Suppression of Anterior Gradient-2 Splice Variant H Decrease *in vitro* Migration of High Metastatic Cholangiocarcinoma Cells

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Abstract

Cholangiocarcinoma (CCA) is the epithelial cell malignancy arising within biliary tree. Many patients were often diagnosed when the cancer progresses into high aggressive stage which generally metastasizes to lung, lymph node or other secondary organs. Previous report demonstrated the mRNA expression of 77 metastatic-associated genes in high metastatic CCA cells (KKU213L5), compared with the parental CCA cells (KKU213), and found that Anterior gradient-2 (AGR2) was the highest up-regulated gene. AGR2 encodes for a disulfide isomerase enzyme which typically expressed in mucus-secreting tissues. In addition, aberrant splicing of AGR2 was reported and maybe used as a surrogate biomarker for prostate cancers. This study explored the alternative splicing of AGR2 and investigated the roles of candidate spliced transcript on the proliferation and migration activities of CCA cells. We found that AGR2vE, AGR2vF and AGR2vH were up-regulated in KKU213L5 cells, but only AGR2vH transcript is predictable to translate into protein isoform. Roles of AGR2vH in the *in vitro* cell proliferation and migration were evaluated using small interfering RNA. The results presented that suppression of AGR2vH had not altered the proliferation but had decreased the migration of KKU213L5 cells. The further investigation will be proposed for performing the AGR2vH overexpression and the functional studies *in vivo*. The better understanding of AGR2 aberrant splicing raises the possibility of using AGR2vH as an alternative prognostic indicator for CCA and might be a pathogenic isoform which allows CCA metastasis.

Keywords**:** *Cholangiocarcinoma, Anterior gradient-2, Alternative splicing*

บทคัดย่อ

โรคมะเร็งท่อน้ำดีเป็นโรคที่มีการเจริญแบ่งตัวอย่างผิดปกติของเซลล์เยื่อบุท่อทางเดินน้ำดีซึ่งมักตรวจวินิจฉัยพบผู้ป่วยในระยะรุนแรงหรือมีการแพร่กระจายของเซลล์มะเร็งไปยังปอดและต่อมน้ำเหลือง มีผลงานวิจัยศึกษาการแสดงออกของยีนที่เกี่ยวข้องกับกระบวนการแพร่กระจายของเซลล์มะเร็งจำนวน 77 ยีนในเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีที่มีการแพร่กระจายสูงชนิด KKU213L5 เปรียบเทียบกับเซลล์มะเร็งท่อน้ำดีชนิด KKU213 พบว่ายีน AGR2 มีการเพิ่มขึ้นของระดับการแสดงออกมากที่สุด โดยยีน AGR2 สามารถสังเคราะห์ได้เป็นเอนไซม์ในกลุ่มไดซัลไฟด์ไอโซเมอเรสซึ่งมักมีการแสดงออกในอวัยวะที่มีการหลั่งเยื่อเมือก นอกจากนี้ยังมีงานวิจัยบ่งชี้ว่าทรานสคริปต์ของยีน AGR2 ที่เกิดจากการตัดแต่งหลังกระบวนการถอดรหัสสามารถใช้เป็นตัวบ่งชี้ทางชีวภาพของโรคมะเร็งต่อมลูกหมากได้ ผู้วิจัยจึงทำการศึกษารูปแบบการตัดแต่งของยีน AGR2 และบทบาทของทรานสคริปต์ที่สำคัญต่อการเจริญแบ่งตัวและการเคลื่อนที่ของเซลล์มะเร็งท่อน้ำดี พบว่าทรานสคริปต์ AGR2vE AGR2vF และ AGR2vH แสดงออกสูงขึ้นในเซลล์ KKU213L5 แต่มีเพียงทรานสคริปต์ AGR2vH ที่ยังคงคุณสมบัติของการแปลรหัสเป็นโปรตีน เมื่อผู้วิจัยยับยั้งการแสดงออกของทรานสคริปต์ AGR2vH และติดตามพฤติกรรมการเจริญแบ่งตัวและการเคลื่อนที่ของเซลล์ KKU213L5 พบว่าการยับยั้งการแสดงออกของทรานสคริปต์ AGR2vH ไม่มีผลต่อการเจริญแบ่งตัวของเซลล์ แต่สามารถลดคุณสมบัติการเคลื่อนที่ของเซลล์ได้ ผลงานวิจัยนี้จะนำไปสู่การศึกษาต่อยอดโดยการเพิ่มระดับการแสดงออกของทรานสคริปต์ AGR2vH และการวิจัยในสัตว์ทดลองเพื่อการประยุกต์ใช้ระดับการแสดงออกของทรานสคริปต์ AGR2vH ในการพยากรณ์โรคมะเร็งท่อน้ำดี รวมถึงเป็นโมเลกุลสำคัญต่อกระบวนการแพร่กระจายของเซลล์มะเร็งท่อน้ำดี

คำสำคัญ: *มะเร็งท่อน้ำดี Anterior gradient-2 การตัดแต่งของยีนหลังกระบวนการถอดรหัส*

1. Introduction

Cholangiocarcinoma (CCA) is the epithelial cell malignancy arising from varying locations within biliary tree. This type of cancer can be divided into intrahepatic and extrahepatic CCA. The worldwide incidence of CCA presents high frequency in Asian countries, especially in Southeast Asia, which is associated with hepatic infections by human carcinogenic liver flukes, *Opisthorchis viverrini* (Ov) (Shin et al, 2010; Sithithaworn et al, 2014). The high prevalence of Ov infection and CCA has been reported in the

North-Eastern and also North part of Thailand. Diagnosis and treatment difficulty of CCA is concerned with the most often of late detection when the cancer progresses into an advanced stage when the metastasis has already occurred at lung, lymph node or other secondary organs (Fidler, 2003; Gores, 2003; Sripa and Pairojkul, 2008; Sripa et al, 2011). A recent report presented the expression profile of metastatic-associated genes in established metastatic cell model of CCA, high metastatic cells (KKU213L5) and parental cells (KKU213). Using customized Real-time RT-PCR array of 77 metastatic-associated genes, Anterior Gradient-2 or AGR2 was up-regulated as the highest expression level in KKU213L5 when compared with KKU213 (Uthaisar et al, 2016). Subsequently, AGR2 was verified in CCA tissues using immunohistochemistry and showed that it increased gradually in intrahepatic mass-forming type, but rarely expressed in normal bile duct. Similar to the previous study in hilar, extrahepatic and a subset of intrahepatic CCA displayed high AGR2 expressions correlated to the mucin producing and no AGR2 detected in typical hepatocellular carcinoma (Lepreux, Bioulac-Sage and Chevet, 2011).

Anterior Gradient-2 (AGR2) was first identified in *Xenopus laevis* as XAG-2 for specifying embryonic ectoderm developed to a cement gland (Aberger et al, 1998). In human, 13,304 bp of AGR2 gene on the seventh chromosome (GenBank accession number: NC_000007) is encoded for 996 bp of 8 exons mRNA (GenBank accession number: NM_006408) and translated into protein with 175 amino acids (GenBank accession number: NP_006399). Human AGR2 belongs to a member of protein disulfide isomerases (PDIs) family which involves the protein modification and structural folding. AGR2 is produced in a restricted number of normal tissues associating with normal processes of mucin secretion. In cancer, AGR2 is upregulated in the primary tumors of liver, pancreas, stomach, colon, urinary bladder, prostate, breast, female reproductive system, and respiratory system (Obacz et al, 2015). Upregulation of AGR2 can promote cell survival and proliferation in pancreatic cancer (Ramachandran et al, 2008), whereas downregulation of AGR2 contributed to the decrease of cell cycle progression and cell death in esophageal cancer (Pohler, Craig and Cotton, 2004).

In addition, AGR2 can be promoted *in vitro* metastasis of breast epithelial cells (Wang, Hao and Lowe, 2008). Therefore, inhibition of AGR2 may be suggested to be the targeted therapeutic strategy of cancers. Moreover, an alternative splicing of AGR2 gene was reported in prostate cancer which 6 spliced transcripts were demonstrated including AGR2wt, -vC, -vE, -vF, -vG and -vH). The clinical impact of these spliced transcripts is that AGR2vG and AGR2vH could be served as the prognostic biomarker in urine specimens and presented their higher sensitivity and specificity than former prostate specific antigen (PSA) (Neeb et al, 2014). The present study aimed to explore the expression profile of AGR2 splicing in metastatic cell model of CCA and the function involves in cell proliferation and migration activities of candidate spliced transcript that upregulated in high metastatic CCA cells using knockdown strategy. This study attempted to achieve the information for research continuing on AGR2 splicing in *in vivo* model as well as for further served as a prognostic biomarker or therapeutic target for CCA.

2. Objectives

This study aimed to explore the alternative splicing pattern of AGR2 in metastatic cell model of CCA and investigate the proliferation and migration changes of high metastatic CCA cells when suppression of the candidate spliced transcript using specific small interfering RNA (siRNA).

3. Materials and methods

Cell culture

KKU213 (parental CCA cell line) and KKU213L5 (high metastatic CCA cell line) were provided from Professor Dr. Sopit Wongkham, Department of Biochemistry, Faculty of Medicine, Khon Kean University, Khon Kaen to Dr. Suchada Phimsen, Department of Biochemistry, Faculty of Medical Science, Naresuan University. Both of them were sub-passaged and cultured in Dulbecco's Modified Eagle's Medium (Gibco, Thermo Fisher Scientific, Waltman, MA) supplemented with 10% w/v fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltman, MA), 100 Unit/ml of penicillin and 100 µg/ml streptomycin (Gibco, Thermo Fisher Scientific, Waltman, MA.), and maintained at 37°C in a humidified 5% CO_2 atmosphere.

RNA extraction and cDNA synthesis

The total RNA was isolated from both KKU213 and KKU213L5 cells using E.Z.N.A.® Total RNA Kit I (OMEGA bio-tek, Doraville, Georgia, USA) according to the manufacturer's recommendations. The 1 µg 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 will be stored in -20°C until use.

Reverse Transcription Polymerase Chain Reaction (RT-PCR*)*

PCR reactions were performed under the optimized condition. The reaction mixture contained 0.2 µg cDNA template, 0.4 µM of each forward and reverse primer in a total volume of 20 µl of 1×MyTaqTM HS Red Mix (Bioline, Taunton, Massachusetts). The PCR condition was initial denaturation 95°C for 5 min, followed by 30 amplification cycles with 30 s at 95°C, 30 s at annealing temperature and 30 s at 72°C and final step with 5 min at 72°C. The beta-actin (ACTB) 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 the intensity of each band was quantitated using ImageQuant TL 7.0 software as a semi-quantitative analysis normalized by the intensity of ACTB bands.

Table 1 Sequence of primers used in this study

Primer name	Forward primer Sequence 5' → 3'	Tm (°C)	Reverse primer Sequence 5' → 3'	Tm (°C)
AGR2ALL	TGAAGAAAGCTCTCAAGTTGCT	54.6	AGCCAGTGTTGTCACACTTCTT	54.2
AGR2wt	CGACTCACACAAGGCAGGT	53.5	TCCACACTAGCCAGTCTTCTCA	55.5
AGR2vC	CACAAGGCAGAGTTGCCATGG	56.5		
AGR2vE	ATCTGGTCACCCATCTCTGA	51.4		
AGR2vF	GGAAATCCAGACCCATCTCTG	52.3		
AGR2vG	AAGGCAGGTACAGCTCTG	51.1		
AGR2vH	CAGACATATGAAGAAAGCTCTCAAGT	56.6		
AGR2vD	GGTGGGTGAGGAAATCCAGCTTTA	56.5	AGATGGGTCAACAAACATAATCCTGG	55.9
ACTB	TCGTGCGTGACATTAAGGAG	53.3	GAAGGAAGGCTGGAAGAGTG	52.9

Silencing of AGR2 spliced transcript by small interfering RNA (siRNA)

The transfection procedure was performed using siAGR2vH (5'-CAGACATATGAAGAAAGCTC TCAA-3') and a negative control siRNA (Ambion, Thermo Fisher Scientific, Waltman, MA) using Lipofectamine™ 2000 (Thermo Fisher Scientific, Waltman, MA) according to the manufacturer's instructions. Briefly, KKU213L5 cells were seeded into 6-well plate for 2.5×10^5 cells/well and cultured in complete media at 37°C in a 5% CO_2 until the cell confluent is approximately 80%. Then, cells were transfected with 75 nmol of siAGR2vH and negative control siRNA in Opti-MEM I reduced serum medium (Gibco, Thermo Fisher Scientific, Waltman, MA) and incubated at 37°C in a 5% CO_2 for 6 h. The media were removed and replaced with fresh complete media. The untransfected cells were cultured in complete media. After 24 h, RNA was extracted for evaluation of the knockdown efficiency using RT-PCR and real-time PCR.

Quantitative real-time PCR

Quantitative real-time PCR was performed for evaluating the efficiency of siAGR2vH under the optimized condition. The 10 µl of reaction mixture contained cDNA template, forward and reverse primers and 1X LightCycler® 480 SYBR Green I Master (Roche Applied Science, Mannheim, Germany). The PCR condition was initial denaturation 95°C for 10 min, followed by 45 amplification cycles with 10 s at 95°C, 10 s at annealing temperature and 10 s at 72°C, followed by melting curve generated from 57°C to 95°C with 0.11°C/s and final step cooling. The beta-actin (ACTB) was used as an internal control for semi-quantitative normalization. All reactions were prepared in triplicate reactions and analyzed using the

LightCycler® 480 systems. The expression levels of the target genes will be normalized with reference to ACTB based on the relative quantification formula of $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Cell proliferation assay

The KKU213L5 cells were assigned into 3 groups for the cell proliferation assay including siAGR2vH transfected, control siRNA transfected and untransfected cells. The cell proliferation was determined by using 3-[4,5-dimethylthiazole]-2,5-diphenyltetrazolium bromide (MTT) assay. After siRNA knockdown for 24 h, cells were seeded into 96-well plate and cultured in complete media at 37°C in a 5% CO_2 for 24, 48 and 72 h. The 0.5 mg/ml final concentration of MTT solution was added and incubated at 37°C in a 5% CO_2 for 3 h. Then the supernatant was removed and 100 µl of dimethyl sulfoxide was added to dissolve the formazan crystals. The absorbance at 540 nm was determined for calculation of cell viability.

Wound healing assay

Three similar experimental cells were assigned in cell proliferation assay. Cells were seeded into 6-well plates and cultured in complete media for 24 h before creating a scratch wound on the monolayer of cells using a 100 µl-pipette tip. Cells were then incubated further in 1% FBS media and incubated at 37°C, 5% CO_2. Phase contrast images were captured at 0, 6, 12 and 18 h. The wound distance was measured as µm under microscope and the relative distance of cell migration change at indicated time points from 0 h was evaluated as previously described (Uthaisar et al, 2012).

Statistical analysis

Quantitative data were examined in triplicate. The mean values were calculated. Data are presented as the mean ± standard deviation. Unpaired Student's t-test (two tailed) was used for comparison between each group by SigmaPlot (SigmaPlot 11.0, Systat Software, San Jose, CA). $P<0.05$ was considered to be significant.

4. Results

Expression of AGR2 spliced transcripts in KKU213 and KKU213L5 sublines

The distribution of 7 spliced transcripts of AGR2 was performed using RT-PCR with a set of the specific primer (Figure 1a). We found that AGR2vE, AGR2vF and AGR2vH isoforms were up-regulated in KKU213L5 when compared with KKU213 (Figure 1b). Although 3 AGR2 spliced transcripts were upregulated, only AGR2vH is predicted to be translatable into protein using bioinformatics tool (web.expasy.org/translate). Therefore, AGR2vH is the candidate spliced transcript that we will further evaluate the functional activities in metastasis of CCA.

Suppression of AGR2vH using specific siRNA.

After transfection with siAGR2vH and control siRNA into KKU213L5, the RT-PCR was performed for evaluating the knockdown efficiency compared with untransfected cells. The mRNA expression level of siAGR2vH transfected KKU213L5 was significantly decreased when compared with control siRNA transfected and untransfected cells (Figure 2a). This suppression was confirmed by using quantitative real-time PCR (Figure 2b).

Suppression of AGR2vH had not effect on *in vitro* cell proliferation

After the cells had been transfected by siAGR2vH, the cell proliferation was analyzed by MTT assays and monitored for 24, 48 and 72 h. After measuring the viability of cells, statistical analysis revealed that the cell proliferation rate was not significant different between siAGR2 transfected, control siRNA transfected and untransfected KKU213L5 (Figure 3). Therefore, the suppression of AGR2vH did not alter the proliferation of KKU213L5 cells (Figure 3).

Suppression of AGR2vH decreased *in vitro* cell migration

After the cells had been transfected by siAGR2vH, the cell migration was examined by wound healing assay and monitored the migrating cells in 6 h-interval until the physically wounded was closed. After measuring the migration distance and analyzing the relative migration activity, the siAGR2vH transfected cells presented a slower rate of migration rate and the wound remained until 18 h monitoring while the wound of untransfected cells was absolutely closed and control siRNA transfected cells was partially closed (Figure 4a). We found that the relative migrating change of siAGR2vH transfected cells was significantly lower than control siRNA transfected and untransfected KKU213L5 cells (Figure 4b). Therefore, the suppression of AGR2vH can decrease the migration activity of KKU213L5 cells.

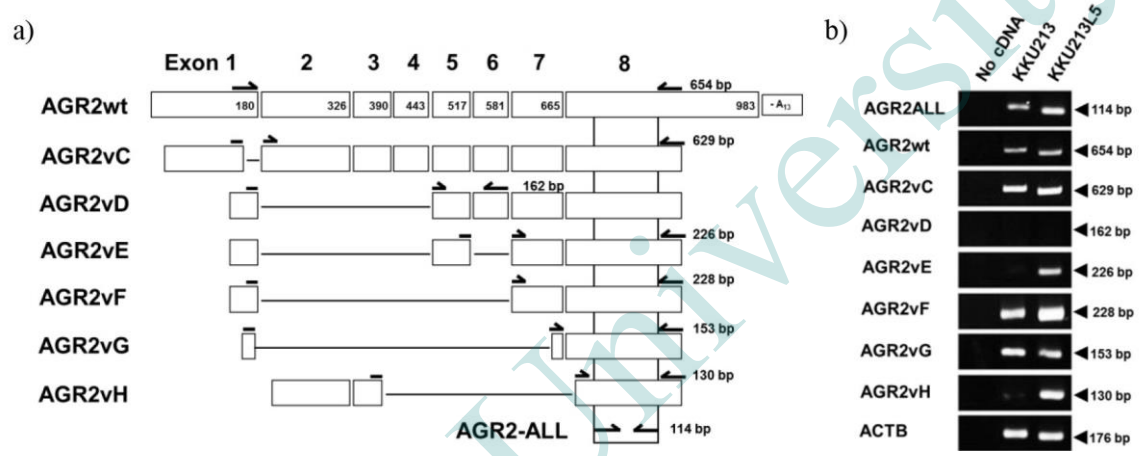

Figure 1 The expression of AGR2 spliced transcripts. Schematic representation of primer design and annealing site of 7 AGR2 spliced transcript (exons are presented as rectangles and each forward-reverse primer pairs are presented as forward and backward arrows) (a). Semi-quantitative of AGR2 spliced transcripts expressed in KKU213 and KKU213L5 sublines using RT-PCR (b).

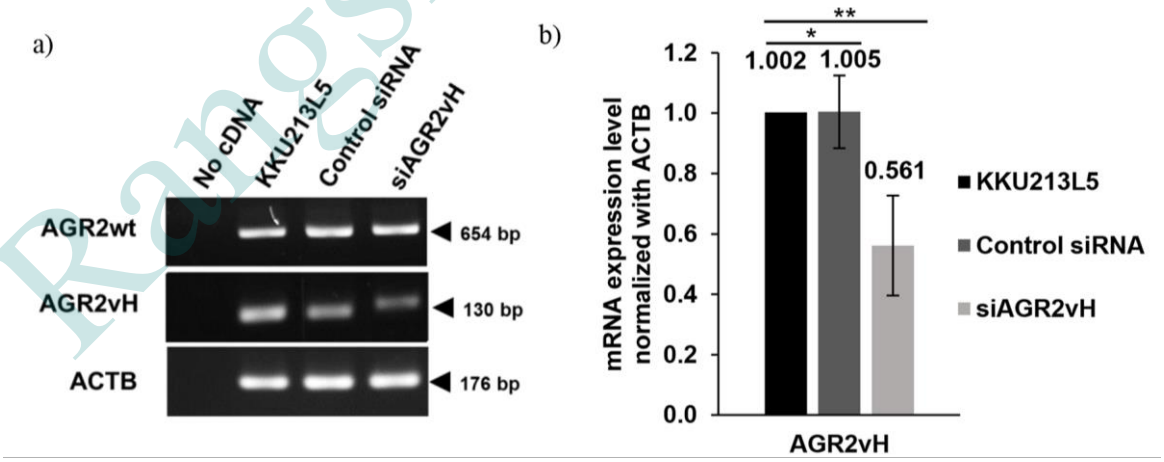

Figure 2 Suppression of AGR2vH using specific siRNA. AGR2vH mRNA was markedly decreased in siAGR2vH transfected cells compared with control siRNA transfected and untransfected cells when determined by RT-PCR (a) and quantitative real-time PCR (b); *P<0.05, **P<0.01.

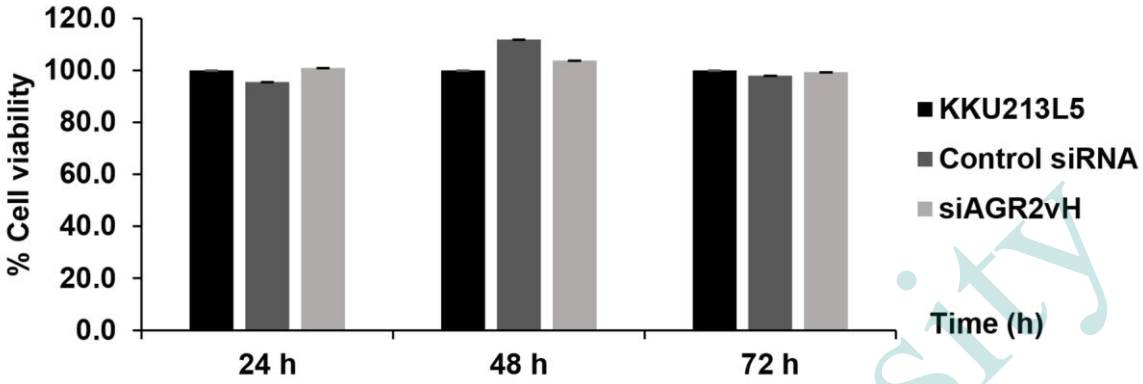

Figure 3 Suppression of AGR2vH had not effected on the proliferation of KKU213L5 cells until 72 h monitored.

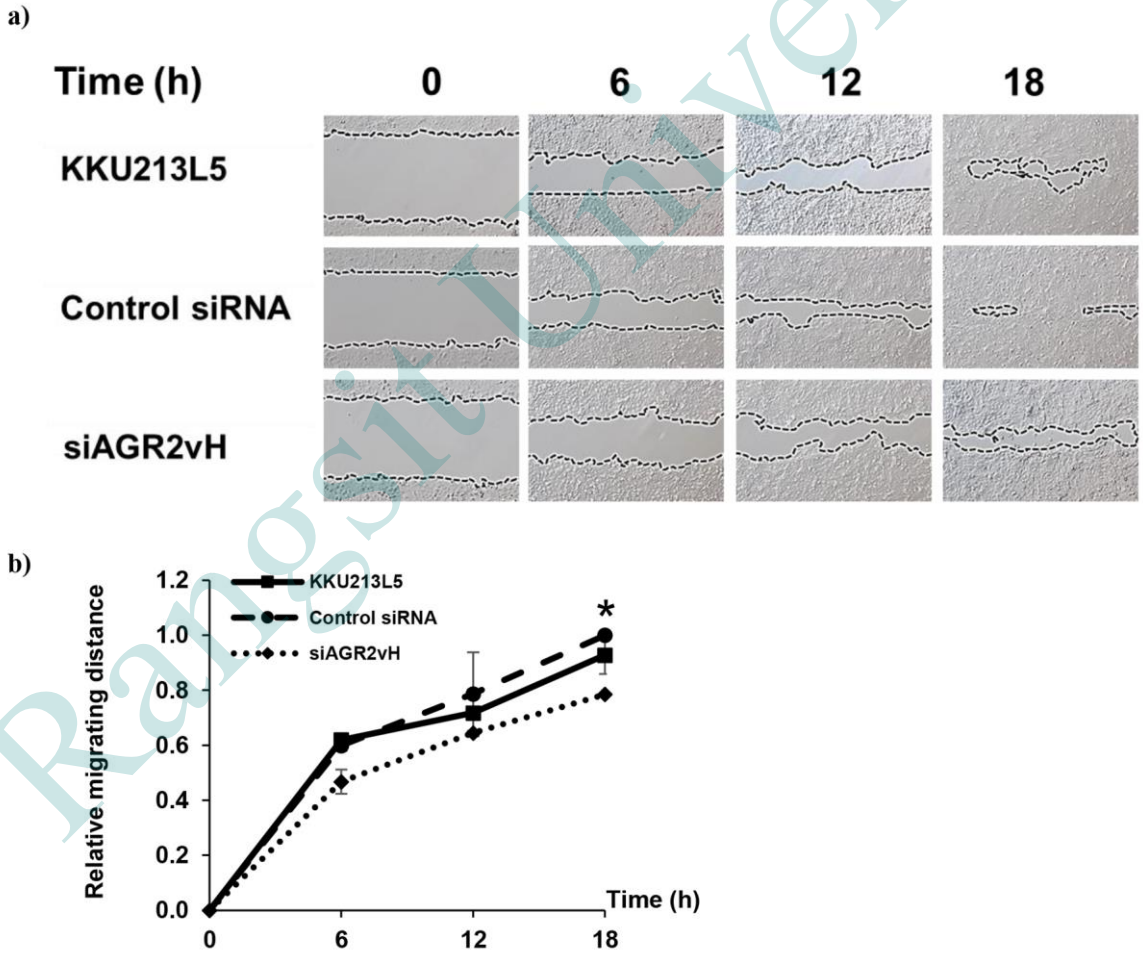

Figure 4 Suppression of AGR2vH inhibited migration activity of KKU213L5 cells. The physically wound was remained until monitored to 18 h in siAGR2 transfected cells whereas the wound of control siRNA transfected and untransfected cell were partial closed. The images of wound healing at 0, 6, 12 and 18 h (magnification, x4) (a) and the migration activities were calculated in relative migrating change (b); *P<0.05.

5. Discussion

CCA is a cancer-related death which is a serious health problem in Asian countries, especially in Thailand. A number of mortality cases are often caused by extensive metastasis of cancer cell to other secondary organs, such as lung and lymph node resulting in very poor prognosis and treatment difficulty. An additional molecular process of gene expression, the alternative splicing, presents the strongly association with many types of cancers (He et al, 2009) and is importantly related to the cancer cell aggressiveness (David and Manley, 2010; Kim and Kim, 2012). In this study, we attempted to demonstrate an association of aberrant splicing of AGR2, the highest upregulated gene in established high metastatic CCA cells, with the basic behaviors of cancer cells, such as the proliferation and migration. Previous study presented 6 AGR2 spliced transcripts in prostate cancer including AGR2wt, -vC, - vE, -vF, -vG and -vH which the AGR2vH can be served as the potential diagnostic biomarker for the non-invasive detection of prostate cancer using urine specimen (Neeb et al, 2014). Our result showed the 3 spliced transcripts of AGR2 including AGR2vE, AGR2vF and AGR2vH were upregulated in high metastatic CCA cells (Figure 1b). The bioinformatics tools were used for prediction of translatable of them and only AGR2vH can be translated into a 67 amino acids-protein isoform. The amino acid substitution occurred at the dimerization motif and PDI domain is spliced out from predicted AGR2vH isoform. Based on these structural changes and the upregulated expression level, we considered depleting AGR2vH using specific siRNA and monitored its biological effect in high metastatic CCA cells.

The result of siRNA knockdown considered siAGR2vH can suppress AGR2vH approximately 50%. About the knockdown efficiency, previous study reported that estimated 50% efficiency of siCLDN4 knockdown could inhibit claudin-4 expression and suppress the migration and invasion in CCA cells (Bunthot et al, 2012). We concerned about the interfering of siRNA to wild-type mRNA (AGR2wt), but our experiment confirmed that siAGR2vH can deplete only AGR2vH and cannot alter the AGR2wt (Figure 2a and 2b). Followed by the 2 assays on cell behaviors, the proliferation of high metastatic CCA cells was not changed after siAGR2vH transfection (Figure 3), but the migration was significantly changed when compared with control siRNA transfected and untransfected cells (Figure 4a and 4b). Therefore, the AGR2vH plays a role in cell migration which can enhance the metastatic potential of CCA cells, but we need to further investigate the involvement of this molecule on the other phenotypes in the multi-step of cancer metastasis.

A number of genes were reported their aberrant splicing in CCA and presented their functional involves with the cancer aggressiveness including WISP1v of Wnt-inducible secreted protein 1 (Tanaka et al, 2003) and PKM2 of Pyruvate kinase (Yu et al, 2015) contributed to neural and lymphatic invasion, CD44v6 and CD44v8-10 of CD44 related to cancer cell proliferation and anti-apoptosis (Yun, Yoon and Han, 2002). In addition, several studies also suggested the association of spliced transcript/wild-type ratio can use as clinical prognostic indicator for CCA patients such as Trefoil factor 2 which low ΔEX2TFF2/wtTFF2 ratio correlated with the shorter survival (Kamlua et al, 2012) whereas Tumor protein 53 which low Δ133p53/TAp53 ratio correlated with longer overall survival (Nuttasirikul et al, 2013). Therefore, we attempted to examine the possibility of AGR2vH/AGR2wt ratio for further applicable to being served as a prognostic indicator. Finally, the research investigated the mechanism of AGR2vH in CCA metastasis and the upstream regulation of this molecule is interesting for our investigation.

6. Conclusion

Anterior gradient-2 or AGR2 is the member of protein disulfide isomerase family which significantly upregulated in established high metastatic CCA cells. A number of research studies present the alternative splicing of many genes produces the spliced transcripts and translate into the oncogenic proteins. To study the alternative splicing of AGR2 and the association with CCA metastasis, we demonstrated the upregulation of AGR2vH in high metastatic CCA cells, KKU213L5, to be a candidate molecule for our study of its functional involving cell proliferation and migration. Suppression by specific siRNA, the knockdown efficiency was achieved before cell behavior assays. The depletion of AGR2vH in KKU213L5 presented the slower cell migration whereas it had not altered the cell proliferation. This is the first report that raises the possibility of applying AGR2 transcript variant H as an alternative diagnostic biomarker for CCA and might be a pathogenic isoform which allows CCA metastasis. The data will be the excellent

preliminary results for other metastatic phenotypes, such as invasion and adhesion as well as the experimental design for overexpression of this molecule in parental CCA cell or in the *in vivo* model.

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