These present results support our previous data that an oncogenic AGR2vH isoform not only promote metastasis-associated phenotypes, but also helps CCA cells to survive and evade apoptosis for persisting and progression of cancer (***Submitted manuscript to Cell Stress & Chaperone, January 28, 2019***). Furthermore, we summarized the aberrant splicing of genes and the functional contributions of the spliced genes, in the carcinogenesis, progression and aggressiveness of cholangiocarcinoma, and factors that influence this aberrant splicing that may be relevant as therapeutic targets or prognosis markers for cholangiocarcinoma. (***Yosudjai J, et al. 2019 in Biomedical Reports***)

Keywords: Anterior Gradient 2, Splicing, Cholangiocarcinoma

Executive Summary

(เนื้อหางานวิจัย)

ความสำคัญ / ความเป็นมา

มะเร็งท่อน้ำดี เป็นมะเร็งที่มีอุบัติการณ์และอัตราการตายของผู้ป่วยที่สูงในประเทศไทย สาเหตุสำคัญที่นำไปสู่ความรุนแรงของการดำเนินโรคคือ ความสามารถในการแพร่กระจายของเซลล์มะเร็งท่อน้ำดีไปยังอวัยวะต่างๆ เช่น ตับ ปอด ต่อมน้ำเหลือง และกระดูก เป็นต้น

ก่อนหน้านี้ ทางกลุ่มวิจัยของ ศ.ดร.โสพิศ วงศ์คำ ได้ศึกษายีนที่มีความสัมพันธ์กับการแพร่กระจายของมะเร็งท่อน้ำดี ในระดับเซลล์เพาะเลี้ยง โดยทำการจำลองเซลล์ให้มีศักยภาพในการแพร่กระจายที่สูงในสัตว์ทดลอง โดยใช้เซลล์มะเร็งท่อน้ำดีจากผู้ป่วยฉีดเข้าที่หางหนู ให้มีการเคลื่อนที่ไปที่ปอด แล้วเก็บเซลล์มาทำซ้ำจนครบ 5 รอบ แล้วศึกษาเปรียบเทียบระดับการแสดงออกของยีนที่เคยมีรายงานว่ามีความสัมพันธ์กับการแพร่กระจายของมะเร็งชนิดต่างๆ กว่า 77 ยีน พบว่ายีน AGR2 เป็นยีนที่มีการแสดงออกสูงเป็นอันดับ 1 ในเซลล์มะเร็งท่อน้ำดีที่มีการแพร่กระจายสูงเมื่อเปรียบเทียบกับเซลล์ท่อน้ำดีจากผู้ป่วย

ผู้วิจัยทำการตรวจยืนยันและพบว่ามีการตัดแต่งของ mRNA ที่สังเคราะห์ได้ในเซลล์มะเร็งท่อน้ำดีที่มีการแพร่กระจายสูงนอกเหนือจาก AGR2 คือ splicing isoform ของ AGR2 ในรูปแบบของ AGR2vH ผู้วิจัยจึงสนใจศึกษาบทบาทของ AGR2vH ต่อการมีคุณสมบัติเป็น Oncogenic isoform ของยีน AGR2 และสัมพันธ์กับการแพร่กระจายของมะเร็งท่อน้ำดี

วัตถุประสงค์ของโครงการ

1. ศึกษาแสดงออกของ AGR2 splicing isoforms ต่างๆ คัดเลือก Splicing isoform (AGR2vH) ที่สนใจแล้วทำการศึกษาบทบาทต่อการแพร่กระจายของมะเร็งท่อน้ำดี
2. ศึกษากลไกทางชีววิทยาโมเลกุลของ AGR2vH ต่อกระบวนการเกิดมะเร็งและการดำเนินโรคของมะเร็งท่อน้ำดีในเซลล์เพาะเลี้ยงและในสัตว์ทดลอง

ผลการวิจัย (สั้น ๆ ที่บ่งชี้ประเด็นข้อค้นพบ กระบวนการ ผลผลิต และการเรียนรู้)

ผู้วิจัยพบการแสดงออกของ AGR2vH สูงขึ้น และเป็น isoform ที่โดดเด่นของ AGR2 ในเซลล์มะเร็งท่อน้ำดีที่มีการแพร่กระจายสูงและรายงานบทบาทของ AGR2vH โดยทำการยับยั้งการแสดงออกอย่างจำเพาะด้วย siRNA ในเซลล์มะเร็งท่อน้ำดีที่มีการแพร่กระจายสูงและเพิ่มการแสดงออกของ AGR2vH ในเซลล์มะเร็งท่อน้ำดีตั้งต้น หลังจากติดตามคุณสมบัติการเจริญแบ่งตัว (proliferation) การเคลื่อนที่ (migration) การบุกรุกเนื้อเยื่อ (invasion) การยึดเกาะ (adhesion) และกลไกของการ

เปลี่ยนแปลงรูปร่างของเซลล์ (Epithelial-Mesenchymal Transition) พบว่า เมื่อยับยั้งการแสดงออกของ AGR2vH ทำให้เซลล์มะเร็งท่อน้ำดีที่มีการแพร่กระจายสูงมีคุณสมบัติเหล่านี้ลดลง ในขณะที่เมื่อเพิ่มการแสดงออกของ AGR2vH ในเซลล์มะเร็งท่อน้ำดีตั้งต้นก็พบว่ามีคุณสมบัติเหล่านี้รุนแรงมากขึ้น โดยผ่านการทำงานของโมเลกุลร่วมคือ Vimentin (ตีพิมพ์เผยแพร่ในวารสารวาการระดับนานาชาติ Yosudjai J, Inpad C, Chomwong S, Dana P, Sawanyawisuth K, Phimsen S, Wongkham S, Jirawatnotai S, Kaewkong W. An aberrantly spliced isoform of anterior gradient-2, AGR2vH promotes migration and invasion of cholangiocarcinoma cell. Biomed Pharmacother. 2018; 107(2018): 109-16.)

ต่อมาผู้วิจัยพบว่า เมื่อเหนี่ยวนำให้เซลล์มะเร็งท่อน้ำดีเข้าสู่สภาวะเครียดของเอนโดพลาสมิกเรติคูลัม (ER-stress) การเพิ่มการแสดงออกเข้าไปของ AGR2vH จะทำให้เซลล์มะเร็งท่อน้ำดีสามารถกู้คืนสภาวะเครียดนี้ได้ ผ่านการกระตุ้น Unfolded Protein Response (UPR) Pathway ทำให้เซลล์มะเร็งท่อน้ำดีมีอัตราการรอดชีวิตสูงขึ้น เข้าสู่กระบวนการตายแบบอะพอพโตสิสน้อยลง (ผลงานวิจัยอยู่ระหว่างการประเมินของวารสารวิชาการระดับนานาชาติ Cell Stress Chaperone โดยยื่นผลงานวิจัยไปเมื่อวันที่ 28 มกราคม 2562)

อย่างไรก็ตาม ระหว่างที่ดำเนินงานวิจัย กลุ่มผู้วิจัยได้รวบรวมข้อมูลที่เกี่ยวข้องและประมวลองค์ความรู้ได้สำเร็จในรูปของบทความทบททวนวรรณกรรม (Review article) ในเรื่อง ความผิดปกติที่เกิดขึ้นภายหลังกระบวนการถอดรหัสของยีนสามารถสังเคราะห์โปรตีนไอโซฟอร์มที่มีคุณสมบัติเกี่ยวข้องกับการพัฒนาและดำเนินโรคของมะเร็งท่อน้ำดี (ตีพิมพ์เผยแพร่ในวารสารวาการระดับนานาชาติ Yosudjai J, Wongkham S, Jirawatnotai S, Kaewkong W. Aberrant mRNA splicing generates oncogenic RNA isoforms and contributes to the development and progression of cholangiocarcinoma. Biomed Report. 2019; 10(2019): 147-155.)

ทั้งนี้ ระยะเวลาของโครงการวิจัยได้สิ้นสุดลง แต่ผู้วิจัยยังค้นคว้าบทบาทหน้าที่ของโมเลกุลนี้ต่อไป โดยภายใต้การสนับสนุนของที่ปรึกษาโครงการวิจัย ดร.ศิวนนท์ จิรวัฒโนทัย (ภาควิชาเภสัชวิทยา คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล) และความร่วมมือกับ ดร.สิทธิรักษ์ รอยตระกูล (สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยี (สวทช.)) รวมไปถึง Professor Dr. Jeeyun Lee (Samsung Medical Center, Samsung Hospital, South Korea) ต่อไป

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว. (เอกสารแนบ)

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

Yosudjai J, Inpad C, Chomwong S, Dana P, Sawanyawisuth K, Phimsen S, Wongkham S, Jirawatnotai S**, Kaewkong W**. An aberrantly spliced isoform of anterior gradient-2 , AGR2 vH promotes migration and invasion of cholangiocarcinoma cell. Biomed Pharmacother. 2018; 107(2018): 109-16.

Yosudjai J, Wongkham S, Jirawatnotai S, **Kaewkong W**. Aberrant mRNA splicing generates oncogenic RNA isoforms and contributes to the development and progression of cholangiocarcinoma. Biomed Report. 2019; 10(2019): 147-155.

Suwanmanee G, Yosudjai J, Phimsen S, Wongkham S, Jirawatnotai S, **Kaewkong W**. Upregulation of AGR2 vH facilitates cholangiocarcinoma cell survival under endoplasmic reticulum stress via activation of the unfolded protein response pathway. (Under review by Cell Stress Chaperone; Submitted 28 January 2019)

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

2. การนำผลงานวิจัยไปใช้ประโยชน์

การนำผลงานวิจัยไปใช้ประโยชน์เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

- ผลิตนิสิตบัณฑิตศึกษา ภาควิชาชีวเคมี คณะวิทยาศาสตร์การแพทย์ มหาวิทยาลัยนเรศวร (จุฑามาศ โยสุดใจ (ป.เอก-ชีวเคมี), จตุรงค์ อินผัด (ป.โท-ชีวเคมี) และ กันธิชา สุวรรณมณี (ป.โท-ชีวเคมี))
- ดำเนินงานวิจัยต่อยอด ร่วมกับ ดร.สิทธิรักษ์ รอยตระกูล สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยี (สวทช.) และ Professor Dr. Jeeyun Lee, Samsung Medical Center, Samsung Hospital, South Korea
- ตีพิมพ์ในวารสารระดับนานาชาติแล้ว จำนวน 2 บทความ (และอีก 1 บทความอยู่ระหว่างการประเมินโดยวารสาร (Under review)) ไปเป็นประโยชน์ด้านวิชาการ นำไปสู่กระบวนการเรียนการสอน เป็นต้นแบบของการศึกษา

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

3. อื่น ๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

- การนำเสนอผลงาน (โปสเตอร์) “An aberrantly spliced isoform of anterior gradient-2, AGR2 vH promotes migration and invasion of cholangiocarcinoma cell” ใน งานประชุมวิชาการ สำนักงานกองทุนสนับสนุนงานวิจัย (สกว.): นักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส (TOAC2019) วันที่ 9-11 มกราคม 2562 จังหวัดประจวบคีรีขันธ์ ประเทศไทย
- การนำเสนอผลงาน (โปสเตอร์) “AGR2 vH retrieves AGR2 from AGR2 dimer under tunicamycin-induced ER stress of cholangiocarcinoma cell” ใน The 6 th Conference on Biochemistry and Molecular Biology (BMB2018) วันที่ 20-22 มิถุนายน 2561 จังหวัดระยอง ประเทศไทย
- การนำเสนอผลงาน (โปสเตอร์) “AGR2vH, aberrantly spliced isoform of Anterior gradient-2 promotes metastasis and diminishes AGR2 homodimer in cholangiocarcinoma cell” ใน The 24th IUBMB Congress and 15th FAOBMB Congress วันที่ 4-8 มิถุนายน 2561 กรุงโซล ประเทศสาธารณรัฐเกาหลีใต้
- การนำเสนอผลงาน (บรรยาย) “Interruption of AGR2 dimerization by overexpression of AGR2 splicing isoform in cholangiocarcinoma cell” ใน Chulaborn Royal Academy International Conference on Innovation in Cancer Research and Care วันที่ 18-20 ธันวาคม 2560 สถาบันวิจัยจุฬาภรณ์ กรุงเทพมหานคร
- การนำเสนอผลงาน (โปสเตอร์) “Knockdown of anterior gradient 2 spliced transcript suppresses in vitro migration and invasion of high metastatic cholangiocarcinoma cells” ใน The 24th Asia Pacific Cancer Conference (APCC 2017) "Building the Asia Pacific Standard of Cancer Care" วันที่ 22-24 มิถุนายน 2560 กรุงโซล ประเทศสาธารณรัฐเกาหลีใต้

ภาคผนวก

ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

Yosudjai J, Inpad C, Chomwong S, Dana P, Sawanyawisuth K, Phimsen S, Wongkham S, Jirawatnotai S**, Kaewkong W**. An aberrantly spliced isoform of anterior gradient-2 , AGR2 vH promotes migration and invasion of cholangiocarcinoma cell. Biomed Pharmacother. 2018; 107(2018): 109-16.

Yosudjai J, Wongkham S, Jirawatnotai S, **Kaewkong W**. Aberrant mRNA splicing generates oncogenic RNA isoforms and contributes to the development and progression of cholangiocarcinoma. Biomed Report. 2019; 10(2019): 147-155.

Suwanmanee G, Yosudjai J, Phimsen S, Wongkham S, Jirawatnotai S, **Kaewkong W**. Upregulation of AGR2 vH facilitates cholangiocarcinoma cell survival under endoplasmic reticulum stress via activation of the unfolded protein response pathway. (Under review by Cell Stress Chaperone; Submitted 28 January 2019)

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An aberrantly spliced isoform of anterior gradient-2, AGR2vH promotes migration and invasion of cholangiocarcinoma cell

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ABSTRACT

Cholangiocarcinoma (CCA) is a cancer of bile duct, considered to be an incurable and lethal cancer. High mortality rate of CCA patients is underlined by cancer metastasis, an ability of the cancer cells that spread to secondary organs. Recently, we have identified Anterior Gradient-2 (AGR2), from a pair of non-metastatic/metastatic cell lines (KKU-213/KKU-213L5), as a gene that is highly and specifically upregulated in the metastatic cell line. AGR2 encodes for a disulfide isomerase enzyme, ubiquitously detected in mucus-secreting tissues. Overexpression of AGR2 has been reported in several types of human cancer. Role of the overexpressed AGR2 in cancer is still unclear. Here, we found that upregulation of AGR2 in metastatic CCA cells coincides with an aberrant splicing of AGR2 mRNA, and that isoforms of AGR2 RNA, such as AGR2vE, AGR2vF, and AGR2vH are specific to the metastatic cells. We demonstrated that the AGR2vH isoform enables metastatic-associated phenotypes in CCA cells. Depletion of AGR2vH by an isoform-specific interfering RNA in metastatic KKU-213L5 cell results in significant reduction of cancer cell migration and invasion, and a slight decrease of cell adhesion. Overexpression of AGR2vH in non-metastatic KKU-213 cells promotes cancer cell migration, invasion, adhesion, and moderate cell proliferation. Moreover, we found that expression of a metastasis-associated gene, vimentin, positively correlates with expression of AGR2vH. Our results support the notion that aberrant alternative splicing of AGR2 facilitates an accumulation of the oncogenic AGR2vH isoform, in turn, contributes to the pathogenesis and severity of CCA.

1. Introduction

Cholangiocarcinoma (CCA) is a malignancy that arising within biliary tree. The worldwide records of CCA indicated a high incidence of the disease in Asian countries, especially in Southeast Asia. CCA in this region is associated with infection by liver fluke, *Opisthorchis viverrini* (Ov) [1,2]. Difficulty in the management of CCA is often attributed by invasive nature of the cancer, which spread very quickly to secondary vital organs [3–6]. A recent report presented an expression profile of 77 metastatic-associated genes significantly and specifically altered in metastatic cells, identified from a matching cell line pair of non-metastasis: metastasis CCA cell lines (KKU-213: KKU-213L5).

AGR2 was the top among the differentially upregulated genes identified [7]. Overexpression of AGR2 was shown to be positively associated with the higher tumor grade in intrahepatic mass-forming type CCA, but barely detected in normal bile duct. An independent report also showed that high AGR2 expressions correlated with more severed and mucin producing subtypes of hilar, extrahepatic, and a subset of intrahepatic CCA [8].

AGR2 was initiailly identified in *Xenopus laevis* as XAG-2, a protein regulating embryonic ectoderm development to cement gland [9]. The human ortholog AGR2 is a member of protein disulfide isomerases (PDIs) family, which involves in the protein modification and folding. The 13,304 bp of AGR2 gene on the seventh chromosome encodes for 996 bp of a 8-exons mRNA, which is translated into a protein with 175 amino acids. In normal tissues AGR2 is known to be involved with mucin secretion.

A number of studies presented a link between AGR2 and

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carcinogenesis. AGR2 is upregulated in several types of cancer, such as cancer of liver, pancreas, stomach, colon, urinary bladder, prostate, breast, female reproductive organs, and respiratory system [10]. Forced overexpression of AGR2 promoted survival and proliferation of pancreatic cancer cells [11], whereas downregulation of AGR2 contributed to reduced cell cycle progression and increased cell death in esophageal cancer [12]. AGR2 was also shown to promote *in vitro* migration of breast epithelial cells [13]. These results implicated AGR2 as an authentic oncogene and that AGR2 may be a good target for cancer therapy.

Emerging evidence has demonstrated significant contribution of aberrant alternative gene splicing in carcinogenesis [14]. Sequence analyses of AGR2 indicated that AGR2 can be spliced and expressed in several isoforms. Alternative splicing of AGR2 gene was detected in prostate cancer, in which 6 spliced transcripts were reported, including AGR2wt, AGR2vC, AGR2vE, AGR2vF, AGR2vG and AGR2vH. AGR2vG and AGR2vH were associated with disease prognosis and suggests to be used as specific cancer prognostic biomarkers in urine specimens [15].

In this study, we set forth to explore the splicing isoforms of overexpressed AGR2 in metastatic cell line models for CCA, and to investigate the contribution of a relevant AGR2 isoform in proliferation, migration, invasion, and adhesion phenotypes by overexpression and depletion of AGR2 expression in non-metastatic and highly metastatic CCA cells, respectively.

2. Materials and methods

2.1. Cell lines

Immortalized cholangiocytes (MMNK-1), and CCA cell lines (KKU-055, KKU-100, KKU-213 and KKU-214) were obtained from Japanese Collection of Research Bioresources (JCRB) Cell Bank. The previously established metastatic CCA cell lines (KKU-213L5 and KKU-214L5) [7,19] were provided from Cholangiocarcinoma Research Institute, Faculty of Medicine, Khon Kaen University. The cell lines were cultured in Dulbecco's Modified Eagle's Medium (Gibco, Thermo Fisher Scientific, Waltman, MA) supplemented with 10% v/v fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltman, MA), 100 Unit/ml of penicillin and 100 µg/ml of streptomycin (Gibco, Thermo Fisher Scientific, Waltman, MA), and maintained at 37 °C in a humidified, 5% CO_2 atmosphere.

2.2. Preparation of RNA and reverse transcription

The total RNA was isolated from all cells using E.Z.N.A.® Total RNA Kit I (OMEGA bio-tek, Doraville, Georgia, USA) according to the manufacturer's protocols. One microgram of total RNA was used to generate cDNA using HisenScriptTM RH[-]RT PreMix Kit (Intron Biotech, Seoul, South Korea) according to the manufacturer's instructions. All cDNA samples were stored in −80 °C until use.

2.3. Polymerase chain reaction (PCR)

PCR reactions were performed under the optimized condition. The reaction mixture contained 0.2 µg of cDNA template, 0.4 µM of each forward and reverse primers in a total volume of 20 µl of 1 × MyTaqTM HS Red Mix (Bioline, Taunton, Massachusetts). A house-keeping gene β-actin was used as an internal control for semi-quantitative normalization. PCR products were analyzed by 2% agarose gel electrophoresis, detected by ImageQuant™ LAS 500 (GE Healthcare Life Sciences, Little Chalfont, UK), and quantitated using ImageQuantTL 7.0 software.

2.4. Quantitative real-time PCR

Quantitative real-time PCR was performed for evaluating the efficiency of siAGR2vH knockdown and the expression level of AGR2vH in AGR2vH-overexpressing cell under the optimized conditions. Reaction mixture (10 µl) contained cDNA template, forward and reverse primers and 1X LightCycler® 480 SYBR Green I Master (Roche Applied Science, Mannheim, Germany). All reactions were prepared in biological triplicate and analyzed using the LightCycler® 480 systems (Roche Applied Science, Mannheim, Germany). The expression levels of the target genes were normalized with reference to β-actin based on the relative quantification formula of $2^{-\Delta\Delta Ct}$ [16].

2.5. Sequencing of AGR2 wild-type and AGR2vH transcripts

Amplified PCR products of AGR2wt and AGR2vH cDNAs from KKU-213L5 cells were separated in 1% agarose gel electrophoresis. The specific bands were isolated and purified using Hiyield™ Gel/PCR DNA fragments Extraction Kit (Applied Biosystems, CA). Concentrations and qualities of the purified-PCR products were determined by the 3500 Genetic Analyzer (Applied Biosystem, Hitachi, Japan). The sequencing results were analyzed using BioEdit software, and amino acid sequences were predicted by ExPASy translate tool (https://web.expasy.org/translate/).

2.6. Depletion of AGR2vH by small interfering RNA

Transfections of siAGR2vH (antisense: 5′-UUGAGAGCUUUCUUCA UAUGUCUG-3′) and a negative control siRNA (Ambion, Thermo Fisher Scientific, Waltman, MA) were performed using Lipofectamine™ 2000 (Thermo Fisher Scientific, Waltman, MA). Briefly, KKU-213L5 cells were seeded into 6-well plate for 25,000 cells/well, and cultured until reaching 80% confluency. Then, cells were transfected with 75 nmol of siAGR2vH, or negative control siRNA in Opti-MEM I reduced serum medium (Gibco, Thermo Fisher Scientific, Waltman, MA) and incubated for 6 h. The media was removed and replaced with fresh complete media. The untransfected cells were cultured in complete media. At 24, 48 and 72 h after transfection, the efficiency of siRNA knockdown was evaluated by RT-PCR and qPCR.

2.7. Establishment of stable AGR2vH-overexpression cell lines and single clone selection

The purified PCR product of AGR2vH was inserted into the PCR 2.1 TOPO cloning vector (Invitrogen, Thermo Fisher Scientific, Waltman, MA), and transformed to the Super HIT-DH5α competent cells (RBC Bioscience, Singapore). After 16 h incubation, white colonies were selected for propagation in sterile LB broth with 100 µg/mL of ampicillin. Positive recombinant plasmids were extracted and verified by *Hind*III and *Eco*RI (Thermo Fisher Scientific, Waltman, MA) digestion before subcloned into p3XFLAG-CMV-14 expression vector (Sigma-Aldrich, St. Louis, MO). Either pCMV14-AGR2vH or pCMV14-Empty vector was transfected into KKU-213 cells by Lipofectamine 2000 and cultured in complete medium for 48 h. The single clones of AGR2vH-overexpressing cells were selected by 2 mg/ml of Geneticin G418 (Thermo Fisher Scientific, Waltman, MA) and subjected for expansion, until sufficient cell numbers were obtained.

2.8. Cell proliferation assay

Six groups of cells were assigned, including, 3 of KKU-213L5 cells (untransfected-, control siRNA and siAGR2vH transfected-cells) and 3 of KKU-213 cells (wild-type control, KKU-213 containing the pCMV-Empty, and AGR2vH overexpressing cells). Cell proliferations were determined using 3-[4,5-dimethylthiazole]-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described [17].

2.9. Cell migration assay

The migration ability of the cells was tested by the wound healing

assay, according to the protocol described previously [18]. The results were monitored at 0, 12 and 24 h for imaging, and calculated for the relative migrating distances.

2.10. Cell invasion assay

Cell invasion was performed using Boyden chamber assay. Polycarbonate membranes transwell inserts with 8 μm-pore size were coated by 0.4 mg/ml Matrigel matrix (Corning Inc., Corning, NY) in upper chamber. The 600 μl of complete media was added in lower chamber. Twenty thousand cells were used in the AGR2vH-depletion groups, and 50,000 cells were used in the AGR2vH-overexpression groups, for seeding into the upper chambers with 100 μl of serum free media. The cells were allowed to invade for 16 h. Invading cells were fixed with 4% paraformaldehyde and stained with 4% crystal violet before they were imaged and counted.

2.11. Cell adhesion assay

For cell-matrix adhesion assay, each well of 96-well plates was coated with 0.4 μg/μl Matrigel matrix and incubated overnight at 37 °C with 5% CO_2 before blocked by 3% bovine serum albumin (BSA) for 2 h. The 20,000 cells of each groups were plated and allowed to adhere. After 1 h incubation, unadhered cells were removed and adhered cells were quantified by MTT assay.

2.12. Protein extraction and Western blot analysis

Protein preparation from cell lysate, SDS-polyacrylamide gel electrophoresis and Western blotting were performed as previously described [19]. Antibodies against E-cadherin (24E10), Claudin-1 (D5H1D), Vimentin (D21H3), Slug (C19G7), β-actin, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG were perchased from Cell Signaling Technology (Danvers, MA, USA).

2.13. Statistical analysis

Experiments were performed in biological triplicate. Data are presented as the mean ± standard deviation (SD). Unpaired *Student's t*-test (two tailed) was used for comparison between each group by SigmaPlot (SigmaPlot 11.0, Systat Software, San Jose, CA). *P* less than *0.05* were considered to be significant.

3. Results

3.1. Expression of AGR2 mRNA and splicing RNA isoforms

The semi-quantitative RT-PCR using AGR2 isoform-specific primers as described in Table 1 and Fig. 1A were performed. We detected differential expressions of AGR2 mRNA isoforms in the cell line models, consisting of a non-cancer cell line (MMNK-1), established CCA cell lines, KKU-055, KKU-100, KKU-213, and KKU-214, and the metatatic counterparts of KKU-213, KKU-214 (KKU-213L5, KKU-214L5). In Fig. 1B, MMNK-1 cells expressed very low level of any of the AGR2 isoforms. Full-length WT (AGR2wt), AGR2vC and AGR2vG isoforms were detected in KKU-213, KKU-214, KKU-213L5, and KKU-214L5. Our analysis did not detect expression of the AGR2vD isoform in any of the cell line tested. Expressions of AGR2vE, and AGR2vF isoforms were weakly detected in KKU-213, KKU-214, and KKU-214L5, but presented the high intensities in KKU-213L5. ARG2vH isoform was weakly detected in all of the cell lines, but was significantly upregulated in the metastatic cell lines, KKU-213L5, and KKU-214L5.

To identifiy the metastasis associated spliced-transcripts of AGR2, the expression of spliced-transcript in non *vs*. metastatic CCA cells were compared. AGR2vE, AGR2vF and AGR2vH were observed specifically upregulated in the metastatic KKU-213L5, but not in the non-metastatic counterpart KKU-213. Because, AGR2vH was consistently upregulated in both metastatic cell lines both KKU-213L5, and KKU-214L5, and the only AGR2vH mRNA sequence can be predictably translatable into a protein (while AGR2vE and AGR2vF can not) we selected AGR2vH for further investigations as a candidate transcript for its role in CCA metastasis.

3.2. Sequencing and analysis of AGR2vH mRNA sequence

The mRNA sequence of AGR2vH was determined. The result demonstrated that AGR2vH uses alternative splice sites within exon-3 and exon-8. Therefore the transcript's structure is skipping out of exon-4 to exon-7. Sequencing analysis that revealed the full sequence of AGR2vH, showed the intersection of nucleotides at the exon boundary (Fig. 2A). The predicted amino acid sequence of AGR2vH consisted of 67 amino acids as presented in Fig. 2B.

3.3. Selective depletion and overexpression of AGR2vH in cancer cells

Semi-quantitative RT-PCR were performed to evaluate the efficiency of siAGR2vH-mediated AGR2vH suppression, and AGR2vH overexpression, in the cell transfected with siAGR2vH and cells ectopically overexpressing AGR2vH, respectively. In Fig. 3A, the expression of AGR2vH was decreased after transfection of siAGR2vH in time-dependent manner, shown as reduced band intensity of the RT-PCR products. Remarkably, the expression of AGR2wt was not interfered. We found that AGR2vH expression was significantly increased in the cells stably containing pCMV14-AGR2vH. Moreover, qRT-PCR confirms the results from RT-PCR of both siRNA efficiency (Fig. 3B) and overexpression (Fig. 3C).

3.4. Effect of AGR2vH depletion and overexpression on CCA cell proliferation

Cell proliferation was monitored by MTT assays at 72, 96 and 120 h post siAGR2vH transfection. Analyses revealed that growth rates of untransfected, control siRNA- and siAGR2vH- transfected KKU-213L5

Table 1
Sequence of AGR2 splicing variants specific primers and internal control.

Name	Forward primer 5′ → 3′	Reverse primer 5′ → 3′
AGR2wt	CGACTCACACAAGGCAGGT	TCCACACTAGCCAGTCTTCTCA[a]
AGR2vC	CACAAGGCAGAGTTGCCATGG	
AGR2vE	ATCTGGTCACCCATCTCTGA	
AGR2vF	GGAAATCCAGACCCATCTCTG	
AGR2vG	AAGGCAGGTACAGCTCTG	
AGR2vH	CAGACATATGAAGAAAGCTCTCAAGT	
AGR2vD	GGTGGGTGAGGAAATCCAGCTTTA	AGATGGGTCAACAAACATAATCCTGG
β-actin	TCGTGCGTGACATTAAGGAG	GAAGGAAGGCTGGAAGAGTG

[a] Common reverse primer for amplification of AGR2wt, AGR2vC, AGR2vE, AGR2vF, AGR2vG and AGR2vH.

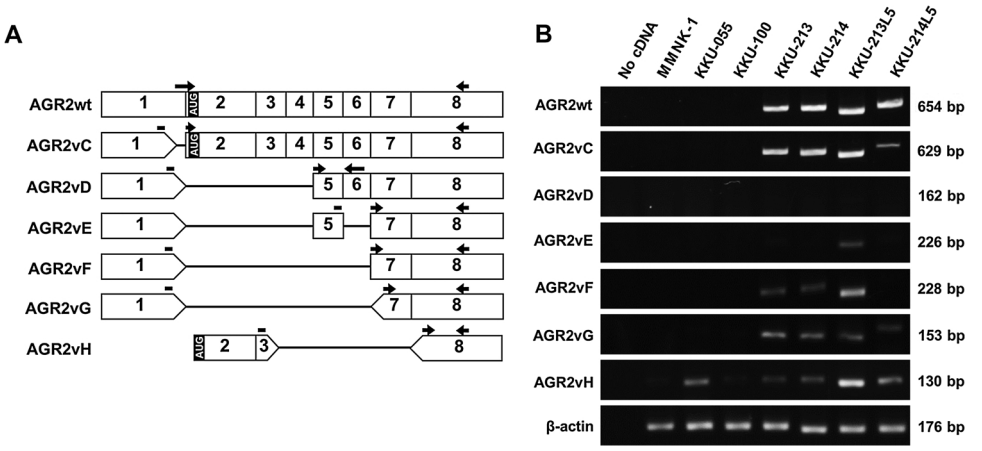

Fig. 1. AGR2 spliced transcripts and expressions in cholangiocyte MMNK-1, and CCA cell lines. (A) Schematic representation of AGR2 spliced transcripts and primers' designs/annealing sites (exons were presented as rectangles, partial of exons as pentagon and each forward-reverse primer pairs were presented as forward and backward arrows). (B) Band intensities presented the semi-quantitative of mRNA expression of AGR2 and its spliced transcripts in cholangiocyte, CCA cells and metastasis sublines by agarose gel electrophoresis.

were not significantly different (Fig. 4A). However, ectopic overexpression of AGR2vH promoted proliferative capacity of the cells. This was clearly observed at the 72 h timepoint, when compared to control cells ($P < 0.05$) (Fig. 4B).

3.5. Effect of AGR2vH depletion and overexpression on cell migration

Role of AGR2vH on cell migration was examined by the wound healing assay. In Fig. 5A, siAGR2vH suppressed the migrating capacity of the highly metastatic CCA cells. We observed that the wound of AGR2vH-depleted cells were still clearly remained at 24 h, when the wounds of untransfected and control siRNA-transfected KKU-213L5 cells were completely closed. On the other hand, overexpression of AGR2vH promotes migration of the non-metastatic CCA cells, as the wound areas of the AGR2vH overexpressing cells were nearly closed at 24 h, whereas that of the control cells were still clearly presented as large wound gaps. The significant differences of migrating capacity at 24 h ($P < 0.05$), analyzed as relative migrating distances were shown

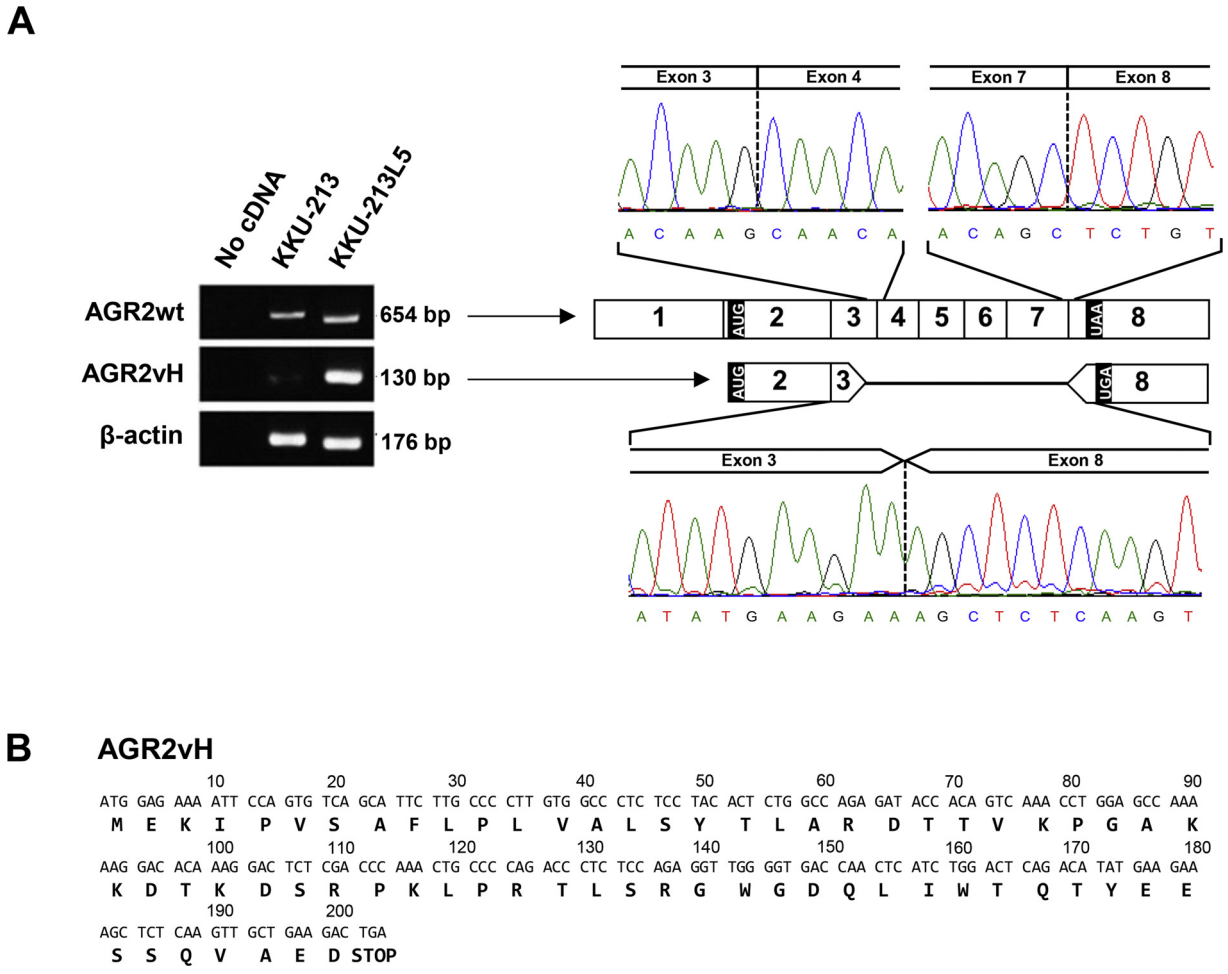

Fig. 2. Nucleotide sequence analyses of AGR2wt and AGR2vH. (A) The full-length AGR2wt mRNA contains the junction of exon-3 to exon-4, and exon-7 to exon-8, whereas AGR2vH mRNA presents the exon boundaries between exon-3 and exon-8. Exons were presented as rectangles and partial of exons as pentagon. (B) Predicted 67-amino acid AGR2vH protein translated from 204 bp of AGR2vH sequence.

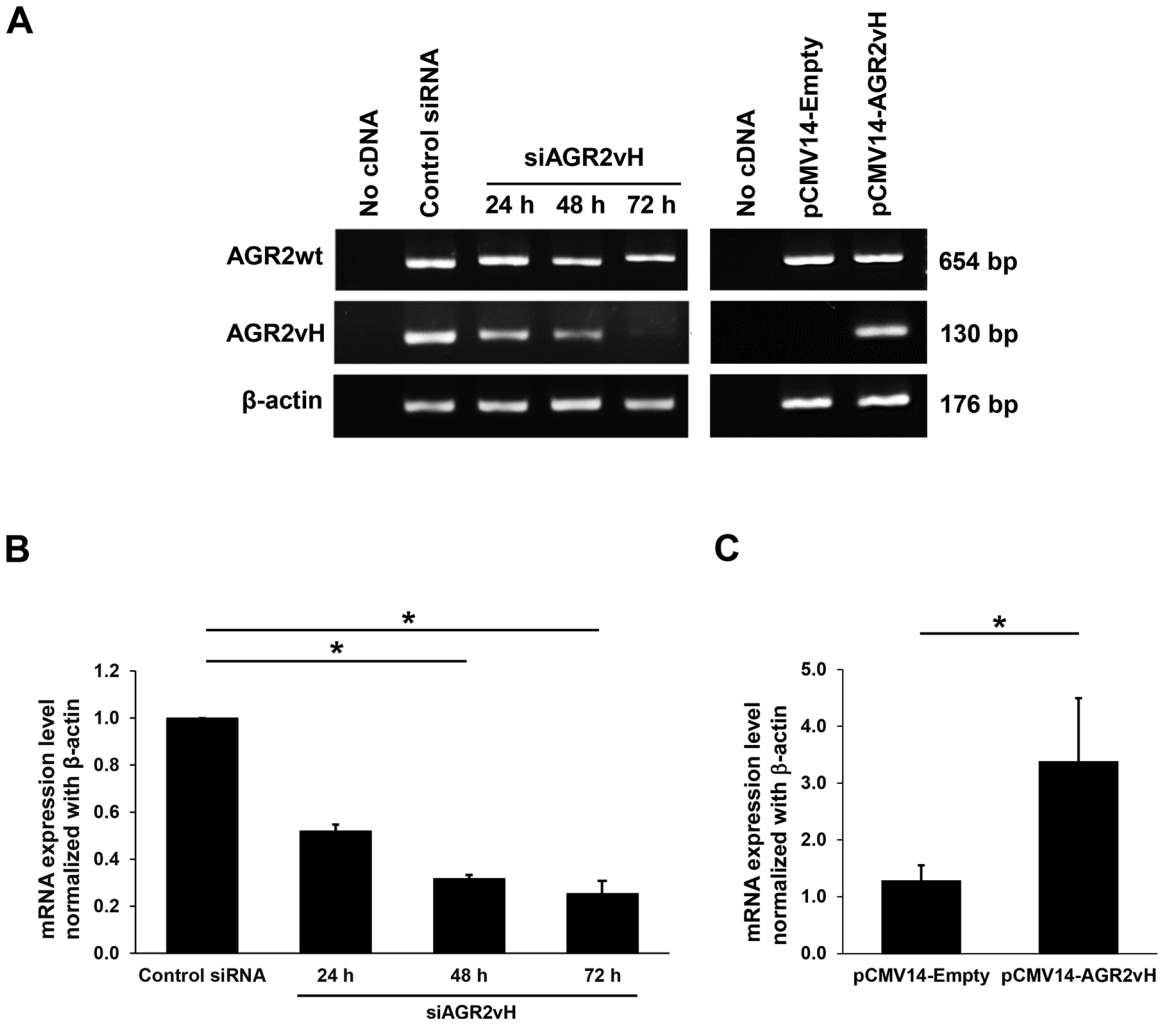

Fig. 3. mRNA expressions of AGR2vH in siAGR2vH-transfected cells, and AGR2vH-overexpressing cells. (A) Using RT-PCR, AGR2vH mRNA was markedly decreased in siAGR2vH-transfected cells, when compared to control (left panel). Increase of the AGR2vH was detected in the AGR2vH-overexpressing cells (right panel). (B and C) The quantitative real-time PCR confirmed the results from siAGR2vH-mediated knockdown, and AGR2vH overexpression in the cells, $*P < 0.05$.

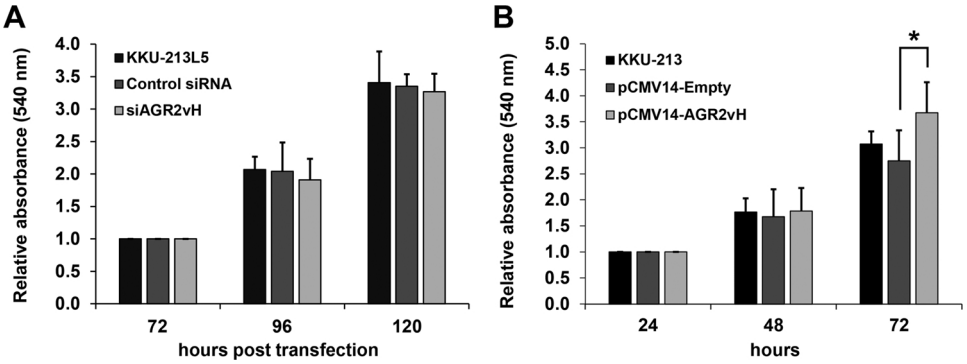

Fig. 4. Effect of AGR2vH on cell proliferation. (A) Reletive cell numbers of AGR2vH-depleted cells, compared to control siRNA transfected- and untransfected cells, measured by MTT assay. (B) Reletive cell numbers of AGR2vH-overexpressing cells compared to control cells, $*P < 0.05$.

in the line graphs. Therefore, expression levels of AGR2vH contributed to the CCA migration ability.

3.6. Effect of AGR2vH depletion and overexpression on cell invasion

Invasion capacity of the cancer cells were determined using Transwell invasion assay. Invading cells at the bottom of upper chamber of each group were captured and the relative invasive capacities (%) of the cell were calculated. In Fig. 5B, siAGR2vH significantly reduced an aggressive capability of the cancer cells to invade the artificial extracellular matrix, compared to untransfected and control siRNA-transfected KKU-213L5 ($P < 0.01$). On the other hand, AGR2vH overexpression significantly increased the invading capability of KKU-213, when compared to the control cells ($P < 0.05$). Therefore, the expression of AGR2vH is associated with the invasion ability of CCA cell.

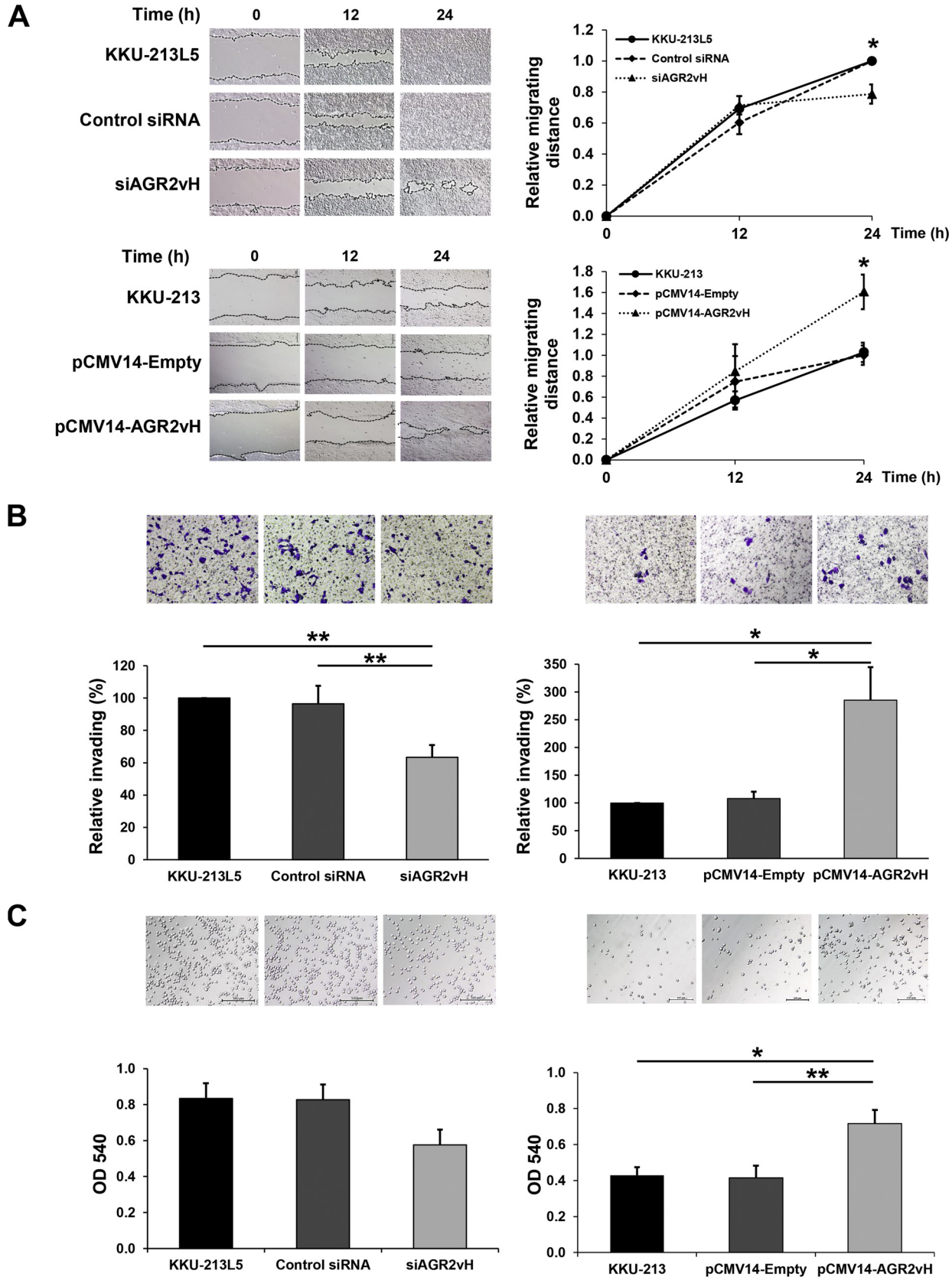

Fig. 5. Effect of AGR2vH on cell migration, invasion and adhesion. (A) The images of wound healing at 0, 12 and 24 h (10× magnification), and the migration activities were calculated as relative migrating distance. Upper panel; KKU-213L5 cells transfected with siARG2vH, or control siRNA, or untransfected KKU-213L5. Lower panel; ARG2vH-overexpressing KKU-213, KKU-213 containing empty vector, and the wildtype KKU-213 cells. (B) Staining of invading cells from Matrigel-coated layer into the bottle of Boyden chamber, and relative invading (%). (C) The adhesion activities were determined using cell-ECM adhesion assay. The adherent cells were analyzed under microscope and measured by MTT assay. All data are shown as mean ± SD of biological triplicate, $*P < 0.05$ and $**P < 0.01$.

3.7. Effect of AGR2vH depletion and overexpression on cell adhesion

To investigate the role of AGR2vH on cell adhesion, we determined the adhesion capacity using a cell-ECM adhesion assay. In Fig. 5C, transfection of KKU-213L5 with siAGR2vH slightly decreased the adhesion ability of the cells compared to the untransfected, or control siRNA-transfected cells, as analyzed under the light microscope, and MTT assay.

Number of the adhered cells of the AGR2vH-overexpressing KKU-213 cell was significantly increased, compared to the control cells ($P < 0.01$). Therefore, these results indicated that expression of AGR2vH regulates adhesion activity of KKU-213L5 cells.

3.8. Relationship of AGR2vH expression and expression of epithelial-to-mesenchymal transition markers

Transformation of the epithelial to mesenchyma cells is known to promote cell migration and metastasis. To identify the mechanism underlying AGR2vH-associated cancer cell migration and invasion, expressions of the proteins in the epithelial-to-mesenchymal transitional (EMT) process were examined. Western blot analyses of epithelial markers (E-cadherin and Claudin-1) and mesenchymal markers (Vimentin and Slug) showed that AGR2vH-depleted cells expressed a lowered level of vimentin, but not the other proteins (Fig. 6A). On the other hand, overexpression of AGR2vH was associated with upregulation of vimentin (Fig. 6B and C). These results indicated that expression of AGR2vH may regulate some mesenchymal properties, which, in turn, supports the metastasis-associated phenotypes that we found.

4. Discussion

Aberrant splicing of the AGR2 in metastatic CCA has not been studied. In this study, we sought to examine, whether splicing isoforms of AGR2 might be involved in metastasis in this cancer. AGR2vH isoform was shown to be associated with cancerous status, and could be served as a highly sensitive and specific biomarker for prostate cancer, better than the prostate specific antigen (PSA) [15]. In this study, we demonstrated that AGR2vH was upregulated in two metastatic CCA cell lines, compared to their non-metastatic counterparts, and that the H isoform of AGR2 regulates metastasis feathers of the cancer cells.

In our study, AGR2vH is the only isoform of AGR2, that can be predicted as a translated protein, because AGR2vH contains the start codon (AUG) on mRNA structure. Sequence analysis showed the prediction, that as H isoform, AGR2 protein was altered at the dimerization domains; 3 important domains were missing, including PDI domain, peptide binding domain, and KTEL-ER retention domain, but retaining 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 linked to the cell migration ability [20,21]. A previous study showed that the mutant AGR2 lacking of the adhesion domain (amino acid residues 21–40) was unable to promote migration of keratinocyte and fibroblast [21]. We speculated that the overexpressed AGR2vH may create a gain-of-function isoform of AGR2, which abberently amplify migrating ability of the CCA cells.

Previous studies in thyroid carcinoma, and ovarian cancer using knowckdown or overexpression of the full-length AGR2wt showed that AGR2wt is involved in several cancer phenotypes such as, cell

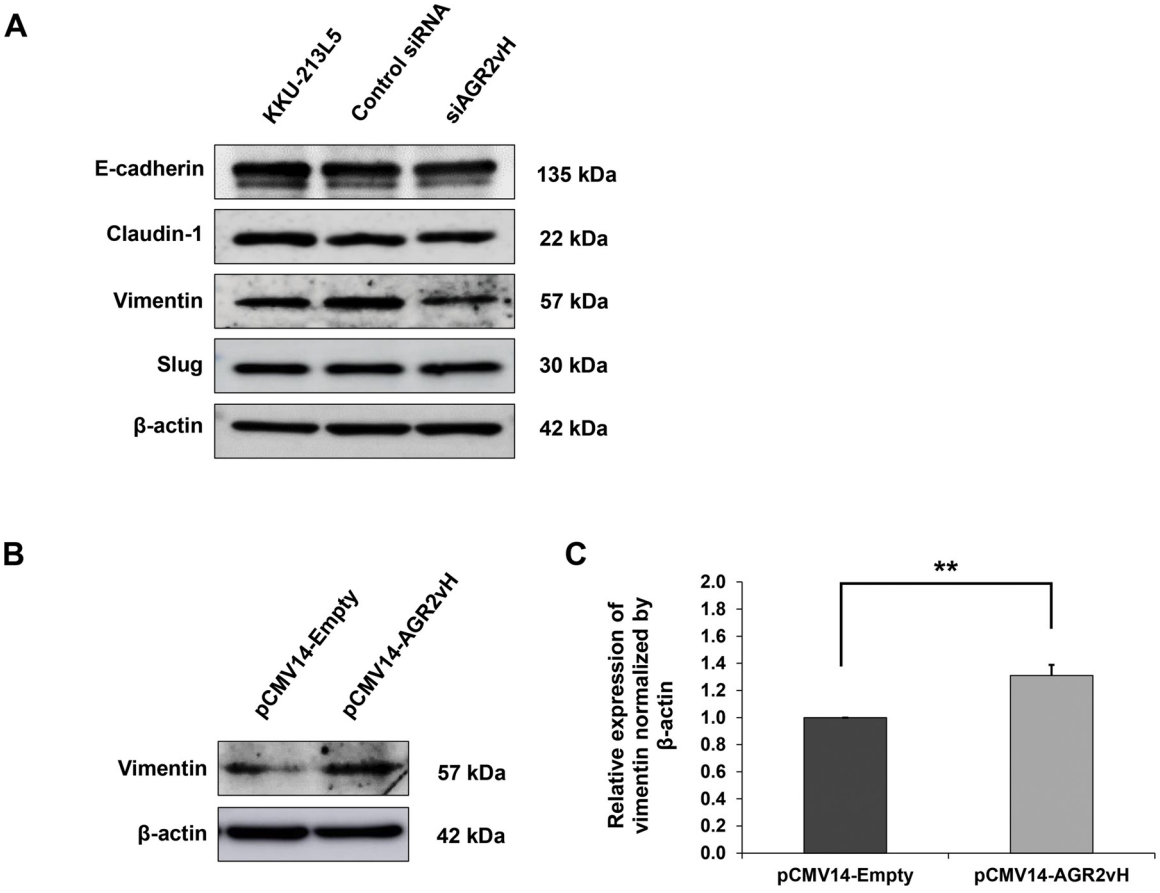

Fig. 6. Effect of AGR2vH on the expression of EMT markers. (A) Expressions of epithelial markers (E-cadherin and Claudin-1), and mesenchymal markers (vimentin, and Slug) in ARG2vH-depleted KKU-213L5 cells were determined by Western blottings. (B and C) Expressions of vimentin in ARG2vH-overexpressing, and control KKU-213 cells were examined by Western blottings, and up-regulation of vimentin were also shown in bar graphs. The data in C are shown as mean ± SD of biological triplicate, $^{**}P < 0.01$.

proliferation, migration, and invasion [22,23]. We found that depletion or ectopic overexpression of the AGR2vH only interfered with metastasis-related phenotypes, but had no, or little effect on cancer proliferation. All in all, these observations suggested that the accumulation of this alternative spliced AGR2vH isoform predominantly facilitate cancer metastasis.

Aberrant splicing of genes were reported from CCA, and suggested as a possible driver for CCA carcinogenesis. It was often associated with aggressiveness of cancer, for instance, WISP1v isoform of Wnt-inducible secreted protein 1 variant [24], and PKM2 isoform of Pyruvate kinase [25], both of which contribute to neural and lymphatic invasion. CD44v6 and CD44v8-10 isoforms of CD44 were shown to be associated with cancer cell proliferative and anti-apoptotic capability [26].

A previous study showed that upregulation of vimentin, a mesemchymal marker, was observed in the KKU-213L5 and KKU-214L5 when compared with their parental cells [27], and suppression of vimentin significantly decreased migration and invasion capabilities of the highly metastasis CCA cell [19]. These suggested that CCA migration is involved with a mesenchymal-like phenotype. Our result showed a reduced expression of vimentin when AGR2vH was suppressed, indicated that AGR2vH may promote mesenchymal features of the cancer cells. As such, AGR2vH-depleted cells were gaining epithelial phenotypes, and became less mobile. Further study is required to elucidate the mechanism how AGR2vH regulates the mesenchymal phenotype.

5. Conclusion

The upregulation of AGR2vH, a 204 bases spliced transcript was identified in metastatic CCA cells. Depletion of AGR2vH using specific siRNA in the highly-metastasis KKU-213L5 resulted in significantly decreased cell migration and invasion, without proliferation defect. Overexpression of AGR2vH in the non-metastatic parental KKU-213 led to increased capacities in cell migration, invasion, adhesion and slightly increased cell proliferation. These results indicated functional involvement of alternative splicing, and oncogenic role of AGR2vH, in CCA metastasis.

Disclosure of conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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Aberrant mRNA splicing generates oncogenic RNA isoforms and contributes to the development and progression of cholangiocarcinoma (Review)

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Abstract. Cholangiocarcinoma is a lethal biliary cancer, with an unclear molecular pathogenesis. Alternative splicing is a post-transcriptional modification that generates mature mRNAs, which are subsequently translated into proteins. Aberrant alternative splicing has been reported to serve a role in tumor initiation, maintenance and metastasis in several types of human cancer, including cholangiocarcinoma. In this review, the aberrant splicing of genes and the functional contributions of the spliced genes, in the carcinogenesis, progression and aggressiveness of cholangiocarcinoma are summarized. In addition, factors that influence this aberrant splicing that may be relevant as therapeutic targets or prognosis markers for cholangiocarcinoma are discussed.

Contents

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Key words: cholangiocarcinoma, alternative splicing, spliced gene

1. Introduction

Cholangiocarcinoma (CCA), is a malignant tumor that arises from the biliary epithelial tissue and is highly aggressive, with no effective pharmacological treatment available. This cancer has a poor prognosis and a high mortality rate (1). The highest worldwide incidence of CCA is found in the North and Northeast of Thailand, at ~85 cases per 100,000 individuals per year (2). The major predisposing factors for CCA in Asia are infection by the liver fluke, *Opisthorchis viverrini* (3,4) and exposure to groups of food-borne carcinogens, especially N-nitrosodimethylamine compounds identified in grilled or fermented foods (5). The only effective treatment for the disease is surgery. For patients who are not eligible for surgical therapy, gemcitabine- or 5-fluoro-uracil (FU)-based treatments are given. These are largely ineffective, since the response rate is only 20-30%.

The molecular pathology of bile duct cancer has been a topic of intense study. The molecular pathogenesis of CCA usually involves abnormal signal transduction and pro-inflammatory secretion, facilitated by gene mutations and epigenetic dysregulations (on a set of oncogenes and tumor suppressor genes) (6). Several lines of evidence also indicate that the abnormal expression of growth factors and receptors, the RAS/RAF/ dual specificity mitogen-activated protein kinase kinase 1 pathway, and the phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin pathway may be involved with CCA initiation, maintenance, and metastasis (7). Several studies reported that specific-target drugs or inhibitors, including epithelial growth factor receptor (EGFR; Lapatinib or Erlotinib), fibroblast (F) GFR and PI3K inhibitor, (8) may be applicable to CCA. A number of novel therapeutics are under evaluation in a phase 2 study (9).

Alternative splicing (AS) is a post-transcription modulation process that can generate a variety of gene isoforms. Spliced mRNA is able to be translated to differential amino acids with various biological functions (10). Pre-mRNA is spliced through the spliceosome; a large macromolecule comprising 5 small nuclear ribonucleoproteins (snRNPs U1, U2, U4/U6 and

U5). The AS generates 5 common splicing patterns, including alternative 5' splice site, alternative 3' splice site, exon skipping, intron retention and mutually exclusive exons. Previous data demonstrates that aberrant alternative splicing also includes exonic regulatory element mutation, splice site mutation and altered splice isoform ratios. The differential expression of splicing factors is implicated in various diseases and linked to hallmarks of cancer (11-15). A number of reports demonstrated a correlation between aberrant AS and tumor initiation/progression (16-20). The truncated oncogenic forms of the proteins, resulted from aberrant AS involved in cancer cell growth, apoptosis, drug resistance and angiogenesis.

Aberrant splicing of macrophage-stimulating protein receptor (RON) (21) and Racl (22) promoted angiogenesis and epithelial mesenchymal transition (EMT) phenotypes. In addition, a BRAF (V600E) spliced isoform, lacking exon 4-8 induced vemurafenib drug resistance in melanoma (23). In the present review, evidence is presented that supports important roles for aberrant splicing and the spliced isoforms of the genes, in CCA carcinogenesis and cancer aggressiveness.

2. Relevance of aberrant AS in cholangiocarcinoma development, progression and aggressiveness of phenotypes

A number of articles have summarized the interconnection between AS and cancer progression, including 17 genes in lung cancer (16), 2 reports in breast cancer in which 7 genes (17) and 9 genes (18), respectively were demonstrated, and 9 genes in hepatocellular carcinoma (19,20). The global cancer-specific transcript variants of five cancers demonstrated protein metabolism and modification are the most prevalent functional processes in cancer (24). As mentioned previously, aberrant AS has been discovered and proven to have functional involvement in the initiation and progression of cancer. In CCA, 623 genes presented with alternative splicing in CCA samples when compared with healthy bile duct tissue samples (25). In this review, atypical splicing of nine genes, which have been investigated at the *in vitro*, *in vivo* and clinical levels, and their relevance to CCA pathogenicity are summarized. The structure of nine pre-mRNAs that undergo alternative mRNA splicing to generate wild-type mRNA or variant transcripts are presented in Fig. 1. The derived-spliced transcripts or protein isoforms are summarized by how they can facilitate various characteristics of a cancer cell, as presented in Fig. 2 and Table I.

Cluster of differentiation (CD)44v6 and CD44v8-10. CD44 is a transmembrane glycoprotein receptor that specifically binds to extracellular hyaluronan and other extracellular matrix (ECM) proteins to activate signal transduction, and serves important roles in tumor proliferation, migration, and invasion (26,27). CD44 pre-mRNA encodes transmembrane and cytoplasmic-tail regions. The AS of CD44 can generate up to 12 isoforms of proteins with different biological functions. The CD44v isoforms participate in cancer progression: CD44v6 promotes EMT and activates the transforming growth factor-β pathway (28,29), and CD44v8-10 is involved in poor cancer prognosis (30,31). Expression of CD44v6 can be linked to CCA proliferation. CD44v6 is a CCA-specific isoform that has never been detected in normal bile ducts (32). Furthermore, the CD44v8-10 transcript was overexpressed in CCA and was demonstrated to stabilize the xCT system, a cysteine/glutamate transporter, to elevate glutathione synthesis and inhibit reactive oxygen species (ROS) accumulation in CCA cells. This function of CD44v8-10 was demonstrated to facilitate cancer cell survival in cases caused by liver fluke-induced inflammation. In addition, upregulation of CD44v8-10 suppressed p38 mitogen-activated protein kinase 1 (MAPK), which is a signaling protein involved in ROS suppression. Although the mechanism by which the CD44 spliced isoform may suppress p38 is still unclear, this observation appeared to be clinically relevant, since patients with CD44v overexpression and negative-phosphorylated (phospho)-p38MAPK have significantly shorter survival times compared with low CD44v expression and positive-phospho-p38(MAPK) (33).

WISP1v. Wnt-inducible secreted protein 1 [(WISP1) also known as CNN4] is a member of the cysteine-rich CCN family of proteins, which are highly expressed in skeletal tissues and has a role in bone formation and maintenance. Functions of this protein involve cell proliferation, osteoblastic differentiation and migration (34,35). WISP1 comprises 4 domains, including insulin-like growth factor-binding protein (IGFBP), VWC, thrombospondin-1 (TSP-1) and CT domains and is known to have variants with biological functions. A WISP1 variant lacking exon 3 (WISP1v) loses its VWC domain, which is thought to participate in protein complex formation. Ectopic expression demonstrated that the WISP1v is a secreted oncoprotein, which drives cellular transformation and rapid cumulative growth. WISP1v overexpression enhanced the invasive phenotype in gastric carcinoma cells, while wild-type WISP1 exhibited no such potential. These findings suggested that the CCN protein WISP1v was involved in the aggressive progression of scirrhous gastric carcinoma (36). In CCA, the aberrant isoform WISP1v was demonstrated to be overexpressed in patient CCA tissues (37). Furthermore, upregulation of WISP1v was associated with shorter overall survival time among patients following surgical treatment (38). In addition, WISP1v was demonstrated to promote cell invasion *in vitro* and this process was demonstrated to be mediated by p38 MAPK (37).

Nek2A and Nek2B. Nek2, or NIMA-related kinase 2, is a serine/threonine kinase that regulates cell division through centrosome separation (39). The spliced isoform of Nek consists of three forms, Nek2A, Nek2B and Nek2A-T (40). Isoforms of NEK are demonstrated to be functionally involved with cancer formation. In patients, overexpression of Nek2a was associated with Ki-67 expression, a cell proliferation marker (41). In addition, NEK2A cytoplasmic expression was positively associated with cancer grade and tumor size in breast invasive ductal carcinoma and metastatic potential (42). Cancer cells overexpressing the NEK2A isoform demonstrated a significant increase in colony formation compared with control cells and small interfering (si)RNA-based depletion of NEK2a resulted in the halting of cancer cell proliferation (43). Nek2A/Nek2A-T were demonstrated to be highly upregulated in CCA cell lines, with the predominant isoform being Nek2A/Nek2A-T and Nek2B being the lesser expressed isoform (44). Furthermore, the expression of Nek2B was demonstrated to positively correlate with proliferation potential in breast cancer cells (45).

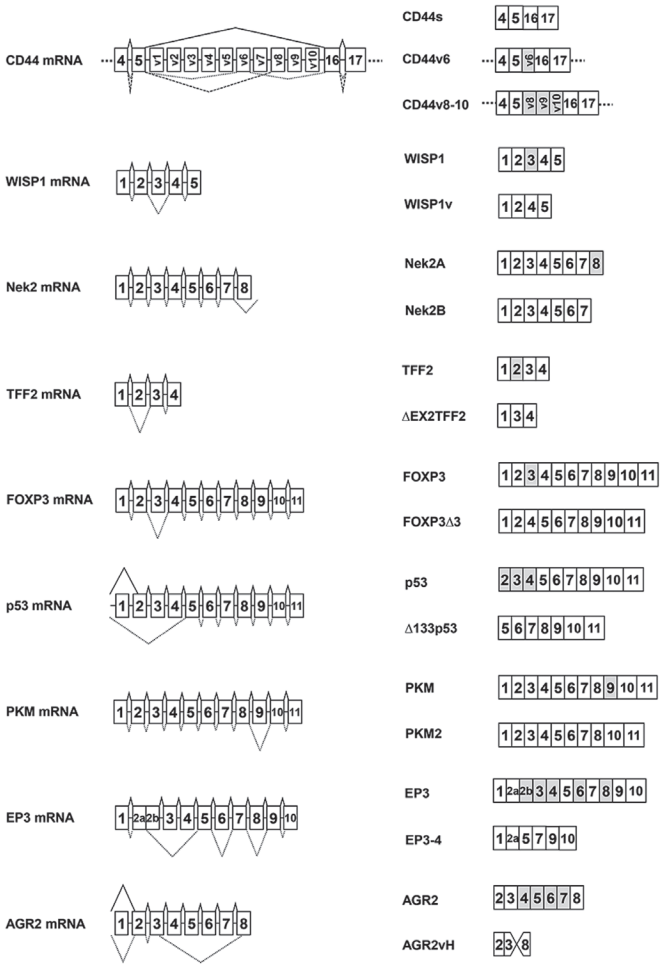

Figure 1. Schematic representation of the alternative splicing events implicated in cholangiocarcinoma development and progression. Exons are represented by boxes and introns by lines. Continuous lines represent the exon inclusion for wild-type mRNA, whereas dotted lines represent the exon inclusion for spliced transcripts. Skipped or included exons from alternative splicing, that differ from wild-type mRNA, are presented in gray.

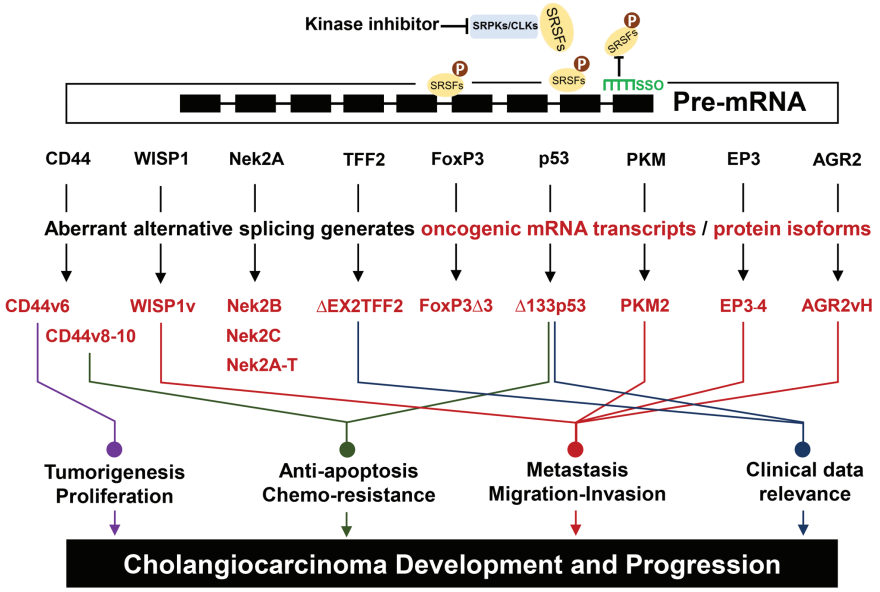

Figure 2. Spliced mRNA transcripts and their functions in cholangiocarcinoma.

ΔEX2TFF2. Trefoil factor 2 (TFF2) is a secreted protein that serves important roles in gastrointestinal restitution (46), chronic kidney disease and pulmonary inflammation, through the induction of cell migration and proliferation. Overexpression of TFF2

Table I. Spliced mRNA transcripts and their functions in cholangiocarcinoma.

Author, year	Gene	Spliced transcript/ isoform	Splicing variants	Function	(Refs.)
Yun *et al*, 2002	CD44	CD44v6	Retained exon v6	Proliferation	(32)
Thanee *et al*, 2016	CD44v8-10	Retained exon v8-10	Anti-apoptosis		(33)
Tanaka *et al*, 2003	Wnt-inducible secreted Protein	WISP1v	Skipping exon 3	Neural and lymphatic invasion	(37)
Kokuryo *et al*, 2007	Serine/threonine-protein kinase Nek2	Nek2B	Skipping exon 8	Function unknown	(44)
Kamlua *et al*, 2012	Trefoil factor 2	ΔEX2TFF2	Skipping exon 2	Independent prognostic marker	(48)
Harada *et al*, 2012	Forkhead box protein 3	Foxp3Δ3	Skipping exon 3	Function unknown	(50)
Nutthasirikul *et al*, 2013	Tumor protein 53	Δ133p53	Exon 1-4 skipping	Independent prognostic marker	(60)
Nutthasirikul *et al*, 2015				5-Fluorouracil resistance	(61)
Yu *et al*, 2015	Pyruvate kinase	PKM2	Mutually exclusive exons; exon 9 skipping and exon 10 retention	Neural invasion	(67)
Du *et al*, 2015	E prostanoid receptor 3	EP3-4	Exon 2b, 3, 4, 6 and 8 skipping	Proliferation migration and invasion	(71)
Yosudjai *et al*, 2018	Anterior Gradient-2	AGR2vH	Alternative 3' and 5' splice site and exon 4-7 skipping	Migration, invasion and adhesion	(74)

is commonly identified in several types of cancer, implicating it in carcinogenesis. TFF2 was reported to exert its pro-proliferative activity through the EGFR-MAPK pathway in CCA (47). Previously, ΔEX2TFF2, an exon 2- skipping isoform of TFF2 with a stop codon (TAG) at exon 1, was uncovered as a spliced isoform of TFF2 (48). Although, the roles of this transcript have not been clarified, the present study demonstrated that a high expression ratio of ΔEX2TFF2/wtTFF2 in patients was significantly associated with a longer survival time (48). Therefore, the spliced isoform may act as a dominant-negative form of TFF2 that counteracts the cancer promoting wtTFF2 activity in CCA.

Forkhead box protein 3 (FOXP3Δ3). FOXP3 is a transcription factor in the forkhead protein family that is involved in $CD25^+$ regulatory T cell (Treg) development. Not only does FOXP3 control Treg development, it is also expressed in colorectal cancer cells, which is associated with poor prognosis in patients (49). Exon 3 skipping of FOXP3, resulting in an amino acid frameshift, has been reported in CCA (50). In addition, a FOXP3 splice isoform was also observed in melanoma cells, suggesting it has a role in suppressing immune activity (51).

Δ133p53. Tumor protein 53 (TP53 or p53) is one of the most important tumor suppressors, indicated by its high mutation rate across all types of cancer. p53 responds to various stress signals and orchestrates processes including cell cycle arrest, DNA repair, cellular senescence and apoptosis in response to specific stress signals (52). AS generates 12 p53 isoforms, including Tap53, Δ40p53, Δ133p53 and Δ160p53 among others (53,54). The differential regulation of p53 isoforms promotes the aggressiveness of several types of cancer. A study demonstrated that Δ133p53b enhanced breast cancer

stemness (55) and protected colorectal cells from camptothecin-induced apoptosis (56).

p53 has been identified as a gene that frequently mutates in a large number of CCA cases (57-59), suggesting that a perturbed p53 pathway facilitates CCA carcinogenesis. A study demonstrated that a high Δ133p53/p53 mRNA expression ratio correlates with a poor overall survival (60). Notably, Δ133p53 is also associated with resistance to certain cancer drugs; an association between Δ133p53 and 5-FU-resistance in CCA cells was demonstrated, and Δ133p53 was upregulated in 5-FU-resistant tumor tissues and CCA cell lines in a dose-dependent manner (61). Given that 5-FU is a cytotoxic drug that interferes with DNA synthesis, the Δ133p53 isoform may act as a dominant-negative p53 that interferes with the activity of wtp53 in the ternary complex (62). Accordingly, suppression of Δ133p53 promoted apoptosis, which correlated with an upregulation of pro-apoptotic Bax and a downregulation of anti-apoptotic Bcl-2 (61).

Pyruvate kinase (PKM2). PKM is a rate-limiting enzyme that catalyzes the conversion of phosphoenolpyruvate to pyruvate during glycolysis. PKM can be generated in 4 isoforms, which are expressed differently in various tissues. One of the isoforms is PKM2, which lacks exon 9 and is a major isoform highly expressed in a number of types of cancer (63). Previously data demonstrate that overexpression of PKM2 is linked to tumor growth, metastasis capability and a poor prognosis in hepatocellular carcinoma, pancreatic ductal adenocarcinoma and gallbladder cancer (64-66). In hilar cholangiocarcinoma, immunohistochemical staining specific to the PKM2 isoform demonstrated a great number of positive-staining cells in the tumor tissue. Patients with high-PKM2-expressing tumors exhibited a higher rate of tumor recurrence and a shorter overall survival time, when compared with patients with low PKM2 expression. However, there is still no conclusive evidence that indicates PKM2 is a cancer driver for CCA. In addition, PKM2 elevation was associated with CCA development and neural invasion (67).

EP3-4. E prostanoid receptor 3 (EP3), or prostaglandin E2 receptor 3 (PTGER3), is a member of a G protein-coupled receptor family, that specifically binds to prostaglandin E2 (PGE2) to activate various responses. EP3 receptor can generate up to 11 spliced isoforms. Previous data demonstrate that EP3-5 and EP3-6 isoforms were associated with cell proliferation in the myometrium in humans (68). Furthermore, overexpression of the EP3-4 receptor promoted cell growth through upregulating FUSE-binding protein 1 in liver cancer (69). In CCA, the truncated EP3-4 isoform, which includes exon 1, 2a, 5 and 10, was detected (70). This EP3-4 isoform is activated through the Src/EGFR/PI3K/AKT/glycogen synthase kinase-3β pathway and promotes cell proliferation, migration, and invasion. This results in enhanced expression of the downstream proteins c-Myc and snail. Therefore, it is believed to serve a regulatory role in CCA cell growth and metastasis (71).

Anterior Gradient-2 (AGR2)vH. The expression profiling of metastasis-associated genes in CCA demonstrated that AGR2 is one of the most-upregulated genes, specific to the metastatic CCA cell line, when compared with the parental cell line (72). The AGR2 gene encodes for a disulfide isomerase enzyme, which is commonly expressed in mucus-secreting tissues. The mRNA splicing of AGR2 was first characterized in prostate cancer (PCa). Spliced isoforms include AGR2vC, AGR2vE, AGR2vF, AGR2vG and AGR2vH. Among the 5 spliced isoforms and the wild-type, AGR2vG and AGR2vH were demonstrated to be significantly upregulated in the exosome from patient's urine sample analysis. These two exhibited high diagnostic value, with higher sensitivity and specificity when compared with the prostate-specific antigen used as a standard clinical biomarker for PCa diagnosis (73). In CCA cell lines, AGR2 RNA isoforms, namely AGR2vE, AGR2vF and AGR2vH, were recently reported that are specific to metastatic CCA cells (74). It was demonstrated that the AGR2vH isoform enables various metastatic-associated phenotypes in CCA cells. Suppression of AGR2vH by the AGR2vH-specific siRNA significantly reduced CCA cell migration and invasion. Concordantly, AGR2vH overexpression promoted cell proliferation, migration, invasion and adhesion potential. In addition, it was demonstrated that the expression of AGR2vH influences metastasis-associated phenotypes through the upregulation of vimentin. Therefore, the results indicated that the metastasis-specific isoform AGR2vH serves an important role in cancer severity (74).

3. Targeting aberrant splicing as a novel concept for cancer treatment

The prominent role of the aberrant AS in carcinogenesis has been demonstrated, indicating that AS may be a good target for cancer therapy. Aberrant AS can be manipulated in several steps: For example, Pre-Trans-Splicing Molecule (PTM) is the artificial sequence that can reprogram mRNA through replacement of the 3'exon, 5'exon and internal exon (75,76). The results demonstrated that the trans-splicing molecule reduced the number of mutant p53 transcripts in the transfected cells, which resulted in cell cycle arrest, apoptosis and tumor xenograft suppression with colorectal cancer and hepatocellular carcinoma cells (77,78). However, the use of PTM for targeting oncogenic AS events is not yet well studied and the PTM modification has limitations for cancer treatment. Therefore, this review discussed the methodologies that may apply to cancer therapy, including small molecule splicing modulators and SSOs, each of which are currently under study in clinical trials.

Small molecules splicing modulators. Splicing factors are key molecules that influence AS regulation and are associated with cancer aggressiveness and pathological phenotypes (79). A previous report demonstrated that an overexpression of serine/arginine-rich splicing factor 1 (SRSF1) can facilitate abnormal splicing of tumor suppressors and proto-oncogenes (80). The results demonstrated that SRSF1 promotes 12A inclusion of an isoform of BIN1, which interferes with the tumor-suppressing activity of this protein. In the same study, the researchers demonstrated an increase in S6K1 isoform 2 expression resulting from SRSF1 overexpression that was associated with colony formation activity (80). An Ov-infected hamster model was used to identify the differentially expressed genes to study

the molecular mechanism of CCA carcinogenesis. The results demonstrated that SRSF9 is one of the genes that are overexpressed in Ov-infected hamsters and may be associated with CCA initiation (81).

Aberrant spliceosomal proteins are important factors associated with carcinogenesis. The data revealed that mutations in splicing factor 3B subunit 1A (SF3B1), which encodes the core component of U2 snRNP, is linked to erroneous 3' splice site selection (82-84). The results demonstrated that the SF3B1 K700E mutation led to differential splicing in uveal melanoma and breast cancer (85,86). In addition, luminal B and progesterone receptor-negative breast cancer patients with additional SF3B1 mutations have significantly shorter survival times (87).

It is possible to modulate aberrant AS based on small molecule inhibitors of splicing factors or mutated spliceosomal proteins: For example, it has been demonstrated that a natural product 'Borrelidin' can bind to a splicing protein, FBP21, leading to a decrease of the vascular endothelial growth factor (VEGF) pro-angiogenic isoform and an increase of the VEGF anti-angiogenic isoform, in RPE cells (88). Previous studies demonstrated that a natural product, FR901464 and its methylated derivative, spliceostatin A, as well as E7107, specifically inhibit spliceosome assembly through SF3B1 and lead to halted splicing reactions (89-91). The results demonstrated that treatment of these small molecules inhibits cell cycle progression and inhibits the tumor angiogenesis through decreasing the levels of VEGF transcripts (92,93).

Not only does the altered expression of splicing regulators affect AS, but the alteration of the phosphorylation status of the splicing factor/modulator was also implicated in cancer progression. In head and neck squamous cell carcinoma, hyperphosphorylation of SRPK2, a serine/arginine-rich protein-specific kinase that phosphorylates SRSF1/2, was detected in cancer cells; the phosphorylation promotes cell proliferation, migration and invasion (94). Alteration to the kinase alters the AS pattern. A previous study demonstrated that CLKs and SRSF protein kinases (SRPKs) are targets for kinase inhibitors to modulate AS; treatment with Cpd-1, Cpd-2, and Cpd-3 significantly reduced the levels of phosphorylated SR proteins, therefore affecting the splicing pattern of multiple genes and inducing cell apoptosis (95). Furthermore, the other kinase inhibitors, including ceramide, affect splice site selection of Bcl-x and increases pro-apoptotic isoforms through the dephosphorylation of the SR protein (96).

SSOs technology. SSOs are single-stranded nucleic acids, usually 15-25 bases, that are complementary to the mRNA target transcripts or the recognition sequence of the splice sites, that leads to modulated splicing. A number of studies demonstrated that SSO can inhibit aberrant RNA translation: I.e., MDM4 is the protein that contributes to embryonic development and is undetectable in adult tissues. An MDM4 isoform with exon 6 is frequently upregulated in cancer cells, impairing p53 tumor-suppressor function. The SSO-mediated skipping of exon 6 results in decreased MDM4 levels and reduced melanoma growth (97). Similarly, SSO targeting exon 26 of HER4 mRNA, named as SSOe26, demonstrated its capacity on HER4 isoform switching from CYT1 to CYP2. This treatment resulted in the depletion of the proliferation of breast cancer cells and tumor growth in mice xenografts (98). Furthermore, SSO targeted B-cell lymphoma (Bcl)-x pre-mRNA, which increased the Bcl-xS isoform, gaining pro-apoptotic activity, which was verified in the models of murine melanoma and in human glioma cell lines (99,100).

Drug development based on targeting aberrant AS, namely small molecule splicing modulators, is an interesting approach for cancer treatment. Splicing regulators are upstream molecules that control the splicing events of multiple genes. Insight into novel target genes of the splicing regulators, can be used to manipulate the effective inhibitor(s) of these upstream molecules to suppress various downstream oncogenic spliced isoforms. However, the off-target effect, toxicity (101,102) and the effects of small splicing factors interfering with the normal splicing patterns of global genes, should be considered. On the other hand, the specificity of SSO technology overcomes more than small splicing modulators by modulating AS through inhibiting only its oncogenic target which leads to effective treatment. The major problems of oligonucleotides include toxicity, instability against nucleases and delivery limitations.

4. Conclusion

The present review summarized the experimental evidence for and clinical relevance of the verification of significant effects of aberrant mRNA splicing of well-characterized genes with respect to CCA initiation and aggressiveness. The nine genes discussed underwent AS and revealed an intercorrelation with cholangiocarcinogenesis and progression. This information will serve as an opportunity to develop novel strategies for CCA detection and intervention. Interestingly, certain of the cancer-specific variants may serve as potential targets for CCA prognosis including Δ2TFF2 and Δ133p53, which demonstrate their clinical impact on patient survival. These oncogenic isoforms may be used as targets for cancer treatment, using specific antibodies, or the construction of SSOs which can modulate aberrant splicing. The regulatory machinery, including splicing factors and regulators, represents alternative targets of precision strategies, regarding the depletion of oncogenic isoforms. Finally, this summarization provides new ideas for the improvement of CCA diagnosis and treatment. Further studies should aim to investigate the unclear linkages between AS and CCA to unlock the molecular mechanisms governing AS regulation in CCA development and progression.

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Authors' contributions

JY and WK designed, performed and wrote the literature review. SJ and SW revised the manuscript for intellectual content.

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Competing interests

The authors declare that they have no competing interests.

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Cell Stress and Chaperones

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

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Abstract:	Cholangiocarcinoma (CCA) is an epithelial cell malignancy arising within the biliary tree in liver. CCA is normally diagnosed when developed into advanced stages with metastasis, resulting ineffective treatment. Recently, anterior gradient-2 (AGR2) was characterized as the top of upregulated gene among 77 metastatic-associated genes in high-metastatic CCA cell lines. AGR2 is generally expressed in mucus-secreting tissues and overexpressed in various types of cancer. Previous reports demonstrated dimeric form of AGR2 is required for triggering unfolded protein response (UPR) pathway to support cancer cell survival especially under imbalance homeostasis of endoplasmic reticulum (ER). Our recent work identified AGR2 short isoform generated by aberrant splicing named as AGR2vH which contributed to metastasis-associated phenotypes of CCA cells. This study, we aimed to determine the roles of AGR2vH on UPR pathway activation to support cancer cell survivability and to evade apoptosis. After experimentally induced ER stress into AGR2vH-overexpressing CCA cell by tunicamycin, UPR pathway was activated by upregulation of 3 UPR markers (ATF6, eIF2a, and XBP1s) and UPR downstream target (GRP94). Under ER stress condition, overexpression of AGR2vH can reduced the number of apoptotic cells, by decreased caspase-3/7 activity and downregulated CHOP expression resulting in higher number of viable cells. These present results support our previous data that an oncogenic AGR2vH isoform not only promote metastasis-associated phenotypes, but also helps CCA cells to survive and evade apoptosis for persisting and progression of cancer.

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

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Short title: AGR2vH overexpression enables CCA cell survival under ER stress via activating UPR pathway

Abstract

Cholangiocarcinoma (CCA) is an epithelial cell malignancy arising within the biliary tree in liver. CCA is normally diagnosed when developed into advanced stages with metastasis, resulting ineffective treatment. Recently, anterior gradient-2 (AGR2) was characterized as the top of upregulated gene among 77 metastatic-associated genes in high-metastatic CCA cell lines. AGR2 is generally expressed in mucus-secreting tissues and overexpressed in various types of cancer. Previous reports demonstrated dimeric form of AGR2 is required for triggering unfolded protein response (UPR) pathway to support cancer cell survival especially under imbalance homeostasis of endoplasmic reticulum (ER). Our recent work identified AGR2 short isoform generated by aberrant splicing named as AGR2vH which contributed to metastasis-associated phenotypes of CCA cells. This study, we aimed to determine the roles of AGR2vH on UPR pathway activation to support cancer cell survivability and to evade apoptosis. After experimentally induced ER stress into AGR2vH-overexpressing CCA cell by tunicamycin, UPR pathway was activated by upregulation of 3 UPR markers (ATF6, eIF2a, and XBP1s) and UPR downstream target (GRP94). Under ER stress condition, overexpression of AGR2vH can reduced the number of apoptotic cells, by decreased caspase-3/7 activity and downregulated CHOP expression resulting in higher number of viable cells. These present results support our previous data that an oncogenic AGR2vH isoform not only promote metastasis-associated phenotypes, but also helps CCA cells to survive and evade apoptosis for persisting and progression of cancer.

Keywords Aberrant splicing · Anterior gradient-2 · Cholangiocarcinoma · Endoplasmic reticulum stress

Abbreviations

AGR2	Anterior gradient-2
AGR2vH	Anterior gradient-2 spliced variant H
ATF6	Activating transcription factor 6
BiP/GRP78	Binding immunoglobulin protein/Glucose-regulated protein 78
CCA	Cholangiocarcinoma
CHOP	C/EBP homologous protein
ER	Endoplasmic reticulum
eIF2	Eukaryotic initiation factor 2
GPR94	Glucose-regulated protein 94
IRE1	Inositol-requiring enzyme 1
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PDIs	Protein disulfide isomerases
UPR	Unfolded protein response
XBP1	X-box binding protein 1

Introduction

Cholangiocarcinoma (CCA) or bile duct cancer is a malignant cancer arising from biliary epithelium in biliary tract. CCA development is associated with the infection of carcinogenic liver flukes, *Opisthorchis viverrini* (Ov) therefore this type of cancer presents the highest incident and mortality rate in Southeast Asia, particularly in Thailand (Shin et al. 2010). Difficult diagnosis and treatment of CCA in most cases, were often detected when the patients achieved advanced stages of cancer, with cells metastasized in liver, lungs, lymph nodes or other secondary organs (Sripa et al. 2008, 2011). A recent model was established for studying the *in vitro* metastasis of CCA is a pair of human CCA cell lines with non- and high-metastatic activities as KKU-213 and KKU-213L5, respectively. The mRNA expression profile of 77 metastatic-associated genes were determined using a real-time PCR array, which revealed that AGR2 was the top among 77 genes that predominantly upregulated in KKU-213L5 with the parental KKU-213 (Uthaisar et al. 2016).

Anterior Gradient-2 or AGR2 was identified as a protein, which localized in the anterior border of the embryonic ectoderm is crucial to cement gland development, in the early stages of birth in *Xenopus laevis* (Aberger et al. 1998). Human AGR2 is classified as an enzyme in protein disulfide isomerases (PDIs) family. The 13,304 bp of AGR2 gene on the seventh chromosome encodes for 996 bp of 8 exons-mRNA which translated into 175 amino acids-protein. AGR2 is typically localized in endoplasmic reticulum (ER) and involved in the production of the cysteine-rich protein, such as the mucin family in mucus-secreting cells/tissues including the respiratory tract, stomach, colon, prostate and female reproductive tissues, and remarkably expressed in various types of cancer tissues (Obacz et al. 2015).

Functional involvement of AGR2 in ER directly acts as the isomerase enzyme for the folding of proteins and corrects the misfolded proteins by catalyzing the cysteine disulfide bond to produce productive functional proteins. Under human abnormalities, upregulation of AGR2 in cancer is associated with development and progression, such as promoting pancreatic cancer cell proliferation and survival (Ramachandran et al. 2008). On the other hand, dimerization of monomeric AGR2 is required particularly when the cells are under ER stress conditions. For example, cancer cells which dramatically increase the ability of protein synthesis for cell proliferation, are stated in the accumulation of proteins in ER for the process of post-translational modification into functional proteins. The cellular ER stress is influenced by an accumulation of unfolded proteins or the presence of mutated proteins which cannot fold correctly, making AGR2 the key enzyme that plays an important role in the protein-folding homeostasis in this ER situation (Higa et al. 2011). A

recent report demonstrated AGR2 homodimer is required to interact with BiP/GRP78 for activating unfolded protein response (UPR) pathway, a cellular stress response mechanism that directly related to ER stress (Ryu et al. 2013). The UPR pathway is initiated by three ER transmembrane-resident proteins including Inositol-requiring enzyme 1 (IRE1), Activating transcription factor 6 (ATF6), Protein kinase RNA-like endoplasmic reticulum kinase (PERK). During unstressed conditions, three ER-transmembrane-resident proteins bind with Binding immunoglobulin protein (BiP) or Glucose-regulated protein 78 kDa (GRP78) to keep them inactive. Upon ER stress, BiP dissociates from these ER-transmembrane sensors resulting to their activation (Oslowski and Urano 2011). Activated IRE1 induces the splicing of X-box binding protein 1 (XBP1) mRNA to XBP1s, which translocates to the nucleus and acts as the transcription factor for upregulation of UPR target genes (Suh et al. 2012). Activated ATF6 translocate to nucleus to be a transcription factor, which modulates the expression of chaperones and enzymes required for ER function (Eizirik et al. 2012). One of downstream of ATF6 is glucose-regulated protein 94 (GRP94), is upregulated for folding of newly synthesized protein and prevents accumulation of unfolded or misfolded proteins (Zhu and Lee 2015). Activated PERK phosphorylates a downstream target which is eukaryotic initiation factor 2 (eIF2), phosphorylated eIF2α promotes expression of transcription factor ATF4, that regulates several UPR pathway target genes involve in ER stress-mediated apoptosis such as C/EBP homologous protein (CHOP) (Harding et al. 2000).

In 2014, a first evidence of AGR2 splicing was revealed in prostate cancer, including 6 spliced variant transcripts which are AGR2vB, AGR2vC, AGR2vE, AGR2vF, AGR2vG and AGR2vH (Neeb et al. 2014). Our previous study reported an aberrant splicing of AGR2 in CCA cells, which characterized the highly upregulated AGR2vH transcript, and its functional roles on the promoting the metastatic-associated phenotypes of CCA cells, including migration, invasion and adhesion capacities. AGR2vH was predicted to be the translatable AGR2 isoform which consist of 67 amino acids which were truncated from 175 amino acids in AGR2 (Yosudjai et al. 2018).

Prospectively, AGR2vH might serve as an alternative partner molecule which contributed the survival of CCA cells. In this study, we aimed to determine the effect of AGR2vH on UPR pathway response and cell viability/apoptosis when upregulation of AGR2vH into CCA cells especially when experimentally inducing ER stress into the cancer cells. The activation of the UPR pathway was investigated by the expression of UPR-sensitive marker genes and UPR downstream gene. In addition, the reduction of dead cells and activity of caspase enzyme in apoptosis pathway and the survival of CCA were determined.

Materials and methods

Cell lines and cell culture

The two CCA cell lines used in this study, including KKU-213 which was obtained from Japanese Collection of Research Bioresources (JCRB) Cell Bank, and KKU-213L5, a highly metastatic CCA cell lines derived from parental KKU-213, which was established in previous study (Uthaisar et al. 2016). Cells were provided from Cholangiocarcinoma Research Institute, Faculty of Medicine, Khon Kaen University. The cell lines were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 10% v/v fetal bovine serum with 100 Unit/ml of penicillin and 100 µg/ml of streptomycin (Gibco, Thermo Fisher Scientific, Waltman, MA), and maintained at 37°C in a humidified, 5% CO_2 atmosphere.

Transfection and overexpression of AGR2vH in CCA cells

AGR2vH-overexpressing in KKU-213 cell was established as in previous publication (Yosudjai et al. 2018). Briefly, AGR2vH mRNA was amplified by specific primer with the relevant restriction sites to clone into the pCR® 2.1-TOPO® cloning vector (Invitrogen, Thermo Fisher Scientific, Waltman, MA). The AGR2vH nucleotide sequences were analyzed and confirmed before sub-cloned into p3XFLAG-CMV-14 expression vector (Sigma-Aldrich, St. Louis, MO). Either pCMV14-AGR2vH or pCMV14-Empty vector was transfected into KKU-213 cells by Lipofectamine 2000 (Thermo Fisher Scientific, Waltman, MA) and the single clones of them were selected by 2 mg/ml of Geneticin G418 (Thermo Fisher Scientific, Waltman, MA) and subjected for expansion and cultured.

Experimental induction of ER stress condition

Tunicamycin was used to block the activity of glycosylase, which resulted in the accumulation of unglycosylated-proteins in ER. Optimal concentration was determined by testing tunicamycin in the ranges of 0.5, 1, 2, 4 and 8 µg/µl in culture mediums for 24 h, to examine cytotoxicity by 3-[4,5-dimethylthiazole]-2,5-diphenyltetrazolium bromide (MTT) assay (Data not show). Then, the expression of ER stress sensitive markers including XBP1s and BiP/GRP78 were determined by RT-PCR and real-time PCR.

Preparation of RNA, Reverse transcription and Polymerase chain reaction

Total RNA was isolated from the cells using E.Z.N.A® Total RNA Kit I (OMEGA bio-tek, Doraville, Georgia, USA). The concentrations of RNA samples were measured, and 1 µg of total RNA was used to synthesize the complementary DNA using HisenScriptTM RH [-] RT PreMix Kit (Intron Biotech, Seoul, South Korea) according to the manufacturer's instructions. All cDNA samples were stored in -80°C until use. For the determination of gene expression by amplification of synthesized cDNA using Polymerase chain reaction (PCR) were performed under optimized conditions. The reaction mixture contained 0.2 µg of cDNA template, 0.4 µM of each forward and reverse primers with a total volume of 20 µl of 1×MyTaqTM HS Red Mix (Bioline, Taunton, Massachusetts). House-keeping gene, β-actin was used as an internal control for semi-quantitative normalization. Primers for the target genes were followed the previous studies including AGR2vH (Yosudjai et al. 2018), XBP1 (Nami et al. 2016), BiP/GRP78 (Oslowski et al. 2011), ATF6, CHOP (Li et al. 2009), eIF2a and GRP94 (Dioufa et al. 2010). PCR products were analyzed by 2% agarose gel electrophoresis, detected by ImageQuant™ LAS 500 (GE Healthcare Life Sciences, Little Chalfont, UK), and quantitated using ImageQuant TL 7.0 software.

Quantitative real-time PCR

Quantitative real-time PCR was performed for relative quantification of the gene expression, including AGR2vH expression in AGR2vH-overexpressing cells and the expression of ER stress sensitive markers. Ten µl-reaction mixture contained cDNA template, forward and reverse primers and 1X LightCycler® 480 SYBR Green I Master (Roche Applied Science, Mannheim, Germany). Primers for the target genes were followed the previous studies including XBP1s (Van Schadewijk et al. 2012) and BiP/GRP78. All reactions were experimentally preformed in biological triplicate and analyzed using the LightCycler® 480 systems (Roche Applied Science, Mannheim, Germany). The expression levels of the target genes were normalized with β-actin using the relative quantification formula of $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001).

Flow cytometry

Cells were plated in a 6-well plate at 2.5×10^5 cells per well for 24 h before being treated with tunicamycin. After 24 h of tunicamycin treatment, 100 µL of Muse™ Annexin V & Dead Cell Reagent (Merck Millipore,USA) and an equal volume of 4×10^5 cells from each of the groups were mixed. After incubating for 20 min at room temperature, the number of live, dead and apoptotic cells were analyzed using Muse® Cell Analyzer and the attached analytical software (Khan et al. 2012).

Caspase 3/7 activity assay

Cells were plated in a 96-well black plate at 2×10^4 cells per well for 24 h before being treated with tunicamycin. After 24 h of tunicamycin treatment, caspase 3/7 activities were analyzed using Apo-ONE® Homogeneous caspase 3/7 assay, according to the manufacturer's instruction (Promega, Madison, USA). The fluorescence signal of each well was measured by a fluorescence microplate reader, EnSpire Multimode Plate reader (Perkin Elmer, Waltham, MA). Regarding the measurement of the fluorescent intensities, the assay suggested that the excitation wavelength be set at 499 nm, and the emission wavelength at 521 nm.

Depletion of AGR2vH by small interfering RNA

AGR2vH-overexpressing cells were transfections of siAGR2vH as in previous publication (Yosudjai et al. 2018). Briefly, AGR2vH-overexpressing cells were plated in a 6-well plate at 2.5×10^4 cells per well for 24 h. Then, cells were transfected with 75 nmol of siAGR2vH, or negative control siRNA in Opti-MEM I reduced serum medium (Gibco, Thermo Fisher Scientific, Waltman, MA). At 48 h after transfection, cells were harvested for used in further experimental.

Cell viability assay

Cells were plated in a 96-well plate at 3×10^3 cells per well for 24 h before being treated with tunicamycin. After 24 h of tunicamycin treatment, 10 µL of Cell Counting Kit-8 (CCK-8) reagent (Sigma-Aldrich, St. Louis, MO) was added to each well. Cells were incubated for 4 h at 37°C, the absorbance at 450 nm was measured using a Synergy HT Multi-Detection Microplate Reader (BioTek, Vermont, USA).

Statistical analysis

Experiments were performed in biological triplicate. Data was calculated and presented as the mean ± standard deviation (SD). Unpaired *Student's* t-test (two tailed) was used for comparison between each group by SigmaPlot (SigmaPlot 11.0, Systat Software, San Jose, CA). If *P* is less than *0.05* it was considered to be significant. *$=P<0.05$, **$=P<0.01$ *and* ***$=P<0.001$.

Results

Expression of AGR2vH on AGR2vH-overexpressing cells

Semi-quantitative RT-PCR was performed to evaluate the expression of AGR2vH after the cells were transfected by pCMV14-Empty and pCMV14-AGR2vH vector. The expression of AGR2vH was significantly increased in AGR2vH-overexpressing cells when compared with empty vector transfected cells but the expression level of AGR2vH in AGR2vH-overexpressing cells were still lower expressed than KKU-213L5 (Fig. 1a). Moreover, qRT-PCR confirms the result from RT-PCR (Fig. 1b).

Experimentally induced ER stress condition

To optimize the different concentrations of tunicamycin, the expression of ER stress markers (XBP1s and BiP/GRP78) were determined after 24 h treatment using less than 4 µg/ml. XBP1s was significantly upregulated at 2 µg/ml, when compared with untreated cells using RT-PCR and confirmed by real-time PCR (Fig.2a). Similar results were presented in BiP/GRP78 in RT-PCR and real-time PCR (Fig. 2b). Therefore, 2 µg/ml of tunicamycin was selected for ER stress induction.

Activation of the UPR pathway and UPR downstream

To investigate UPR response after AGR2vH overexpressed into the CCA cells and UPR response under ER stress inducing condition. The expressions of UPR pathway and UPR downstream markers were determined, including unspliced and spliced forms of XBP1, ATF6 and eIF2a. We found that XBP1s and eIF2a were upregulated in AGR2vH-overexpressing cells under ER stress inducing condition, while ATF6 was upregulated in AGR2vH-overexpressing cells in normal condition of CCA cells and under ER stress inducing condition (Fig. 3a). In addition, the expression of GRP94, an ER chaperones that downstream of UPR pathway, was upregulated in AGR2vH-overexpressing cells, especially under ER stress inducing condition (Fig. 3b).

The effects of AGR2vH on cell apoptosis

The apoptosis of CCA cells were determined using flow cytometry. The apoptotic cells were decreased in AGR2vH-overexpressing cells under ER stress inducing condition when compared with empty vector transfected cells (Fig. 4a and b). The apoptotic cells were also confirmed by caspase 3/7 activities, which significantly decreased in AGR2vH-overexpressing cells under ER stress inducing condition when compared with empty vector transfected cells (Fig. 4c). In addition, the mRNA expression of CHOP, ER stress-induced

apoptosis, was downregulated in AGR2vH-overexpressing cells, especially under ER stress inducing condition (Fig. 4d).

The effects of AGR2vH on cell survival

The mRNA expression of AGR2vH was upregulated in AGR2vH-overexpressing cells under ER stress inducing condition, and downregulated in AGR2vH-overexpressing cells with depleted of AGR2vH (Fig. 5a). The expression of AGR2vH correlated with the survival of CCA cells, which were determined by CCK-8. The survival cells was increased in AGR2vH-overexpressing cells under ER stress inducing condition when compared with empty vector transfected cells, while the cell viability of AGR2vH-overexpressing cells with depleted of AGR2vH under ER stress inducing condition was decreased when compared with AGR2vH-overexpressing cells under ER stress inducing condition (Fig. 5b).

Discussion

AGR2vH form aberrant splicing of AGR2 promotes the metastatic phenotypes of CCA cells. AGR2vH is predicted to be translatable a 67 amino acids protein isoform. In addition AGR2vH presented to contribute with the migration and invasion of CCA cell (Yosudjai et al. 2018). The dimerization of AGR2 is required to activate the UPR pathway by interaction with BiP/GRP78 for recovery of cellular ER stress and increases the survival of cancer cell (Ryu et al. 2013). Prospectively, AGR2vH might serve as an alternative partner molecule, which may interact easier with BiP/GRP78 for activating UPR pathway when ER stress occurs in cancer cells.

For verification of ER stress, BiP/GRP78, protein chaperone which activated UPR pathway, was upregulated as well as XBP1 will be spliced, which removes a 26 nucleotides intron from XBP1mRNA, to XBP1s leading to activate the expression of chaperon protein (Wang et al. 2014) as also in our study that both BiP/GRP78 and XBP1 were upregulated. In addition, AGR2 was upregulated in ER stress condition for facilitation of protein folding in the cell (Dumartin et al. 2017) as in our study reported that AGR2 is induced.

The activation of UPR pathway under ER stress condition can follow by three ER transmembrane receptors, including IRE1, ATF6 and PERK (Ron and Walter 2007). In our study, we investigated the expression of XBP1u/XBP1s (XBP1-unspliced/XBP1-spliced) downstream of IRE1. AGR2vH-overexpressing cells downregulated of XBP1u while upregulated of XBP1s, that induces expression of genes involved in restoring protein folding such as BiP/GRP78, protein disulfide isomerase (PDIs) (Suh et al. 2012). A previous

study showed the expression levels of eIF2a, downstream of PERK, was upregulated after tunicamycin treatment (Dioufa et al. 2010) as in our study reported that eIF2a was upregulated under ER stress induction by 2 µg/ml of tunicamycin and significantly upregulated in AGR2vH-overexpressing cells. Moreover, in this study the expression of GPR94 that downstream targets of ATF6 (Yoshida et al. 1999; Yamamoto et al. 2007) was upregulated correlated with the expression of ATF6.

In addition, the expression of CHOP, a molecule involved in ER stress-induced apoptosis, was low expression under non-stressed conditions but overexpression under ER stress condition (Nishitoh 2012). In our study, the expression of CHOP in normal condition of CCA cells was low expression and upregulation under ER stress inducing condition, but downregulation in AGR2vH-overexpressing cells that describable AGR2vH involved about CCA cells survival.

In conclusion, the upregulation of AGR2vH activated UPR pathway and UPR downstream markers expression for decreasing apoptotic cells via decreased caspase-3/7 activity and contributed to the survival of CCA cells especially under ER stress inducing condition by activated of the UPR pathway and UPR downstream. These studies could form the basis of knowledge which supported the possibility for applying this molecule to be an alternative therapeutic targeted for CCA.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Figure legends

Fig. 1 The expression of AGR2vH in AGR2vH-overexpressing cells. **a** Semi-quantitative RT-PCR **b** Quantitative real-time PCR of AGR2vH in KKU-213 (untransfected control, empty vector transfected- and AGR2vH-overexpressing cells) and KKU-213L5. The data in b are shown as mean ± SD of biological triplicate, **P<0.05*

Fig. 2 Effects of tunicamycin on expression of ER stress markers. **a** Expression of XBP1 (XBP1u and XBP1s) using semi-quantitative RT-PCR and expression of XBP1s using quantitative real-time PCR. **b** Expression of BiP/GRP78 after 24 h tunicamycin treatment with 0.5, 1, 2, and 4 μg/ml using semi-quantitative RT-PCR and quantitative real-time PCR. All data are shown as mean ± SD of biological triplicate, **P<0.05, **P<0.01*

Fig. 3 Activation of the UPR pathway and UPR downstream markers by overexpression of AGR2vH. **a** mRNA expression of three ER stress markers, including XBP1 (XBP1u and XBP1s), ATF6 and eIF2a, were upregulated in AGR2vH-overexpressing cells, especially under ER stress induction by 2 μg/ml of tunicamycin. **b** mRNA expression of GRP94, an ER chaperones that downstream of UPR pathway, was upregulated in AGR2vH-overexpressing cells, especially under ER stress induction by 2 μg/ml of tunicamycin

Fig. 4 Overexpression of AGR2vH decreased cancer cells apoptosis in ER stress inducing condition. **a** Comparative determination of apoptotic cells of AGR2vH-overexpressing cells. Reduction of apoptotic cells population, particularly in a cell population in late apoptosis, was presented in AGR2vH-overexpressing cells under ER stress inducing condition. **b** Quantitative analysis of the total apoptotic cells of AGR2vH-overexpressing cells. **c** Detection of caspase 3/7 activity of AGR2vH-overexpressing cells. Decreasing of caspase 3/7 activity was measured in AGR2vH-overexpressing cells under ER stress inducing condition. **d** mRNA expression of CHOP, an ER stress-induced apoptosis, was downregulated in AGR2vH-overexpressing cells, especially under ER stress induction by 2 μg/ml of tunicamycin. The data in b and c are shown as mean ± SD of biological triplicate, ***P<0.01, ***P<0.001*

Fig. 5 Overexpression of AGR2vH contributed cancer cell survival. **a** mRNA expression of AGR2vH was upregulated in AGR2vH-overexpressing cells under ER stress induction by 2 μg/ml of tunicamycin, and

downregulation in AGR2vH-overexpressing cells with depleted of AGR2vH. **b** The cell viability of AGR2vH-overexpressing cells under ER stress inducing condition was increased when compared with empty vector transfected cells; while the cell viability of AGR2vH-overexpressing cells with depleted of AGR2vH under ER stress inducing condition was decreased when compared with AGR2vH-overexpressing cells under ER stress inducing condition. The data in b are shown as mean ± SD of biological triplicate, ***P<0.01, ***P<0.001*

Figure 1

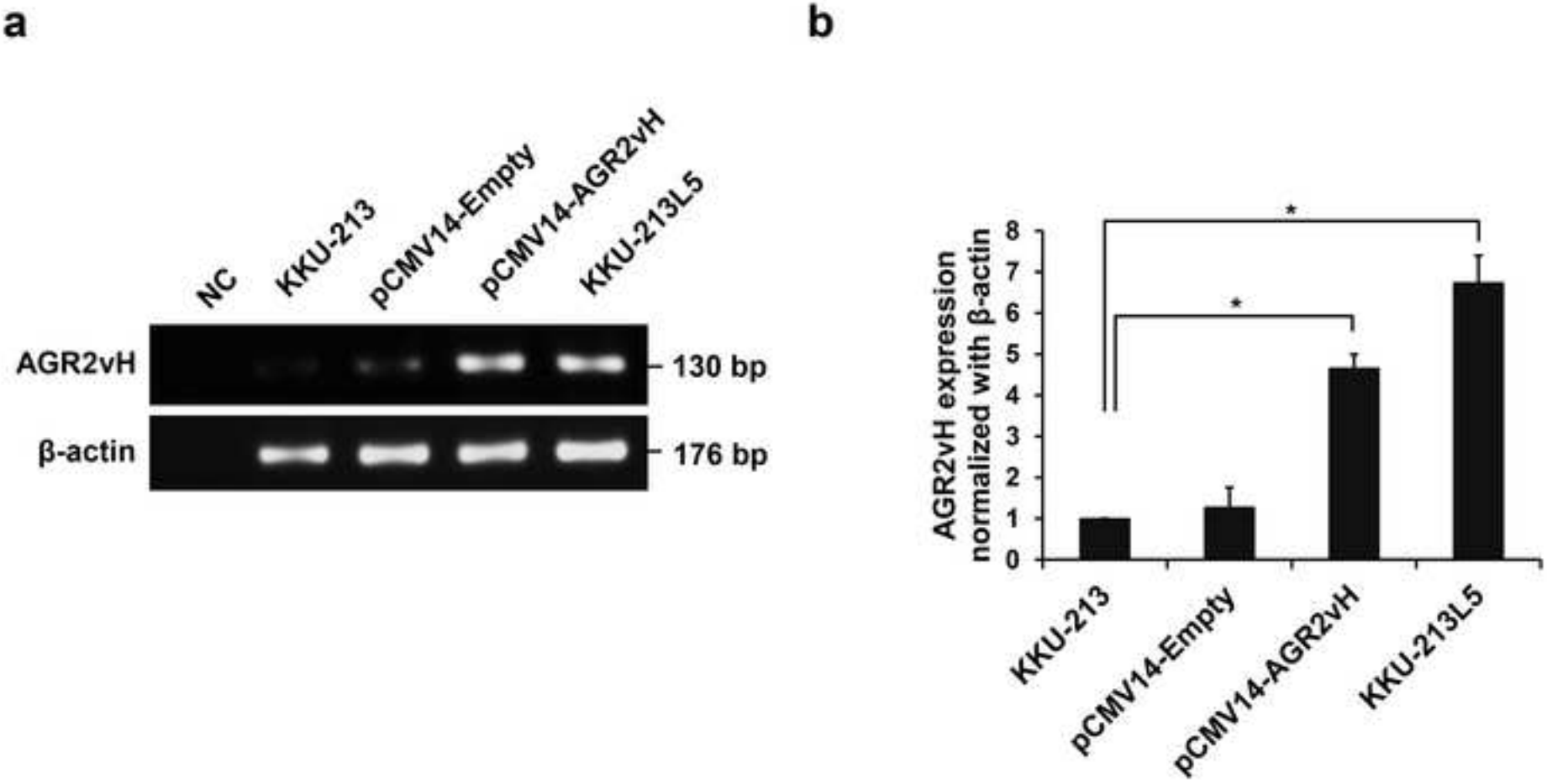

Figure 2

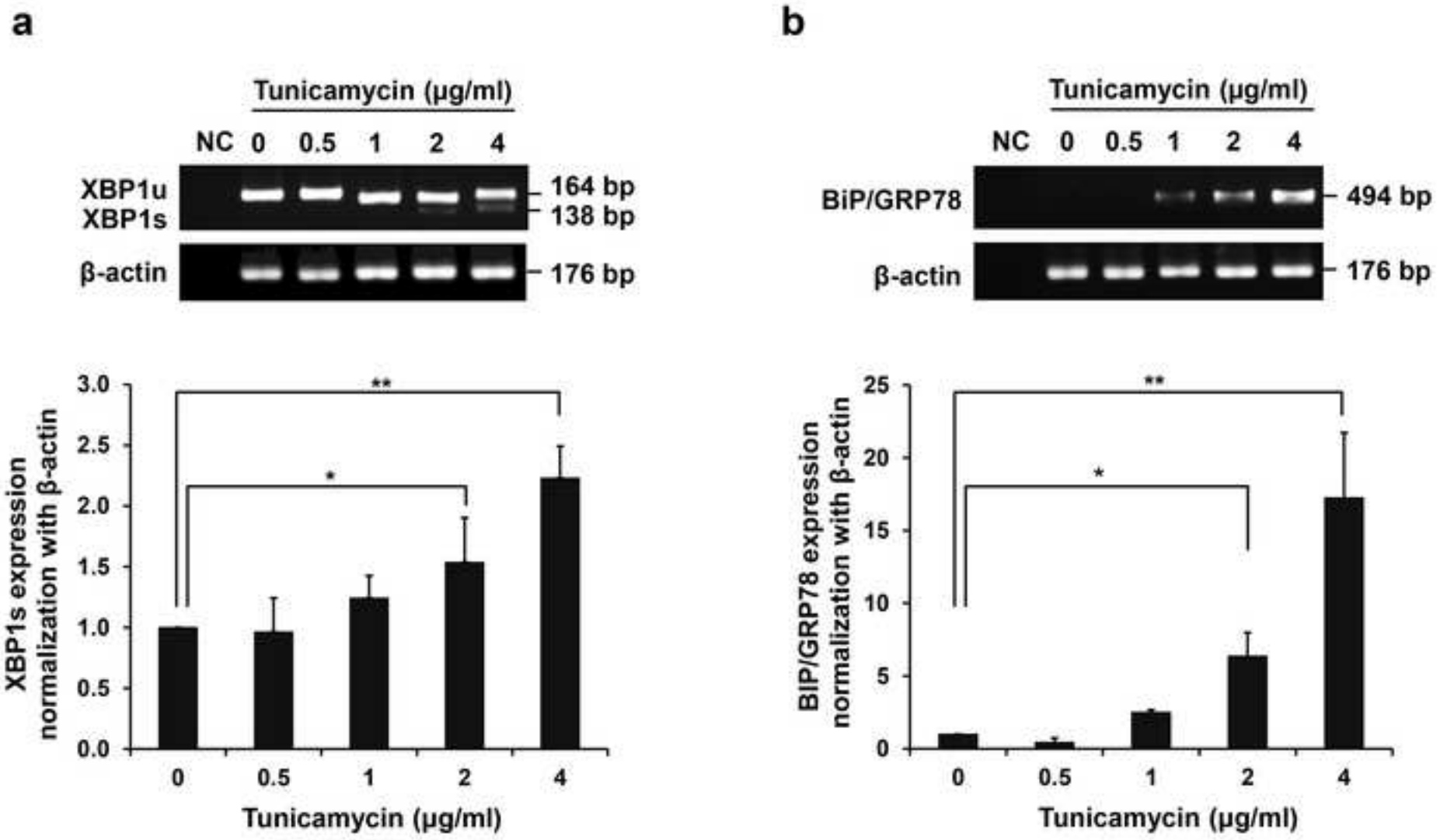

Figure 3

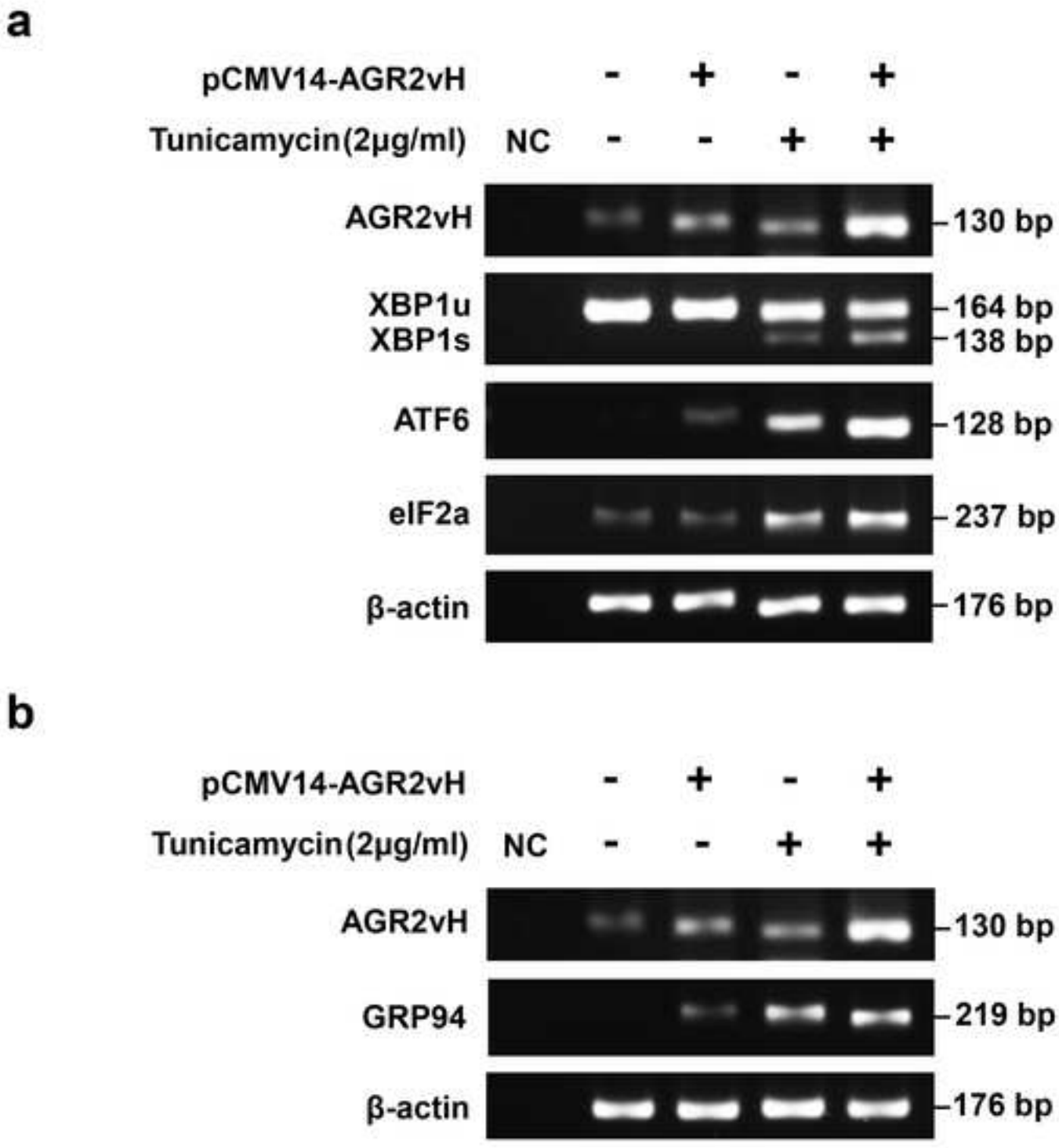

Figure 4

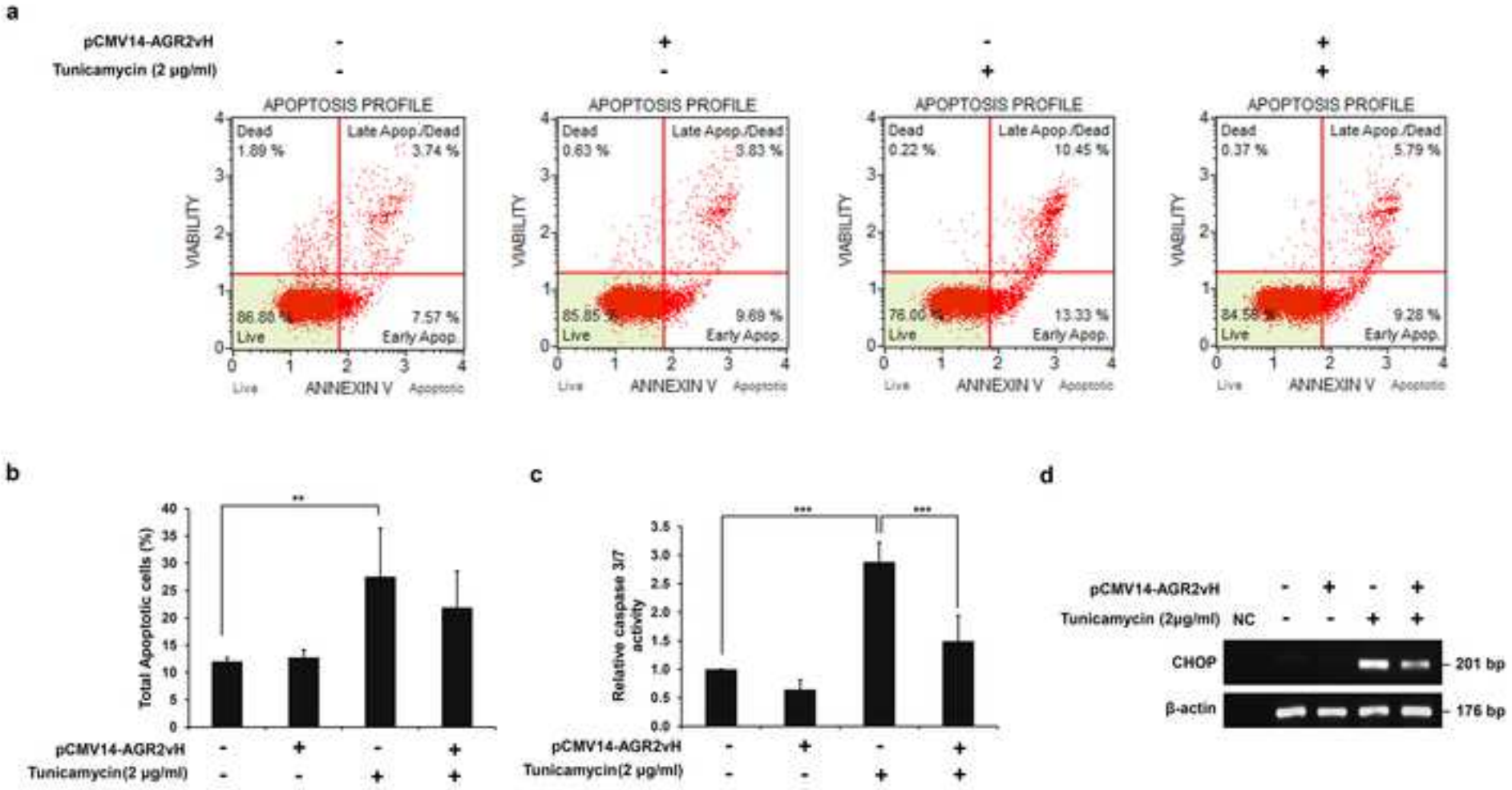

Figure 5