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โครงการวิจัย เรื่อง “บทบาทของ ANTERIOR GRADIENT-2 ต่อการแพร่กระจายของมะเร็งท่อน้ำดี”

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ABSTRACT

Cholangiocarcinoma (CCA) is a high metastatic and poor prognostic cancer. Thailand has the highest incidence of CCA in the world. Most of CCA are diagnosed at the late stage when tumor has been metastasized, resulting in an effectiveness of treatment and the short survival of patients. Anterior gradient 2 (AGR2) protein, a disulfide isomerase regulates protein folding in endoplasmic reticulum, is highly expressed in various cancers, and it has been described to promote aggressive tumor features. However, little is known about the roles of AGR2 in CCA metastasis. In this study, we investigated the expression of AGR2 in metastatic CCA cell lines. We found that expression of AGR2 was strongly increased in highly metastatic CCA cell line (KKU-213L5) compared with its parental cell line (KKU-213). To explore the functional roles of AGR2 on metastasis of CCA, we generated the transient and stable knockdown of AGR2 in KKU-213L5 cells. The results showed that the level of AGR2 in knockdown cells was significantly suppressed compared with the control cells. Knockdown of AGR2 in KKU-213L5 significantly decreased cell proliferation. In addition, cell migration and invasion were also significantly reduced in AGR2 knockdown cells. Furthermore, the activities of matrix metalloproteinase2 and 9 were not altered in AGR2 knockdown cells. Taken together, our data showed that AGR2 plays an important role in progression of CCA by promoting cell proliferation, migration and invasion. Thus, AGR2 would be strongly considered to be a target for CCA therapy.

Keywords Cholangiocarcinoma, Anterior gradient 2, Metastasis

บทคัดย่อ

มะเร็งท่อน้ำดีเป็นมะเร็งชนิดแพร่กระจายสูงและมีการพยากรณ์โรคไปในทางแย่ พบอุบัติการณ์สูงสุดในโลกที่ประเทศไทย
โดยผู้ป่วยมะเร้งท่อน้ำดีส่วนใหญ่จะถูกวินิจฉัยโรคในระยะท้าย ซึ่งเป็นมะเร็งมะยะแพร่กระจายแล้ว ส่งผลให้ประสิทธิภาพของการรักษาและอัตราการรอดชีวิตของผู้ป่วยต่ำ โปรตีน Anterior gradient 2 หรือ AGR2
เป็นโปรตีนในกลุ่มเอนไซม์ไดซัลไฟด์ไอโซเมอเรสที่ควบคุมการม้วนพับของโปรตีนภายในเอนโดพลาสมิกเรติคูลัม พบว่ามีการแสดงออกสูงในมะเร็งหลายชนิด และมีการรายงานว่าสามารถส่งเสริมลักษณะความรุนแรงของมะเร็งได้
อย่างไรก็ตามยังไม่มีการศึกษาที่แน่ชัดถึงบทบาทของ AGR2 ต่อกระบวนการแพร่กระจายของมะเร็งท่อน้ำดีได้ ในงานวิจัยนี้จึงสนใจศึกษาการแสดงออกของ AGR2 ในมะเร็งท่อน้ำดีชนิดแพร่กระจายต่างๆ ผลการศึกษาพบว่าระดับการแสดงออกของ AGR2 สูงขึ้นในมะเร็งท่อน้ำดีชนิดที่ถูกพัฒนาให้มีความสามารถในการแพร่กระจายสูง (KKU-213L5) เมื่อเปรียบเทียบกับเซลล์มะเร็งท่อน้ำดีชนิดต้นแบบซึ่งมีความสามารถในการแพร่กระจายต่ำกว่า (KKU-213) ต่อมาผู้วิจัยทำการศึกษาบทบาทหน้าที่ของ AGR2 ต่อกระบวนการแพร่กระจายของเซลล์มะเร็งท่อน้ำดี
โดยทำการสร้างเซลล์ที่เกิดการยับยั้งการแสดงออกของ AGR2 ด้วยกระบวนการ RNA interference ทั้งแบบชั่วคราวและแบบถาวร
ผลการทดลองพบว่าระดับการแสดงออกของกลุ่มเซลล์ที่ถูกยับยั้งการแสดงออกของ AGR2 ลดลงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ
จากนั้นผู้วิจัยถึงนำเซลล์ที่ได้มาทำการศึกษาถึงคุณสมบัติต่างๆของเซลล์ที่เกี่ยวข้องกับกระบวนการแพร่กระจาย พบว่า ความสามารถในการเจริญเติบโต การเคลื่อนที่และการบุกรุกของเซลล์ในกลุ่มเซลล์ที่ถูกยับยั้งการแสดงออกของ AGR2 ลดลงอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับกลุ่มควบคุม
แต่ไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติ เมื่อทดสอบการทำงานของเอนไซม์ matrix metalloproteinase ชนิด 2 และ 9 การศึกษาครั้งนี้แสดงให้เห็นว่า AGR2 เป็นโมเลกุลที่มีความสำคัญต่อการพัฒนาความรุนแรงของมะเร็งท่อน้ำดี
โดยการส่งเสริมกระบวนการเจริญเติบโต การเคลื่อนที่ การบุกรุกของเซลล์ ดังนั้น AGR2 จึงอาจจะใช้เป็นโมเลกุลเป้าหมายในการพัฒนาการรักษาผู้ป่วยมะเร็งท่อน้ำดีได้

คำสำคัญ มะเร็งท่อน้ำดี , Anterior gradient 2, การแพร่กระจาย

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ABBREVIATIONS

%	=	percent
β	=	beta
κ	=	kappa
°C	=	degree Celsius
µg	=	microgram
µl	=	microliter
µm	=	micrometer
µM	=	micromolar
CCA	=	cholangiocarcinoma
cDNA	=	complementary DNA
Cp	=	crossing points
CO_2	=	carbon dioxide
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleotide triphosphate
ECL	=	enhanced chemiluminescent
EDTA	=	ethylenediaminetetraacetic acid
ECC	=	extrahepatic cholangiocarcinoma
et al.	=	alii/alia (Latin), and other people
FBS	=	fetal bovine serum
g	=	gram
h	=	hour
ICC	=	intrahepatic cholangiocarcinoma
kDa	=	kilodalton
mRNA	=	messenger ribonucleic acid
mg	=	milligram
mL	=	milliliter
mM	=	millimolar in minute
M	=	molar
ng	=	nanogram
nm	=	nanometer

ABBREVIATIONS (CONT.)

OV	=	*opisthorchis viverrini*
PBS	=	phosphate-buffered saline
PVDF	=	polyvinylidene fluoride
RNA	=	ribonucleic acid
RT-PCR	=	reverse transcription-polymerase chain reaction
SDS	=	sodium dodecyl sulfate
siRNA	=	small interfering RNA
shRNA	=	short hairpin RNA

CHAPTER I

INTRODUCTION

Background and rationale of the study

Cholangiocarcinoma (CCA) is a malignant tumor originated from bile duct epithelial cells and classified into intrahepatic and extrahepatic bile ducts. CCA incidence is the second most common liver cancer (Khan et al., 2002; Shaib et al., 2005). The WHO database indicated a global increase in CCA related mortality in countries in Europe, America, Australia and Asia (Patel, 2001). CCA is highly prevalent in the Northeast region of Thailand. It is generally accepted as the region with the highest incidence of CCA in the world and is a major public health problem in Thailand. Several risk factors have been identified for the development of CCA, including primary sclerosing cholangitis, liver fluke infection, choledochal cysts, and thorotrast exposure. Primary sclerosing cholangitis is the major risk factor in the western world (Yachimski & Pratt, 2008). In Asian countries, liver fluke infection is the major risk for CCA whereas infection of *Clonorchis sinensis* (CS), a liver fluke that is endemic in China, Korea, Japan and Taiwan, has been proposed as a probable cause of CCA (Hou, 1956). *Opisthorchis viverrini*, OV infection is prominent in Laos, Malaysia and the Northeast region of Thailand. The infection is acquired by eating raw freshwater and salt-fermented fish contaminated with encysted metacercariae of liver fluke leading to chronic inflammation associated with carcinogenesis and development of CCA (Haswell-Elkins et al., 1992). CCA is a highly invasive tumor which leads to an extremely poor prognosis. Early onset CCA cannot be detected. Most patients are diagnosed at an advanced or metastasis stage, where chemotherapy and surgery do not guarantee a better chance of survival. Metastasis is mostly the cause of death for CCA patients. Thus, finding specific targets for treating CCA patients at metastasis is important in extending life and reducing the mortality rate.

Current research seeks to identify key molecules involved in cancer metastasis. This is a complex mechanism containing several steps, including cell

growth, cell adhesion, cell migration, cell invasion, and angiogenesis. These processes depend on the cooperation of several molecules from synthesis by cancer cells to promote metastasis. Understanding of the molecular functioning is essential in the metastatic process of CCA. This could lead to the development of better treatments and a better chance of survival for CCA patients. Recently, highly metastatic CCA cell line (KKU-213L5) was established from the five cycles of lung metastasis post injection of parental CCA cell line (KKU-213). The result showed that the metastatic potential, namely the proliferation migration and invasion ability of KKU-213L5 was higher than the parental cell (KKU-213). The investigation of a gene potentially involved in the metastatic process of these two CCA cell lines using real-time PCR array showed that the Anterior gradient 2 (AGR2) gene was highest upregulated among 77 metastatic-associated genes in highly metastatic CCA cells compared with the parental cells (Uthaisar et al., 2016). It has been shown that AGR2 may be an important molecule that plays a functional role in promoting metastasis for CCA and could be a molecular target for CCA treatment.

AGR2 is classified as a member of the protein disulfide isomerase family (PDI), a group of endoplasmic reticulum (ER) resident proteins (Persson et al., 2005). AGR2 functions as a molecular chaperone in protein folding and maintains ER homeostasis. Several studies have reported that AGR2 was overexpressed in various cancers. It played an important role in promoting aggressive tumor features associated with a phenotype of cell proliferation, migration and invasion ability. Moreover, AGR2 protein has been detected in the serum of mucinous ovarian tumor patients by Western blot and ELISA methods. These results suggested that serum AGR2 could be used as a biomarker for diagnosis and prediction of mucinous ovarian cancers (Park et al., 2011). Wang et al., 2008 reported that conditioned media from an esophageal adenocarcinoma cell line expressing higher levels of AGR2 enhanced the migration of cells. It has been shown that AGR2 is overexpressed in both intracellular and extracellular spaces, suggesting that AGR2 might be molecular based, and involved in promoting cancer metastasis. However, little is known about the relationship between AGR2 and CCA. The Preliminary study of AGR2 involved with CCA was reported to be frequently expressed in hilar cholangiocarcinoma and found general expression in the epithelial cells of intrahepatic and extrahepatic

biliary tracts observed by immunohistochemistry (Lepreux et al., 2011; Liau et al., 2014). However, the functional role of AGR2 is associated with metastatic potential in CCA and has not yet been clarified. Therefore, this study aims to analyze the expression of AGR2 in both intracellular and extracellular spaces of CCA. Additionally, to investigate the functional roles of AGR2 in promoting metastasis in CCA. In this study, the functional roles of AGR2 were determined using AGR2 deleted cells with transient and stable knockdown. Then, the important metastatic related phenotypes of CCA were characterized including cell proliferation, migration, invasion and MMP-2 MMP-9 activities. These data will be the basic information of the molecular mechanism of AGR2 in metastatic CCA leading to the development of potentially targeted metastatic CCA.

Hypothesis

AGR2 is overexpressed in highly metastatic CCA cells and has significant roles in promoting metastatic phenotypes of the CCA cells.

Objectives of the study

1 . To determine the AGR2 expression of highly metastatic CCA cells, KKU-213L5 cell line, compared to parental cells, KKU-213

2 . To examine the metastatic activities of AGR2 associated phenotypes in metastatic CCA cells by knockdown of AGR2 expression

Conceptual framework and research design

This study was divided into two parts. The first part was to demonstrate the AGR2 expression of mRNA levels and protein levels in both intracellular and extracellular fractions of highly metastatic CCA cells (KKU-213L5) as compared with its parental cells (KKU-213). The second part was to investigate the functional roles of AGR2 in promoting the metastasis of the CCA cell line using transient and stable knockdown of AGR2, followed by determining the metastatic phenotypes, including cell proliferation, cell migration, cell invasion and MMP-2, MMP-9 activity.

Anticipated outcomes

1. The findings of this thesis would provide further information related to the role of AGR2 in promoting metastasis in CCA

2. The findings from this thesis would provide information for the further study of *in vivo* metastatic activities of AGR2 in CCA.

3. The findings of this thesis would give further information for the development of AGR2 as a targeted therapy of CCA, that is, the final goal of this work

Experimental design

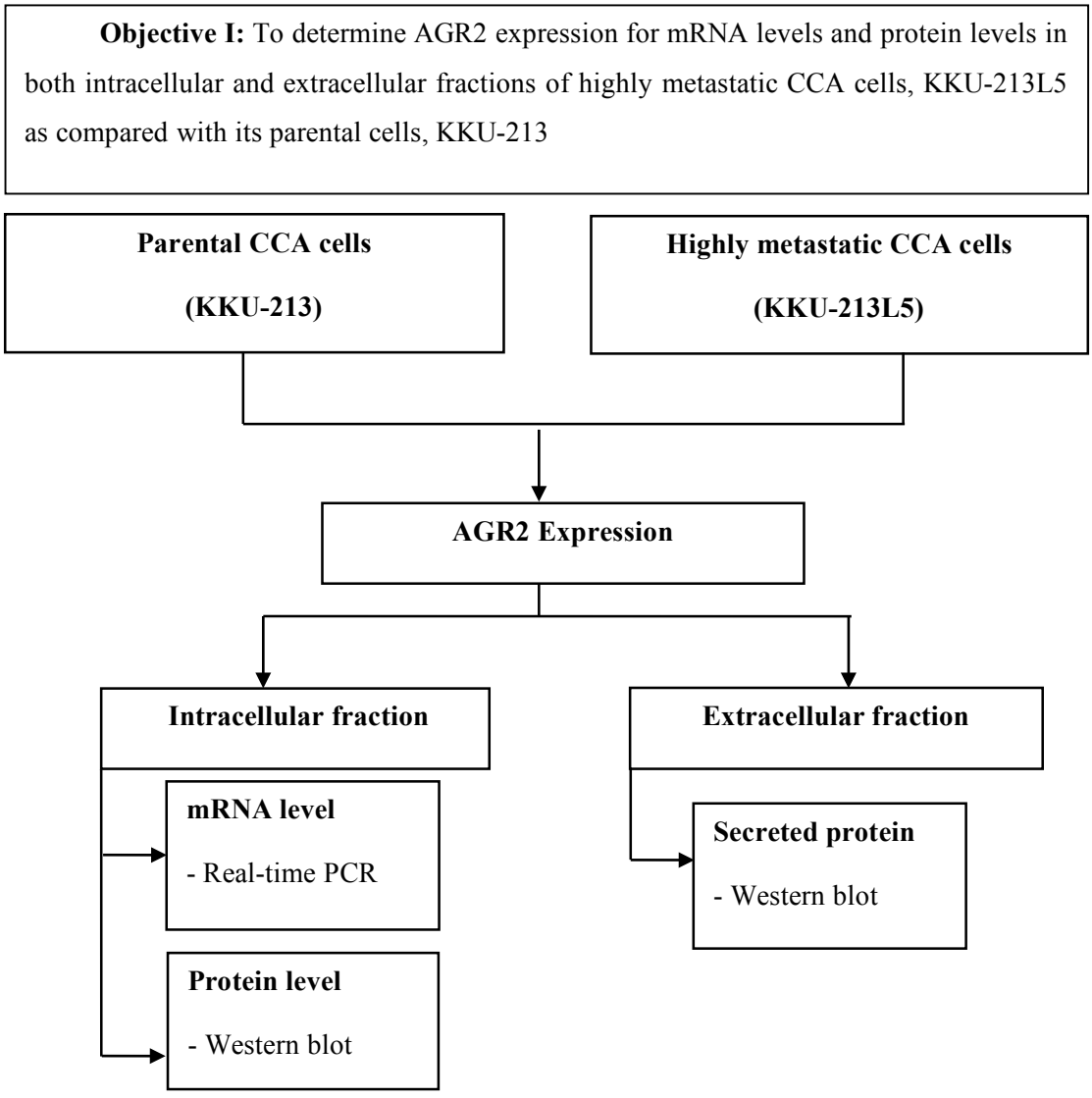


Figure 1 This schematic diagram shown experimental design of part one.

Objective II: To examine the functional roles of AGR2 in promoting CCA metastasis, after transient knockdown of AGR2 using small interfering RNA (siRNA) and stable knockdown of AGR2 using short hairpin RNA (shRNA), then subsequently characterizing the metastatic phenotypes which are cell proliferation, cell migration, cell invasion and MMP-2, MMP-9 activity

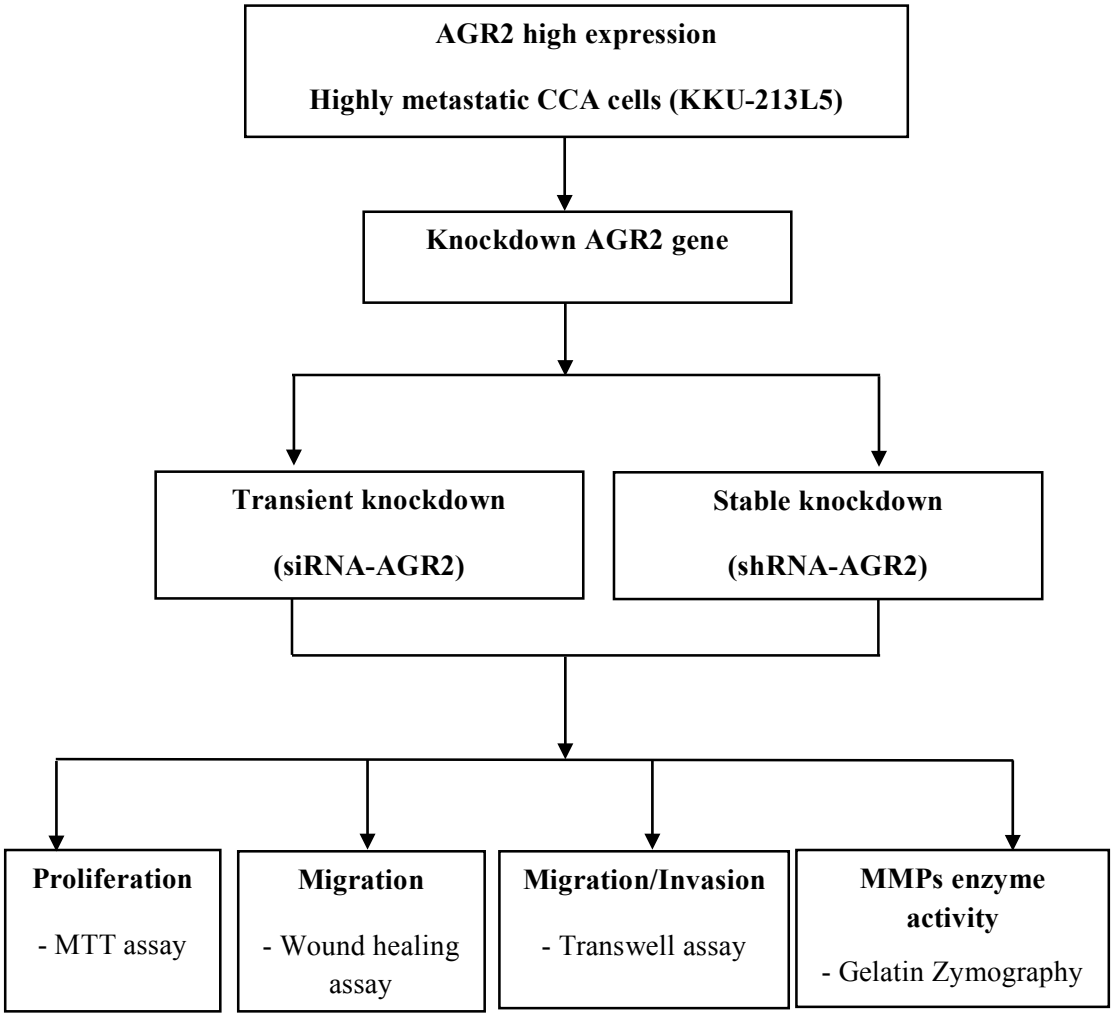


Figure 2 This schematic diagram shown experimental design of part two.

CHAPTER II

LITERATURE REVIEW

Cholangiocarcinoma

1. Definition and classification

Cholangiocarcinoma (CCA) also known as bile duct cancer is malignant tumor from epithelial cells that originate of the bile ducts can develop in any part of the bile duct and may cause different symptoms in different locations. Based on the location of the CCA is classified into two types, as shown in figure 3 (Malhi & Gores, 2006).

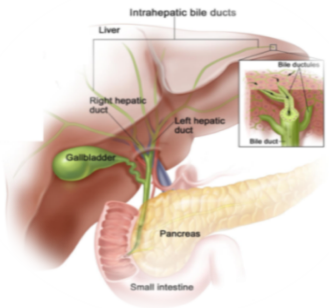
1. Intrahepatic bile duct cancer develops in the smaller bile duct branches inside the liver, as shown in figure 3A

2. Extrahepatic bile duct cancer develops in the ducts outside of the liver and ends in the small intestine, as shown in figure 3B. They are made up of two regions including

2.1 Perihilar bile duct cancer develops at the hilum region, where the left and right hepatic ducts have joined and are just leaving the liver.

2.2 Distal extrahepatic bile duct cancer is found in the distal region made up of the common bile duct which passes through the pancreas and ends in the small intestine.

A



B

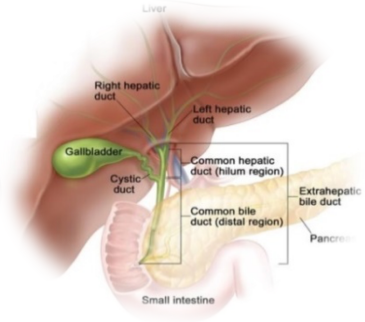


Figure 3 Definition of cholangiocarcinoma is classified into two types A. Type of the intrahepatic bile ducts B. Type of the extrahepatic bile ducts.

Source: Malhi & Gores, 2006

Further classification of both intrahepatic and extrahepatic bile ducts is details of the gross morphology, histology, mode of spreading, and clinical manifestations are outlined in figure 4. CCA have been identified based on their growth pattern; mass-forming (MF), periductal-infiltrating (PI), or intraductal-growing (IG) CCA (Lim & Park, 2004).

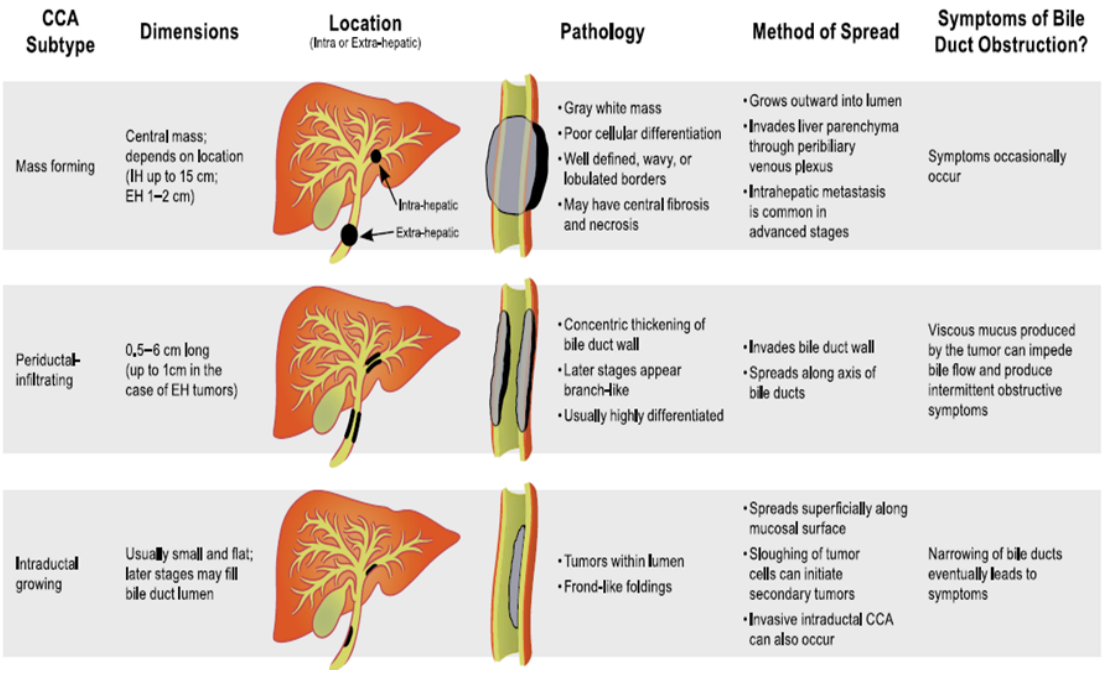


Figure 4 Classification of cholangiocarcinoma.

Source: Lim & Park, 2004

2. Epidemiology and etiology

There are several important risk factors for the development of CCA include primary sclerosing cholangitis (PSC), liver fluke infection, choledochal cysts, and exposure of carcinogen. PSC is the major risk factor for CCA in Western countries (Rosen et al, 1991) whereas the etiology of CCA in Asian countries appears to be mostly linked to infections, especially infections with the liver flukes *Clonorchis sinensis* (*C. sinensis*) and *Opisthorchis viverrini* (*O. viverrini*) (Kullavanijaya et al., 1999; Sithithaworn et al.,1994). *C. sinensis* a liver fluke endemically infected

in China, Korea, Japan and Taiwan, has been proposed to be a propable cause of CCA, (Hou, 1956) while *O. viverrini* is found mainly in Thailand, Lao, Cambodia and Vietnam (Kullavanijaya et al., 1999; Sithithaworn et al., 1994). The prevalence of CCA and liver fluke infection in these regions associated the traditional habit of eating raw freshwater and salt-fermented fish contaminated with encysted metacercariae of liver fluke on a daily basis are strong predisposing factors for the genesis of CCA (Khan et al., 2008). CCA is a major public health problem in Thailand especially Northeast region is generally accepted as the country with the highest incidence of CCA in the world. Epidemiological studies have revealed a correlation between CCA and *O. viverrini* infection in this region and often found to be higher in men than women, as shown in figure 5. This has been attributed to people within these regions to eat raw or undercooked fish that are infected with liver fluke cysts leading to the effects serious risk of carcinogenesis and development to CCA (Sithithaworn et al., 2012).

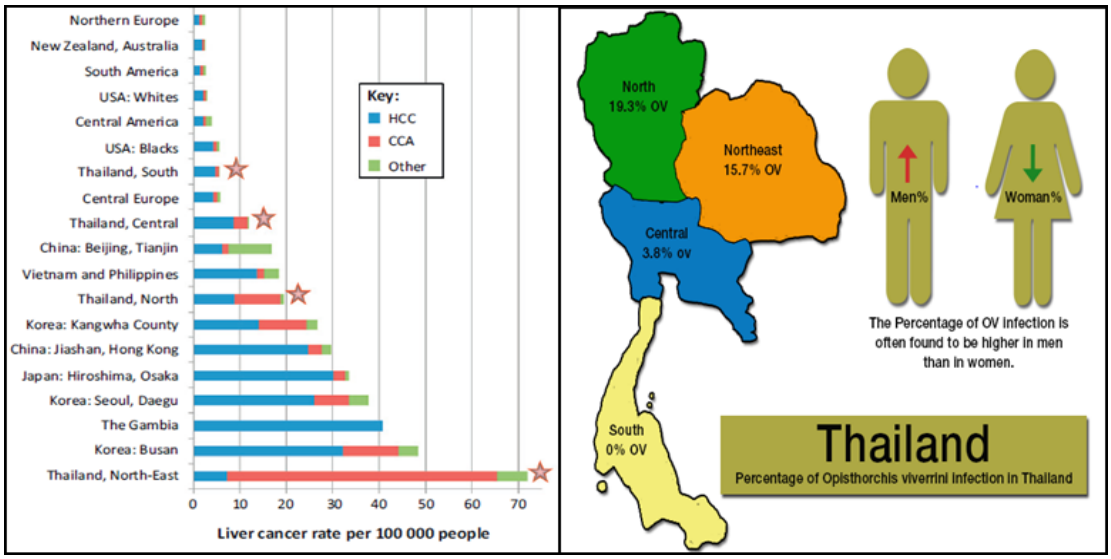


Figure 5 Incidence of liver cancer and prevalence of liver fluke infections. Liver cancer rates are divided into the major subtypes of hepatocellular carcinoma (HCC), cholangiocarcinoma (CCA).

Source: Sithithaworn et al., 2012

3. Clinical features

Cholangiocarcinoma is often referred to being a silent disease as symptoms may go unnoticed until the cancer is already in an advanced stage. Sometimes symptoms can appear sooner and lead to an early diagnosis. Symptoms of CCA are usually more severe and gradually worsen caused by bile duct obstruction lead to severe abdominal pain, the stool may become paler. Patients feel tired, uncomfortable and may feel a mass in their abdomen. However, symptoms of CCA are not common and these are more likely to be caused by something other diseases such as hepatitis c infection, cirrhosis, or gallstones (Malhi & Gores, 2006).

4. Diagnosis for cholangiocarcinoma

Diagnosis of cholangiocarcinoma is based on several techniques and used combination for accurate diagnosis and treatment.

Ultrasound is usually recommended as the first choice test for the investigation of patients with suspected cholangiocarcinoma used in the clinical diagnosis for tumor mass detection (Boberg & Schrumpf, 2004). In some cases, ultrasound can allow the tumor itself to be visualized as a rounded mass and also detect the presence of liver metastases as single or multiple rounded lesions in different return signals from the ultrasound.

Computed tomography scan (CT scan) uses x-rays to make detailed cross-sectional images of your body and very sensitive for detecting intrahepatic CCA larger than 1 cm. CT scan can often help diagnose CCA by showing tumors in the area and find out cancer has spread to distant organs (Teefey et al., 1988; Zhang et al., 1999).

Magnetic resonance imaging (MRI) with MR Cholangiopancreatography (MRCP) uses radio waves and strong magnets to create a three-dimensional image of the biliary tree, liver parenchyma, and vascular structures. It has mostly replaced CT in diagnosis and staging evaluation of CCA (Angulo et al., 2000; Oberholzer et al., 1998; Textor et al., 2002). They provide a great deal of detail and can be very helpful in looking at the bile ducts and nearby organs.

Tests of liver and gallbladder function from blood sample to find out how much bilirubin, albumin, and liver enzymes e.g. alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT) and certain other substances in your blood. These tests can indicate

bile duct, gallbladder, or liver disease. If levels of these substances are higher, it might point to blockage of the bile duct, but they can't show if it is due to cancer or some other reason (Bhudhisawasdi et al., 2004).

Several tumor markers are substances made by cancer cells that can sometimes be found in the blood may support the diagnosis of CCA. Patient of CCA may have high blood levels of the carcinoembryonic antigen (CEA) and tumor markers carbohydrate antigen (CA19-9) are most common have been suggested for diagnosis of CCA (Chen et al., 2002). Recently, serum MUC5AC mucin has been shown to be a potential marker for CCA with high specificity and sensitivity (Bamrungphon et al., 2007; Boonla et al., 2003; Wongkham et al., 2003). High amounts of these substances often mean that cancer is present. These tests can sometimes be useful after a person is diagnosed with CCA. If the levels of these markers are found to be high, they can be used a targeted therapy for diagnostic, follow-up, and treatment. In order to improve the accuracy of diagnosis therefore using the combination of several markers has been suggested for diagnosis of CCA. However, the recent gold standard for diagnosis of CCA is the histologic diagnosis, which determines the pathological change of the liver using the section or biopsy from tumor tissue.

5. Treatments for Cholangiocarcinoma

Treatment of cholangiocarcinoma will depend on the position and size of cancer and whether it has spread beyond the bile duct. There are several strategies used for CCA treatment as mentioned below.

Surgical resection is the preferred treatment to remove the cancer completely, but most patients present with advanced disease that is not amenable to surgical treatment. The choice to perform surgery with curative intent depends on the location of the tumor. However, only a few patients are suitable for surgery and therefore early coordination with a specialist surgical team is recommended (Khan et al., 2002).

Radiation therapy uses high-energy rays or particles to destroy cancer cells. The postoperative radiation therapy alone or in combination with chemotherapy following complete surgical resection is the strategy to control the local recurrence of CCA (Anderson et al., 2004).

Chemotherapy is drugs used to treat cholangiocarcinoma administered to those patients who are not operative candidates, and the results are largely disappointing. In some cases, two or more of these drugs may be combined to try to make them more effective. The responses to chemotherapy and median survival are summarized in Table 1 (Olnes & Erlich, 2004).

Table 1 Selected systemic chemotherapy trials for cholangiocarcinoma

Regimen	Response	Median survival	Reference
5-FU	1/12 (8%) PR	6.5 months	(Falkson et al.,1984)
5-FU, meCCNU	2/12 (17%) PR	2 months	(Falkson et al.,1984)
5-FU, Strepto	0/10 (0%)	3 months	(Falkson et al.,1984)
dFUR, LV	4/22 (18%) PR 1/22 (5%) CR	8 months	(Colleoni et al.,1984)
5-FU, LV, CBP	2/10(20%) PR 1/10 (20%) CR	4 months	(Sanz-Altamira et al.,1984)
5-FU, LV, MMC	5/20 (25%) PR	9.5 months	(Raderer et al.,1984)
5-FU, LV	7/28 (25%) PR 2/28 (7%) CR	8 months 15 months	(Choi et al.,1984)
5-FU, MMC, Dox	4/14 (28%) PR	8 months	(Harvey et al.,1984)
5-FU, IFN α-2b	19/24 (38%) PR	12 months	(Patt et al.,1984)

CBP = Carboplatin; CR = complete response; dFUR = doxifluridine; Dox = doxorubicin; 5-FU = 5-fluorouracil; LV = Leucovorin; meCCNU = methyl-CCNU; MMC = mitomycin C; PR = partial response; strepto = streptozotocin

However, CCA is a slow growing cancer with poor prognosis and is extremely aggressive. Early stage CCA often goes undetected. The majority of CCA patients have short survival due to diagnosed at the metastatic stage, of which current conventional treatment options for CCA are limited to chemotherapy and surgery are do not provide a better survival. Cancer metastasis is mostly the cause of death of CCA at advanced stage or metastasis stage.

Cancer metastasis

Metastasis is one of the most characteristic biological behaviors of cancer for circulation and the development of metastatic cancers at secondary sites. This is the most insidious and life-threatening aspects of cancer. Metastasis is the process consists of a series of sequential, interrelated steps. The initial step of cancer cells must be able to leave the primary tumor, invade the local host tissue, and survive at the secondary sites and while in transit, in order to proliferate successfully at the metastatic site. The capacity for metastasis may not be expressed initially or in all tumors hence our ability to cure most in situ lesions with a local intervention. However, despite significant improvements in diagnosis, surgical techniques, general patient care, and local and systemic adjuvant therapies, most patients deaths from cancer are due to metastases of cancer cells that are resistant to conventional therapies. The main barrier to the treatment of metastasis is the biological heterogeneity of cancer cells in the primary neoplasm and in metastases. Furthermore, the specific organ microenvironment can modify the response of metastatic cancer cells to systemic therapy. Therefore, Understanding the cellular pathogenesis and molecular mechanisms underlying of cancer metastasis is important to prevent metastasis and to search for an effective new targeted therapy for metastatic cancers.

Routes of dissemination

Cancer cells can spread through two main routes for leading to specific organ or secondary sites: blood vessels and lymphatic vessels are shown in figure 6.

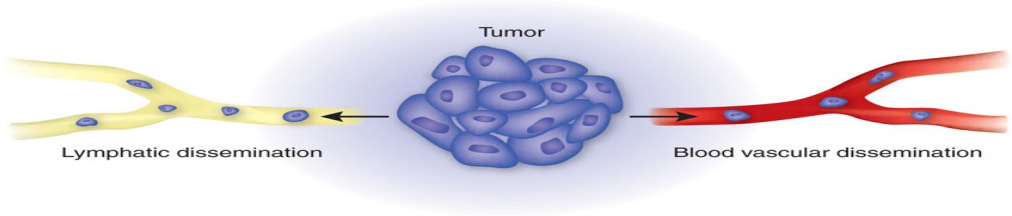


Figure 6 Routes of cancer cells often spread through blood vessels or lymphatic vessels and colonize in other organs.

Source: Cao, 2005

1. Blood metastasis

Travel via the blood circulation is often called hematogenous metastasis. If metastasizing cells survive these initial dangers and gain access to the larger vessels in the venous system, they will travel with the blood through the heart by must first pass through the right ventricle of the heart and thence through the lungs before it passes through the left ventricle and is pumped into the general arterial circulation, as shown in figure 7A. The lung is the most common organ of metastasis spreads because the metastatic cells can travel through the blood circulation from the other organs to the lungs where is abundant of oxygen and nutrients.

2. Lymphatic metastasis

Lymphatic system contains with small lymphatic vessels merge into larger ones and these large vessels eventually empty into lymph nodes. Lymph nodes are kidney bean shaped tissues that are found in grape-like clusters in several locations around the body. Lymphatic vessels have larger diameters than blood vessels and lacks tight junctions is present on a discontinuous layer of basement membrane. These structural features of lymphatics make them more accessible for cancer cells invasion and enable access to regional lymph nodes. Invasion of cancer cells into lymphatic vessels might therefore be easier than invasion into blood vessels.

If metastasizing cells survive these initial dangers and gain access to the larger vessels in the lymphatic system, they will travels through a network of small and large lymphatic vessels that are in some ways similar to the blood vessels are shown in figure 7B (Cao, 2005).

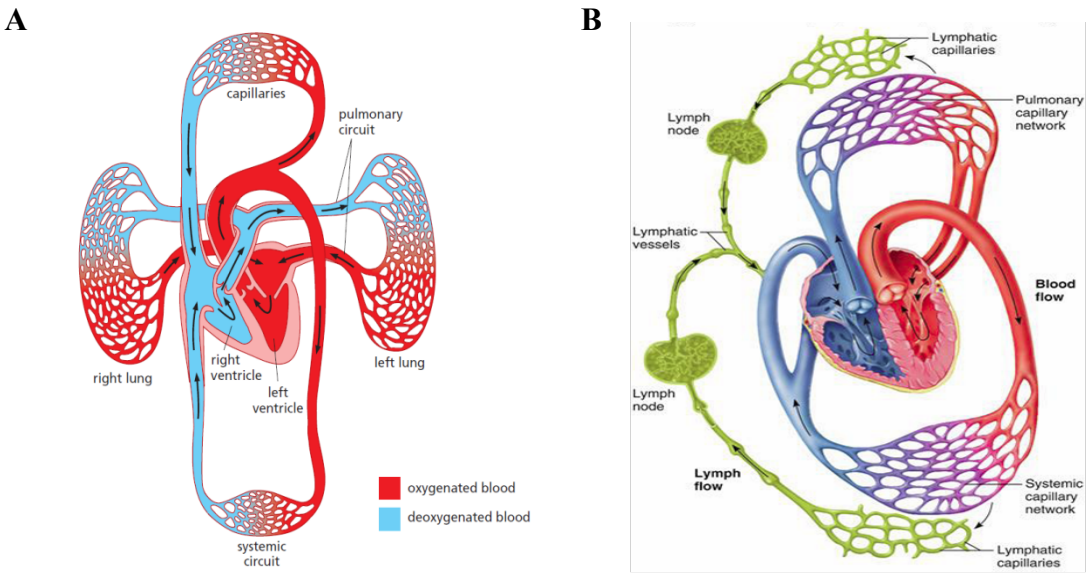


Figure 7 Major routes of blood (A) and lymphatic (B) circulation through the body.

Source: Raven et al., 2005

Steps of cancer metastatic process

To metastasize, cancer cells must complete all steps of the metastasis cascade (Fidler, 2002). Metastasis cascade describes seven distinct steps to the overall process. These include 1. Transformation; cancer cells growing within the primary tumor must be progressive, with nutrients for the expanding tumor mass initially supplied by simple diffusion. 2. Angiogenesis; a tumor mass larger than 0.125 mm^2 has outgrown its capacity to acquire nutrients by initiate angiogenesis through host vessel initiation of capillary sprouts in the direction of the tumor. Many autocrine growth factors for tumors may also act as synthesis and secretion of angiogenic factors establish a capillary network from the surrounding host tissue. 3. Invasion; cell or group of cells able to leave the primary tumor, they are localized invasiveness.

4. Intravasation; cancer cells move through the walls into either lymphatic or blood vessels. Then, cancer cells release of single cells or metastasizing cells in the circulation, they will travel with the vessels and gain access to the larger vessels. 5. Adhesion; cancer cells have survived the circulation they become trapped in the vessels beds of distant organs by adhering either to basement membrane that might be exposed. 6. Extravasation; once lodged in the vessels of various tissues, cancer cells must escape from the immune system and penetrate into the surrounding tissue. 7. Micrometastases; the latter then transport these cancer cells to secondary sites and form dormant micrometastases, in order to proliferate successfully at the metastatic site. Steps of cancer metastatic process are shown in figure 8

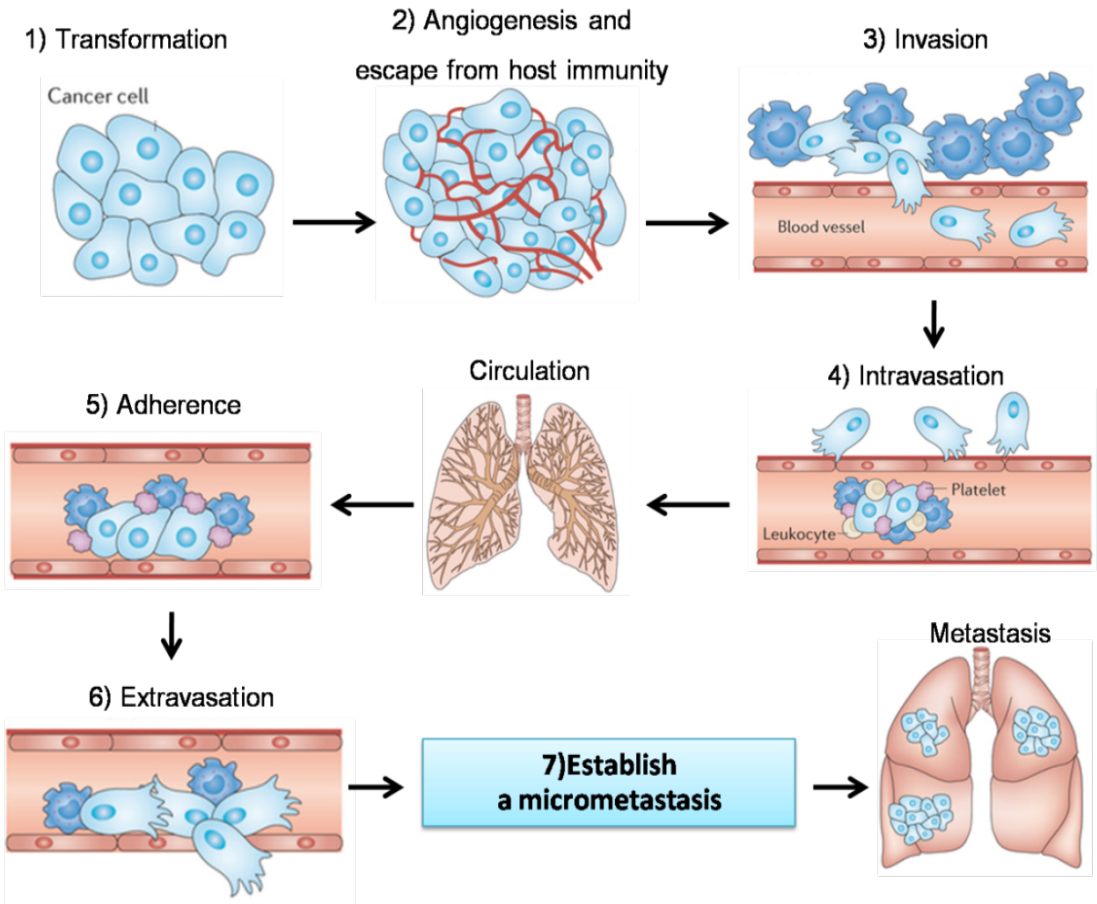


Figure 8 The main step in the formation of a metastasis.

Source: Fidler, 2002

1. Transformation

The transformation of a tumor is the complex process that usually proceeds over a period of decades. The first step in the process, tumor formation is thought to be the result of a genetic alteration leading to an abnormal proliferation of a single cell. Additional mutations followed by selection for more rapidly growing cells within the population then result in progression of the tumor to increasingly rapid growth and malignancy are shown in figure 9 (Weinberg, 2007).

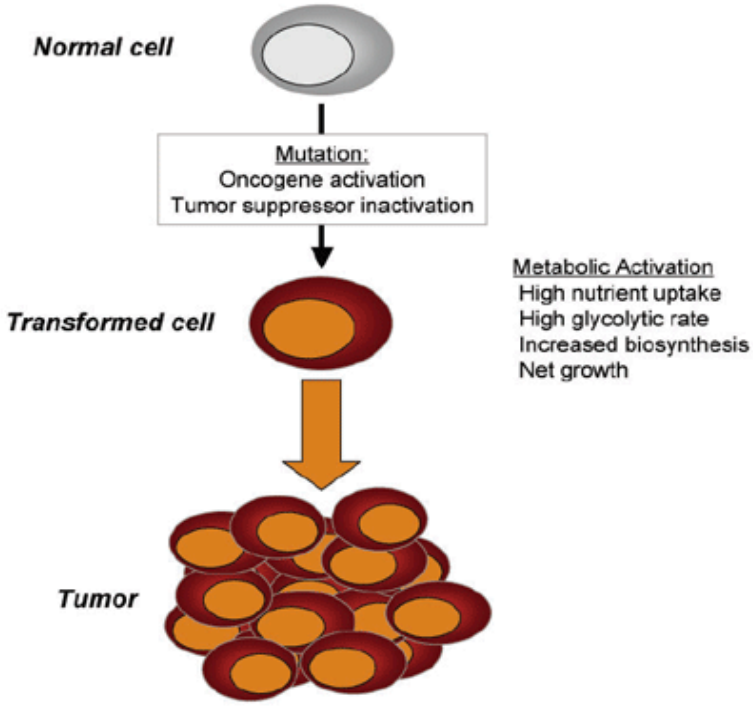


Figure 9 The development of cancer initiates when a single mutated cell begins to proliferate abnormally and continues as additional mutations occur within cells of the tumor population then result in progression of the tumor.

Source: Weinberg, 2007

2. Angiogenesis

The growth of cancer cells can be progressive, with nutrients for the expanding tumor mass. A tumor mass larger than 0.125 mm^2 has outgrown its capacity to acquire nutrients by simple diffusion and must initiate angiogenesis through host vessel initiation of capillary sprouts in the direction of the tumor. The process of blood vessel formation consists of multiple steps step cascade, as shown in figure 8, which is tightly regulated through the balance of a number of pro- and anti- angiogenic factors. Tumor cells are frequently the production of pro-angiogenic factors causing activating endothelial cells, with the independent events of adhesion, proteolysis, and migration that characterize the spreading of cancer cells. Some of the common pro-angiogenic factors include basic fibroblast growth factor (bFGF), which induces the proliferation of a variety of cells and has also been shown to stimulate endothelial cells to migrate, to increase production of proteases, and to undergo morphogenesis, vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), which has been shown to promotes the growth and chemotaxis of endothelial cells. Also included are interleukin-8 (IL-8), a cytokine produced by a variety of tissues and blood cells, platelet-derived endothelial cell growth factor, which has been shown to stimulate endothelial cells DNA synthesis and to induce production of FGF, hepatocyte growth factor (HGF), or scatter factor, that increase endothelial cell migration, invasion, and the production of proteases, platelet derived growth factor (Risau et al., 1992).

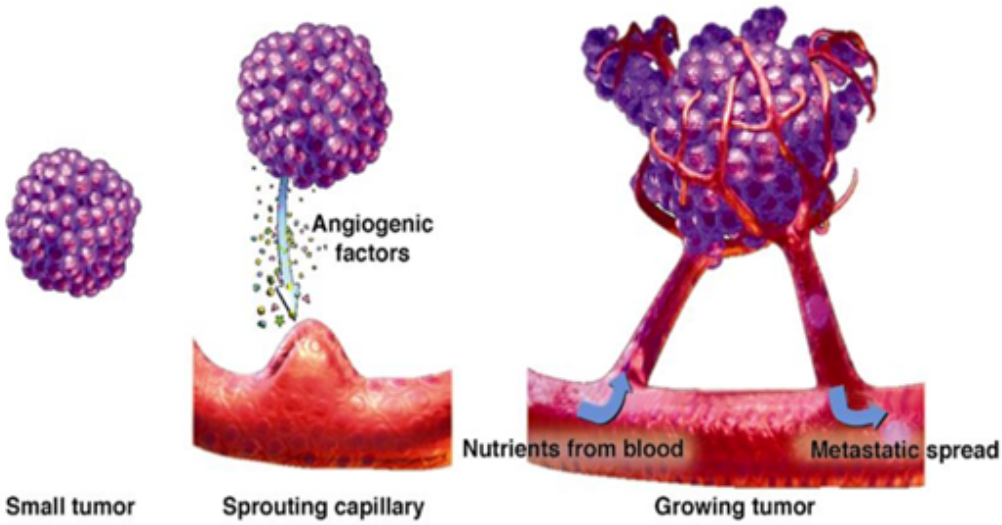


Figure 10 Angiogenesis in cancer can produce angiogenesis signaling molecules resulting in new blood vessels formation. These new vessels in turn supply growing tumors with oxygen and nutrients, allowing the cancer cells to invade nearby tissue, to move throughout the body, and to form new colonies of cancer cells.

Source: Risau et al., 1992

3. Invasion

The mammalian organism is divided into a series of tissue compartments separated by the extracellular matrix (ECM) unit consisting of the basement membrane and its underlying interstitial stroma. Extracellular matrix (ECM) is a dense meshwork of type IV collagen, glycoproteins, such as laminin, fibronectin, and proteoglycans. During the transition from in situ to invasive carcinoma do tumor cells have to degrade the ECM in order to invade successfully. Tumor cell invasion of the extracellular matrix can be separated into three steps: The first step is invasive tumor cell change its adhesive interaction with of the matrix such as laminin (for the basement membrane) and fibronectin (for the stroma). Next, invasive tumor cell is local degradation of the matrix by tumor cell-associated proteases. Such proteases may degrade both the attachment proteins as well as the main structural collagenous proteins of the matrix. Finally, tumor cell is locomotion into the region of the matrix

modified by proteases. The direction of locomotion may be influenced by chemotatic factors. Once the tumor cells invade the extracellular matrix, they gain access to the lymphatics and blood vessels for distant dissemination.

The factors that enable cancer cells to acquire the attributes of invasiveness by excavating passageways through the extracellular matrix (ECM) is the matrix metalloproteinases (MMPs) are a family of neutral metalloenzymes secreted as latent proenzymes. They require activation through proteolytic cleavage of the amino-terminal domain, and their activity depends on the presence of Zn^{++} and Ca^{++}. MMPs have been defined, grouped according to substrate specificity, including collagenases, gelatinases, stromelysins, and matrilysin are summarized in Table 2.

Table 2 Matrix metalloproteinases and extracellular matrix substrates

Name of MMP	Alternative name of MMP	Extracellular matrix substrates
MMP-1	Collagenase-1	Various collagens, gelatin, entactin, aggrecan, tenascin
MMP-2	Gelatinase A	Elastin, fibronectin, various collagens, laminin, aggrecan, vitronectin
MMP-3	Stromelysin-1	Proteoglycans, laminin, fibronectin, gelatin, various collagens, fibrinogen, entactin, tenascin, vitronectin
MMP-7	Matrilysin	Same as MMP-3
MMP-9	Gelatinase B	Same as MMP-2
MMP-11	Stromelysin-3	Inactive serpin

Source: Bast Jr R. C. et al., 2000

The function of MMPs activation will through proteolytic of urokinase plasminogen activator (uPA) which secreted by stromal cells as an inactive pro-enzyme. This form of uPA proceeds to bind to cell surface receptor (termed uPAR), which is displayed by cancer cells and thereby becomes catalytically resulting

creates an activated protease leading to cleave and thereby activate the pro-enzyme forms of yet other extracellular proteases, notably, the matrix metalloproteinase (MMPs) are shown in figure 11. Numerous studies have suggested that the matrix metalloproteinases (MMPs), including MMP-2 and MMP-9 are gelatinases, degrade type IV collagen as a primary substrate of ECM are involved primarily in host tissue degradation. Increased MMP activity has been detected and shown to correlate with invasion and metastatic potential in cancers.

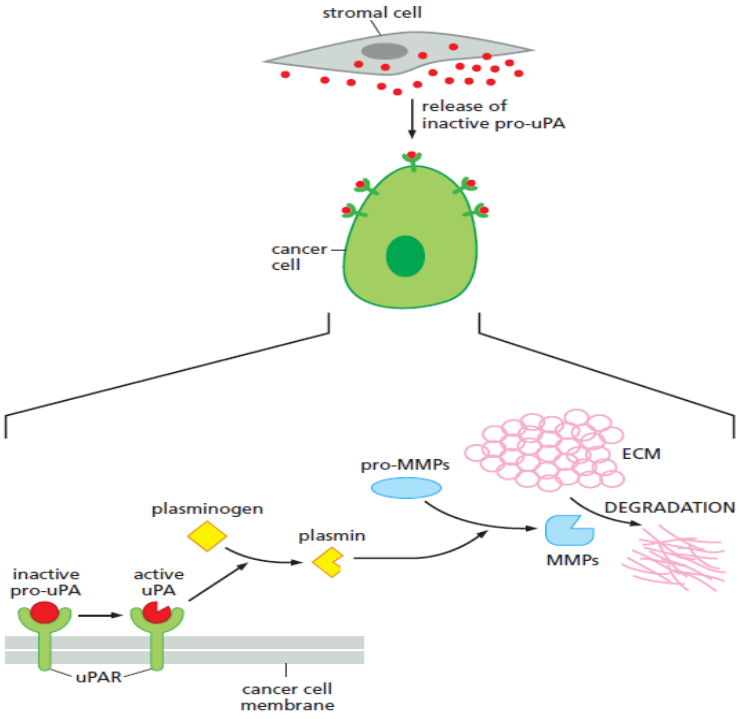


Figure 11 MMPs activation through proteolytic of urokinase plasminogen activator (uPA) is released by stromal cells it binds to its cognate receptor, uPAR, which is displayed on the surface of epithelial cells; this binding converts uPA into a catalytically active protease. Active, receptor-bound uPA can then convert inactive, soluble plasminogen to the activate plasmin form; the latter functions as a protease to cleave pro-enzyme forms of matrix metalloproteinases (pro-MMPs) into active MMPs.

Soucre: Blasi & Carmeliet, 2002

Moreover, MMPs activity has been detected and shown to correlate positively with the metastatic ability of a primary tumor, suggesting that MMPs like this one can act at several stages of metastasis cascade, including local invasion of the primary tumor, intravasation, and extravasation.

4. Intravasation

Cancer cell entering the blood and lymphatic vessels is intravasation. The process intravasation is functionally similar to tumor cell invasion and can be considered a form of regulated invasion. Once they have arrived within the lumen of a blood or lymphatic vessel, individual cancer cells may travel with the blood or lymph to other areas in the body. However, cancer cells entering the blood vessels, they will travel through circulation and are rapidly trapped in the capillary beds, because capillaries usually have internal diameters in the range of 3 to 8 µm, while most cancer cells, in contrast, are more than 20 µm in diameter and are not especially deformable. Moreover, if cancer cells in the blood are coated with platelets, their effective diameters are greatly increased, causing them to be trapped in vessels far larger than capillaries, e.g. the small arteries known as arterioles, as shown in figure 12. In addition, some many do not stay at the local capillaries, they will move through to other capillaries or move within tissues (Weinberg, 2007).

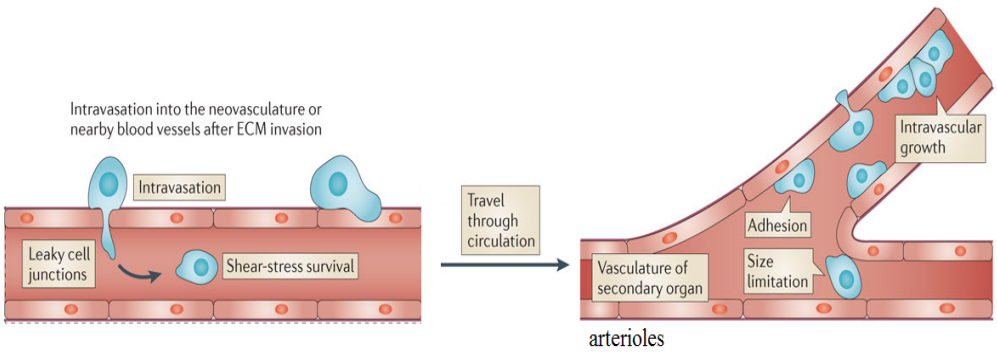


Figure 12 Cancer cell entering the blood vessels is intravasation. This schematic diagram indicates the cancer cells enter the blood vessels, they travel through circulation and are rapidly trapped in the arterioles.

Source: Brabletz et al., 2002

5. Adhesion

Both cell–cell interactions and cell–stroma interactions play an important role during the invasive cascade. Connections through cell adhesion molecules, integrins, and cadherins stabilize tissue integrity, whereas loss or alteration of these cell surface proteins has been shown to be associated with increased metastatic potential. Cell polarity and organization during spreading and migration is regulated by cell interaction with extracellular matrix proteins through the integrin family and with other cells, through the transmembrane glycoprotein cadherins. Activation of these cell surface receptors passes signals from the outside into the cell and thus directs cell behavior. Some experiments suggest that cancer cells use specific cell surface receptors, such as integrins, to initially adhere to the luminal walls of arterioles and capillaries in certain tissues (Weinberg, 2007).

6. Extravasation

Once lodged in the vessels of various tissues, cancer cells must escape from vessels and penetrate into the surrounding tissue, the step termed extravasation. The process of extravasation is functionally similar to tumor cell invasion and depends on complex interactions between cancer cells and the walls of the vessels in which they have become trapped. Cancer cells can use several alternative strategies to extravasate. However, metastasizing cancer cells into the blood circulation and time to leave of vessels have suggested that the process is extravasation often proceeds through the following sequence of steps, as shown in figure 13. A Metastasizing cell is trapped physically in a capillary. Within minutes, a large number of platelets become attached to the cancer cell, forming a microthrombus. Some of these have not yet degranulated and released growth factors, proteases, etc. The cancer cell pushes aside an endothelial cell on one wall of the capillary, thereby achieving direct contact with the underlying capillary basement membrane. Within a day, the microthrombus is dissolved by the proteases in the blood that is usually responsible for dissolving clots. The cancer cell begins to proliferate in the lumen of the capillary. Within several days, sometimes earlier, the cancer cells break through the capillary basement membrane and invade the surrounding tissue (Weinberg, 2007).

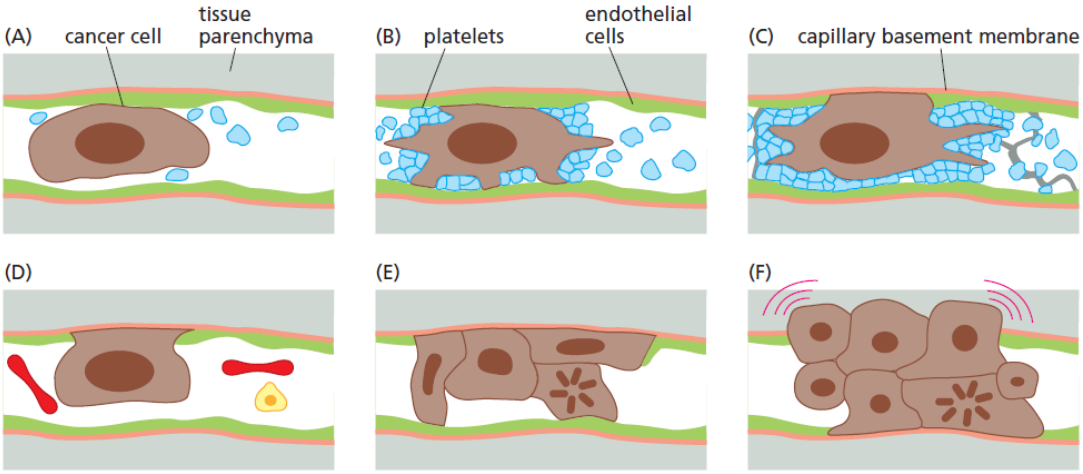


Figure 13 Steps leading to extravasation. This schematic diagram indicates the cancer cells moving out into the blood vessels, they escape from vessels and penetrate into the surrounding tissue.

Source: Menter et al., 1988

7. Establish a micrometastasis

Once they have arrived within the parenchyma of a tissue, metastasizing cancer cells may begin forming a tumor mass in their newfound homes, the process often termed colonization. This step is perhaps the most difficult step of all, ostensibly because the foreign tissue environments do not provide cancer cells with the collection of familiar growth and survival factors that allowed their progenitors to thrive in the primary tumor. Without these various types of physiologic support, the metastasizing cells may rapidly die or, at best, survive for extended periods of time as single cells or small clumps of cancer cells so-called micrometastases that can only be detected microscopically. These micrometastases may be widely disseminated throughout the tissues of a cancer patient, occasionally leading to disastrous outcomes (Weinberg, 2007).

Furthermore, There are other processes that necessary at the metastatic cascade, suggesting that cell migration one can act at several stages of metastasis cascade, including invasion, angiogenesis, intravasation, adhesion, and extravasation.

Cell migration involves continuous restructuring of the actin cytoskeleton in different parts of a cell as well as the making and breaking of attachments between the migrating cell and the extracellular matrix (ECM).

The process of cellular movement; to begin a cell will extend its cytoplasm in the direction of intended movement. This extension involves the protrusion from the cell surface of pseudopodia (termed lamellipodia) protruding in response to chemoattractants may serve multiple functions, including acting as sense organs for the migrating cell to locate directional clues, to secrete motility-stimulating factors, to provide propulsive traction for locomotion, and to induce matrix proteolysis to assist in the penetration of the matrix are shown in figure 14. The detailed management of cell shape and motility is under the control of members of a group of Ras-related proteins.

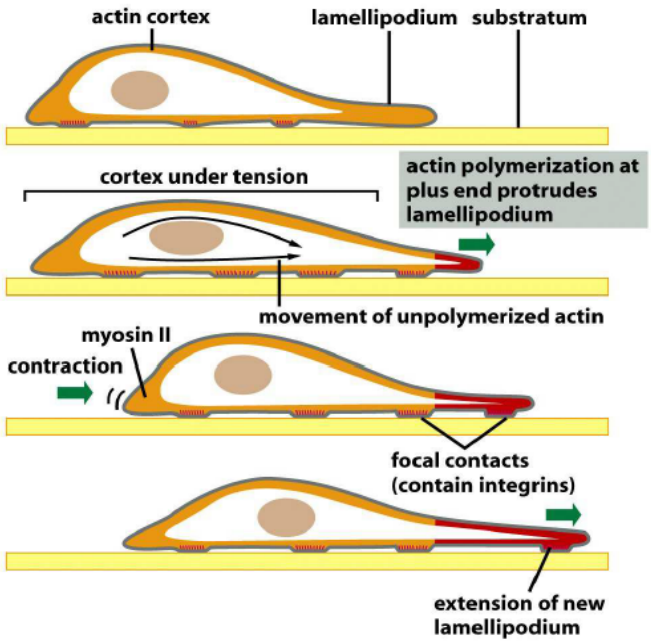


Figure 14 Locomotion of cells on solid substrates. This schematic diagram indicates the cell extends its cytoplasm (termed lamellipodia) in the direction of intended movement and depends on the coordination of a complex series of changes in the cytoskeleton.

Source: Lewis et al., 2002

Metastasis is very complicated multi-step processes. Consequently, one gene product is not sufficient for metastasis. In order to exhibit the metastatic phenotype, individual tumor cells must have either a deficiency in the negative factors (loss of function) and/or an augmentation in the positive factors (gain of function). The metastatic phenotype may require additional genetic changes over and above those resulting in uncontrolled proliferation. To understand the molecular basis for metastasis, investigation of gene expression profile of primary and metastatic tissues are necessary.

Gene expression profile of primary and metastatic cancers

The progression of the primary tumor towards invasive carcinoma and dispersion of cancer cells through the lymphatic or blood vessels. Circulating cancer cells that survive could infiltrate distant organs. Infiltrated cells in the new microenvironment might proceed towards overt metastasis with or without an intervening period of latency. These steps are supported by functions of the cancer cells themselves or of the tumor stroma. In addition to the tumor-initiating events that produce an incipient carcinoma, metastasis requires functionally distinct classes of genes that provide metastasis initiation, progression and virulence functions, as shown in figure 15. These functions can be acquired through distinct genetic or epigenetic alterations, and might collectively endow circulating cancer cells with the competence to infiltrate, survive in latency and colonize distant organs.

Genes that allow transformed cells to invade the surrounding tissue and attract a supportive stroma also facilitate the dispersion of cancer cells and probably continue to do so after cancer cells infiltrate distant tissues. The genes that determine these activities can be defined as metastasis initiation genes. These genes could promote cell motility, epithelial–mesenchymal transition (EMT), extracellular matrix degradation, bone marrow progenitor mobilization, angiogenesis or evasion of the immune system. For example, EMT is mediated by developmental programs that are under the control of aberrantly regulated transcription factors, such as TWIST1, SNAI1 and SNAI2 (also known as SLUG). Other determinants of invasion are components and modulators of the hepatocyte growth factor (HGF)–HGF receptor (HGFR) pathway, such as metadherin in breast cancer and the metastasis-associated

in colon cancer 1 (MACC1) gene in colorectal carcinoma. Metastatic growth is also initiated by the suppression of non-coding RNAs, such as miR-126 and miR-335 in breast and colorectal carcinoma. The expression of these metastasis initiation genes and their targets predicts a poor prognosis in particular types of cancer (Nguyen et al., 2009).

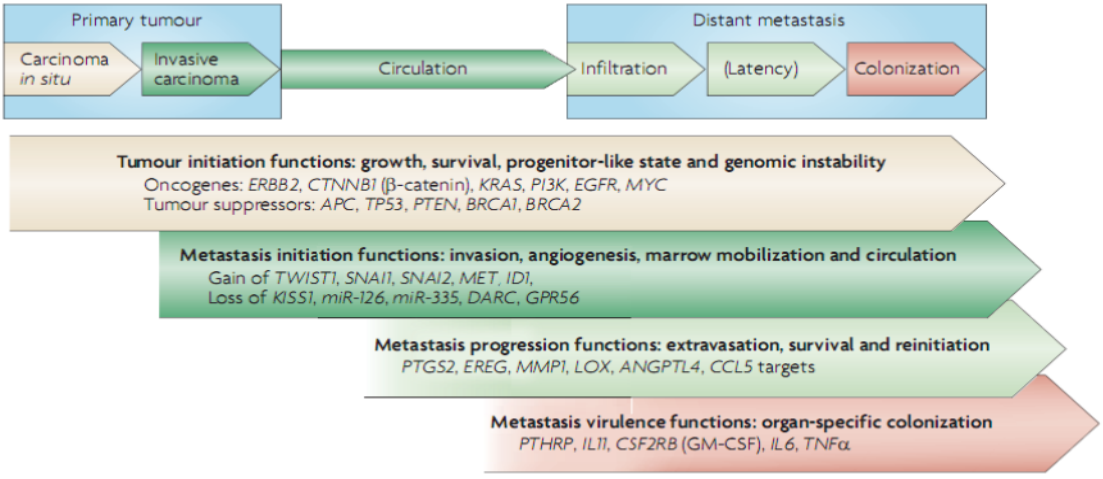


Figure 15 Basic steps of metastasis and hypothetical classes of metastasis genes. Different set of genes and key molecules play role in each step of carcinogenesis and metastasis. The examples of key genes in each step are given

Source: Nguyen et al., 2009

Gene expression profile of primary and metastatic CCA

Cancer phenotype of CCA reflects changes in the expression patterns of hundreds or even thousands of genes that occur as a consequence of the primary mutation of an oncogene or a tumor suppressor gene.

To date, studies of a comprehensive analysis of gene expression profile in CCA have been many reported. In 2003 Hansel et al, exported global gene expression of primary resected tumors, biliary cell lines and non-neoplastic biliary epithelial using Affymetrix oligonucleotide microarrays have been reported two hundred and eighty-two known genes are up-regulated three fold or more in cancer

compared to normal epithelium, including proliferation and cell cycle antigens (cyclins D2 and E2, cdc2/p34, and geminin), transcription factors (homeobox B7 and islet-1), growth factors and growth factor receptors (hepatocyte growth factor, amphiregulin, and insulinlike growth factor 1 receptor), and enzymes modulating sensitivity to chemotherapeutic agents (cystathionine synthase, dCMP deaminase, and CTP synthase). In 2005 Obama et al, studied gene expression profiles of intrahepatic CCA using tumor cell populations purified by laser microbeam microdissection were analyzed. Fifty-two genes are commonly up-regulated and Four hundred and twenty-one genes are down-regulated in intrahepatic CCA compared with noncancerous biliary epithelial cells. Among the Fifty-two up-regulated genes, P-cadherin and survivin were investigated for enhanced expression in cancer tissues by immunohistochemical staining. One hundred and thirty-seven genes are differentially expressed, and among them, ANXA1, ANXA2, AMBP and SERPINC1 were verified in human ICC cell lines and tissues by semi-quantitative RT-PCR and immunohistochemical staining (Wang et al., 2006). Significant progress has been made over the past decade in defining molecular alterations associated with cholangiocarcinoma. Table 3 summarizes the most studied molecular alterations that have been described to date for human cholangiocarcinoma in relation to dysregulation of cell growth and survival pathways, aberrant gene expression, invasion and metastasis, and tumor microenvironment (Alphonse & Sirica, 2005).

Table 3 Molecular alterations in cholangiocarcinoma

Name of molecular	Alternative
Autonomous growth signaling	
Hepatocyte growth factor (HGF)	Overexpression
Interleukin-6 (IL-6)	Overexpression
Receptor tyrosine-protein kinase (ErbB-2	Overexpression
K-ras	Overexpression
Serine/threonine-protein kinase B- rafBRAF	Overexpression
Cyclooxygenase-2;COX-2	Overexpression

Table 3 (Cont.)

Name of molecular	Alternative
Tumor suppressor genes	
p53	Mutation/ Inactivation
APC	Mutation/ Inactivation
DPC4/Smad4	Mutation/ Inactivation
Cell cycle	
Cyclin D1	Up-regulation
$p16^{INK4a}$	Down-regulation
$p21^{waf1/cip1}$	Down-regulation
$p27^{kip1}$	Down-regulation
$p57^{kip2}$	Down-regulation
Antiapoptosis	
Bcl-2	Up-regulation
Bcl-XL	Up-regulation
Mcl-1	Up-regulation
Fas	Down-regulation
Angiogenesis	
vascular endothelial growth factors (VEGF)	Up-regulation
basic fibroblast growth factor (bFGF)	Up-regulation
transforming growth factor (TGF-β)	Up-regulation
Invasion and metastasis	
E-Cadherin	Down-regulation
α-Catenin	Down-regulation
β-Catenin	Down-regulation
Human aspartyl (asparaginyl) β-hydroxylase	Up-regulation
WISP1v	Up-regulation

Source: Alphonse & Sirica, 2005

Various routes of tumor spreading are seen in CCA. The recognized patterns are direct invasion, infiltration along the biliary tree, vascular and lymphatic permeation, and perineural and intraneural invasion (Sripa & Kaewkes, 2000). There are many molecular alterations and variations associated with metastasis of CCA.

Cell adhesion molecules, E-cadherin and catenin families are downregulated and associated with invasion and metastasis of CCA (Sirica, 2005). Increased c-erbB-2 expression contributes to the development of CCA into an advanced stage and is associated with tumor metastasis (Aishima et al., 2002). Expression of thymidine phosphorylase, an important regulator of angiogenesis, shows a significant correlation with vascular invasion, lymphatic permeation, perineural invasion and lymph node metastasis of in trahepatic CCA (Aishima et al., 2002). In addition, Sialyl-Lewis is related to vascular invasion and poor prognosis of CCA (Juntavee et al., 2005). On the other hand, expression of bcl-2 is inversely related to lymph node metastasis, vascular invasion and perineural invasion of CCA (Ito et al., 2000). Moreover, the expression of Smad4, TGFβ-1 and TGFβ-RII correlate with the histological grading, clinical staging and metastasis of the lymph node and liver in CCA (Zhang et al., 2005). Intrahepatic CCAs of the periductal infiltrating type were further K-ras mutations with the incidence have been reported to be higher in CCA patients with lymph node metastasis than in those without lymph node metastasis (Isa et al., 2002).

The incidence rate of lymph node metastasis, which found commonly in CCA, has been shown to increase in proportion to an increase in the expression of matrix metalloproteinase-9 (MMP-9) in intrahepatic CCA (Shirabe et al., 1999). Over-expression of aspartyl (asparaginyl)-hydroxylase correlated in cell motility of CCA (Maeda et al., 2003) and Cyclin D1 over-expression was more frequently observed in CCA with lymph node metastasis (Ito et al., 2001). Likewise, WISP1v expression associated with lymphatic and perineural spread of CCA and poor clinical outcome (Tanaka et al., 2003).

Recently, Hilar and extrahepatic CCA and mucus-excreting intrahepatic CCA also displayed AGR2 expression. In addition, Up-regulation of AGR2 expression in highly metastatic compare with its metastatic parental CCA cell

(Uthaisar et al., 2016). Suggesting that AGR2 might be molecular basis involved in CCA metastasis. Thus, the functional roles of AGR2 in metastasis of CCA need to be further explored.

Anterior Gradient 2 (AGR2)

The anterior gradient genes were first discovered in 1997 by virtue of expression in *Xenopus laevis* In X. laevis the xAG family is highly expressed in the ectoderm during the early stages of the embryo development (Aberger et al., 1998). Certain members are xAG1, xAG2 and xAG3. xAG-2 is involved in the anterior specification of the Xenopus embryonic ectoderm giving rise to forebrain and cement gland. Recent studies identified AGR-2, termed as nAG, as a key player in limb regeneration in adult salamander. nAG interact with the surface protein Prod-1 and is a key player in limb regeneration (Kumar et al., 2007). Prod-1 is expressed at the cell surface as a glycosylphosphatidylinosotol (GPI)–anchored and mediates cell-to-cell interaction and positional identity (Kumar et al., 2007). In zebrafish, *Dario renio*, zAG-2 was identified in most organs containing secretory cells, including epidermis, olfactory bulbs, otic vesicles, pharynx, oesophagus, pneumatic duct, swim bladder, and intestine (Shih et al., 2007). Gene silencing studies, utilising AGR2-specific siRNA knockdown have described AGR2 as performing a role in the maintenance of intestinal homeostasis, with respect in particular to goblet and paneth cells (Zhao et al., 2010). AGR2 depletion, results in the loss of the goblet cells function to secrete intestinal mucus and significantly changes the morphology of these cells (Zheng et al., 2006). The study in 2010 by Zhao et al. describes additional aberrations causing symptoms in the small intestine of AGR2 knockout mice, the paneth cells morphology was drastically altered, and severe intestinal inflammation reported. Interestingly, phenotypically, AGR2 knockout mice present symptoms resembling human Crohn's disease, (Park et al., 2009) suggesting a role of AGR2 in the aetiology of inflammatory bowel disease. In addition, AGR2 has been implicated in the formation of mixed disulfides in the maturation of cysteine rich glycoprotein, intestinal mucin, MUC2 and MUC1 (Park et al., 2009). AGR2 was analysed to investigate whether there was a relationship in the synthesis of the airway mucins MUC5AC and MUC5B (Schroeder et al., 2012).

AGR2 was shown to be co-expressed in the same cells as MUC5AC and MUC5B and shown to co-immunoprecipitation with MUC5AC (Schroeder et al., 2012).

The hAGR-2 gene was first described in the MCF-7 breast carcinoma cell line, and found to be co-expressed with the Estrogen Receptor α, in ERα-positive breast cancer cell lines (Thompson et al., 1998).

1. Structure of Anterior Gradient 2 (AGR2) protein

The human agr2 gene was spanned 50 kb of genomic DNA, located to chromosome band 7p21.3 containing eight exons and seven introns and product translates to a 175 amino acid residue protein, (Petek et al., 2000) with a predicted molecular mass of 19,979.2 Da and a predicted pI of 9.03 (http://web.expasy. org/compute_pi/) The primary structure and recent research into the structural motifs of AGR2 highlight several important characteristics of the expressed protein. Further bioinformatics analysis of the primary protein structure identifies a 20 amino acid residue the N-terminal hydrophobic leader sequence incorporating a signal peptidase cleavage site at Ala-20/Lys-21 (Zhang & Henzel, 2004) giving rise to a 17,949 Da is a mature protein and sequence of 60-64 amino acid residue is the dimerization motif which importance the interaction of dimer structure (Patel et al., 2013). Another key motif of AGR2 is the CXXS motif or "thioredoxin fold" has a sequence of 80-44 amino acid residue which is a member of the protein disulfide isomerase (PDI) family (Persson et al., 2005). A sequence of 104-111 amino acid residues is the substrate binding loop fine mapped for the interaction. The extreme C-terminus includes another classic linear peptide motif; the endoplasmic reticulum retention motif containing tetrapeptide sequence of lysine (K), threonine (T), glutamic acid (E), leucine (L) abbreviated as KTEL which is ER-retention signal (Gupta A et al., 2012), as shown in figure 16.

Figure 16 Structure of Anterior Gradient 2 (AGR2) protein.

Source: Modified from Patel et al., 2013

AGR2 is novels members of protein disulfide isomerase (PDI) family. The PDIs play an important role as they catalyze oxidation, reduction and isomerization of disulfide bonds in the endoplasmic reticulum to correct folding and processing of the proteins targeted to secretion (Persson et al., 2005) as showne in figure 17. AGR2 has been described as performing a pivotal role in the control of endoplasmic reticulum homeostasis (Higa et al., 2011). A luciferase reporter gene construct driven by the AGR2 promoter assay indicated that the forkhead box transcription factors FOXA1 and FOXA2 increased luciferase activity thus may regulate the transcription of AGR2 mRNA (Zheng et al., 2006). The increased transcription of AGR2 in response to physiological stresses of serum starvation, coupled with oxygen restriction induced AGR2 transcription fivefold. Conversely, this study also interrogated specific inhibitors to known transcriptional regulatory pathways ERK1/2, JNK, p38 and PI3K and only ERK1/2 inhibition resulted in a reduction of AGR2 mRNA expression. (Zweitzig et al., 2007) Tunicamycin (an inhibitor of N-linked glycosylation), DTT (reducing agent) and thapsigargin (a SERCA, sarco/ER Ca2+ ATPase inhibitor), all promoters of ER stress, were uncovered as inducers of AGR2 transcription (Zhang et al., 2005; Higa et al., 2011). Further, it was shown that AGR2 expression could be controlled by the unfolded protein response (UPR), and that silencing of AGR2 disrupts the components of the ER-associated degradation machinery, resulting in a reduction in cells ability to cope with acute ER stress (Higa et al., 20011). Complementary to this, data suggest AGR2 induction regulate the expression of several other ER chaperones, namely PDI, CALU and RCN1, proteins of the ubiquitin-proteosome degradation pathway (HIP2, PSMB2, PSMA3, PSMC3 and PSMB4) and lysosomal proteases (cathepsin B and cathpsin D) (Dumartin et al., 20011). Further mechanisms incorporating AGR2 to the UPR signaling pathway arises in the recent publication by Ryu et al., 2013 who describe AGR2 forming a homodimer, via an intermolecular disulphide bond, and show it to be essential in the interaction with BiP/GRP78, and resultant attenuation of ER stress induced cell death.

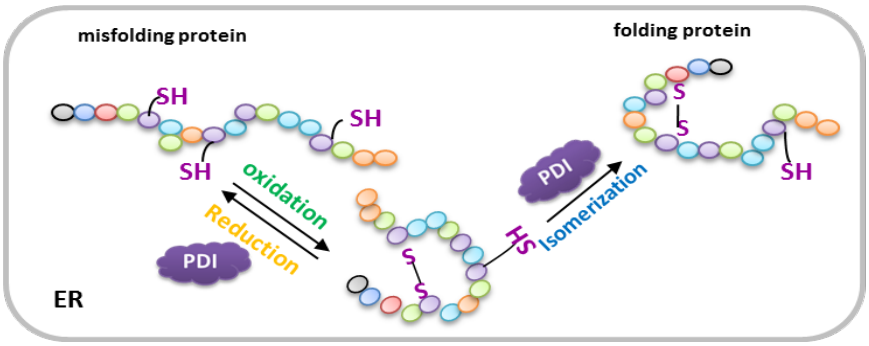


Figure 17 The function of protein disulfide isomerase (PDI). This schematic diagram shows that the function of PDI controls misfolded protein to folding protein by catalyze oxidation, reduction and isomerization of disulfide bonds in the endoplasmic reticulum.

Soure: Modified from Persson et al., 2005

Indeed, possible roles of AGR2 are complex since AGR2 subcellular localization is highly disputed, with ER (Higa et al., 2011), nucleus (Fourtouna et al., 2009 & Gupta et al., 2012), cell surface and extracellular space (Dumartin et al., 2011) all being reported are shown in figure18. The different location of AGR2 may other functions in these compartments.

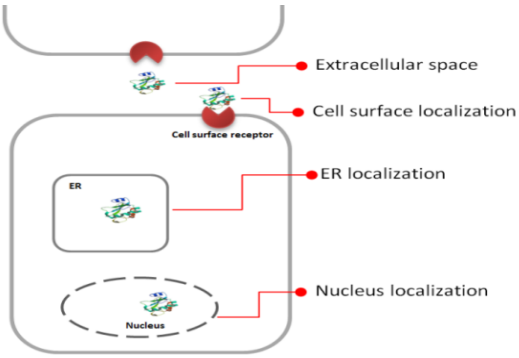


Figure 18 Localization of AGR2. This schematic diagram the different location of AGR2 are reported that nucleus, ER, cell surface, and extracellular localization.

Source: Modified from Fourtouna et al., 2009; Gupta et al., 2012

Human AGR2 is strongly expressed in tissues of the colorectal (Kim et al., 2002), esophageal (Wang et al., 2008), lung (Pizzi et al., 1997), ovarian (Park et al., 1997), pancreatic (Ramachandran et al., 2008), prostate (Maresh et al., 1996) and organs which contain mucus secreting cells, or function as endocrine organs as shown summarized in Table 4 (Brychtova et al., 2011). AGR2 may also be involved in epithelial barrier function as the AGR2 promoter regulators are grouped into a family which are typical for epithelial goblet cells (Zheng et al., 2006).

Table 4 The expression of AGR2 in normal human tissues.

Normal Tissue Staining by Immunohistochemistry	Cell Type	Protein Expression
Appendix	Glandular cells	Strong
Breast	Glandular cells	Weak
Bronchus	Respiratory epithelial cells	Strong
Cereblal cortex	Neuronal cells	Moderate
Cervix, uterine	Glandular cells	Strong
Corpus, uterine	Glandular cells	Strong
Duodenum	Glandular cells	Strong
Epididymis	Glandular cells	Strong
Oviduct	Glandular cells	Strong
Gall Bladder	Glandular cells	Strong
Kidney	Cells in tubules	Moderate
Lung	Alveolar cells	Moderate
Nasopharynx	Respiratory epithelial cells	Strong
Pancreas	Exocrine glandular cells	Moderate
Prostate	Glandular cells	Strong
Rectum	Glandular cells	Strong
Seminal vesicle	Glandular cells	Strong
Small intestine	Glandular cells	Strong

Source: Brychtova et al., 2011

2. Correlation of AGR2 expression in human cancer

AGR2 exhibits the basic features of an oncoprotein, due to pro-growth and cell survival enhancing activity (Pohler et al., 2004; Hrstka et al., 2010). The overexpression and suppression of AGR2 in cancer cell lines has been used to determine whether it has growth-suppressive or progrowth functions. Clonogenic assays were used to demonstrate that AGR2 can enhance cancer cell survival, rather than inhibit cell growth (Hrstka et al., 2010). AGR2 has also been described as an oncogene supporting a role in cellular transformation and adenocarcinoma growth. In the premalignant Barrett oesophagus and oesophageal cancer models, AGR2 overexpression induces colony formation and transformation (Pohler et al., 2004; Wang et al., 2008). Conversely, short interfering RNA or short hairpin RNA-mediated AGR2 knockdown inhibits colony and subcutaneous growth in oesophageal and pancreatic cancer models (Ramachandran et al., 2008; Wang et al., 2008). AGR2 silencing was shown to inhibit proliferation, survival *in vitro* in breast cancer cell lines and identified modulation of cancer-signaling pathways, such as cyclin D1, c-Myc, p-Src and survivin (Vanderlaag et al., 2010). Intriguingly, deletion of the 10 C-terminal amino acid residues, those harbouring the ER-retention site, prevented clonogenic growth stimulation indicating a role in tumorigeneses of the ER localized protein and survival signaling. Coupled to this, the inhibition of p53 and the promotion of p53 nucleus exclusion in UV-induced DNA damaged cells (Pohler et al., 2004). In breast cancer, AGR2 expression is associated with estrogen receptor (ER)-positive tumors; its overexpression is a predictor of poor prognosis (Hrstka et al., 2010) and a malignant metastatic phenotype (Barraclough et al., 2009). High expression levels of AGR2 are also reported in prostate carcinomas both intracellularly and secreted into the blood (Kani et al., 2013) and urinary secretions of patients and studies are now ongoing as to whether AGR2 could be used as a minimally invasive prognostic marker of prostate cancer (Bu et al., 2010). Recent reports suggest that the FOXA1 and FOXA2 transcription factors are likely to be involved in AGR2 expression in prostate cancer, coupled to the inverse correlation of AGR2 levels with ErbB3 binding protein 1 (EBP1), an endogenous negative regulator of androgen receptor signaling, which may allow the invasive character of prostate tumors (Bu et al., 2010). AGR2 is associated with poor survival of prostate cancer patients (Kristiansen et al., 2005)

and silencing of AGR2 in prostate cancer cell lines redacts the survival enhancement, allowing cells to enter senescence (Hu et al., 2012). AGR2 protein have been documented as being overexpressed in a wide range of human cancers involved in both the onset and in progression of cancers as shown summarized in Table 5

Table 5 Correlation of AGR2 expression in human cancer

Cancer Type	AGR2 Manifestation	Reference
Breast	Overexpression/upregulation	Fletcher et al.,2003
	Metastasis	Liu et al.,2005; Barraclough et al.,2009
Colorectal	Poor Prognosis	Valladares-Ayerbes et al.,2012
Gastric	Metastasis	Lee et al.,2011
Lung	Overexpression	Fritzsche et al., 2007; Chung et al., 2012
Pancreatic	Overexpression/upregultion	Barry et al., 2012& Ramachndran.,2008
Prostate	Overexpression in serum	Kani et al.,2013
	Overexpression/Upregultion	Kristiansen et al.,2005
	Metastasis	Hu et al.,2012
Ovarian (mucinous)	Upregulation	Park et al., 2011
	High plasma concentration	Edgell et al., 2010

Source: Modified from Brychtova et al., 2013

3. Signaling mechanisms of AGR2 in cancers

The cellular mechanism by which AGR2 expression has been reported to be induced by estrogens or androgens, possibly through an AKT signaling pathway (Hrstka et al., 2013) or an EGRR-dependent pathway to stimulate cell growth, proliferation, and differentiation in hormone-dependent cancer modal (Dong et al., 2011). In addition, Ma et al., 2016 propose the induction of AGR2 can be mediated by TGF-β. As TGF-β responsiveness and EMT is altered in salivary adenoid cystic carcinoma a modal can be switched on and coupled to aggressive cancer metastasis suggesting that gain of TGF-β can be induced AGR2 and activating EMT pathway to promote cell migration and invasion. The treatment of cancer cells with tunicamycin or thapsigargin has been shown that the endoplasmic reticulum stress induced expression of AGR2 via ATF6 and IRE1-dependent signaling (Higa et al., 2011). The molecular of AGR2-dependent functions are identified in yeast-two hybrid assays (Y2H) have driven our most comprehensive discovery of AGR2 binding proteins. Such studies have shown that AGR2 protein interact the membrane receptor and pro-metastatic protein Dystroglycan/C4.4a can mediate secretion of receptors linked to adhesion and cell growth (Fletcher et al., 2003). In addition, it have shown that AGR2 can bind nuclear DNA binding protein as the AAA^{+} protein Reptin can form an immune complex with each other in breast cancers (Maslon et al., 2010). Dong et al., 2011 propose that the functionally linked AGR2 and AREG in lung adenocarcinomas. AREG may stimulate the EGFR signaling pathway regulated by the Hippo pathway acts to dephosphorylate Yap-1, to regulation of cell proliferation and anchorage-independent growth (Zeng et al., 2008). A key clinical model used to evaluate cancer progression mechanisms is oesophageal adenocarcinoma. AGR2 was found to attenuate p53 activation in UV-damaged cancer cells, leading to the suggestion that AGR2 functions as a survival factor through inhibition of p53 (Pohler et al., 2004). In addition, the introduction of the AGR2 to HCT116 colon cancer cells resulted in the redistribution of p53 from the nucleus to the cytoplasm in ultraviolet-irradiated cells (Fourtouna et al., 2004). Further, AGR2 silencing was shown to inhibits cell growth, cell-cycle progression and induces cell death of breast cancer and identified modulation of cyclin D1 supporting inhibitory effects on cell growth and cell cycle (Vanderlaag et al., 2010). This observation

was recently substantiated by a study showing that AGR2 was a cell surface antigen-promoting tumor cell metastasis through the activation of cathepsins B and D (Dumartin et al., 2011) Physiological/pathophysiological of AGR2 in cancers as shown summarized in figure 19.

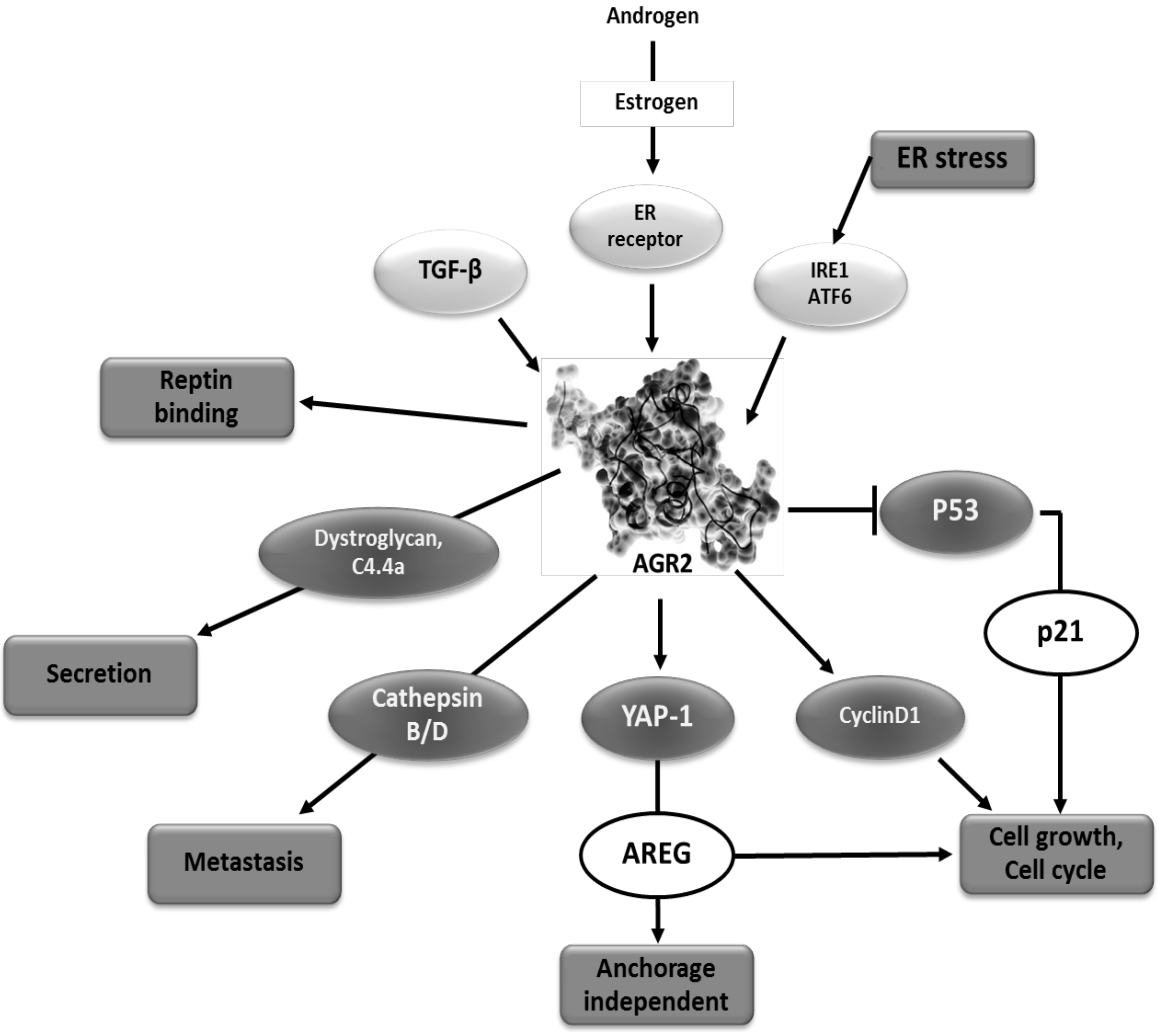


Figure 19 AGR2 biological pathways. This schematic diagram pathway indicates several intermediates to regulate AGR2 expression and AGR2-dependent functions.

Source: Modified from Chevet et al., 2013

4. The function of AGR2 in metastasis of cancers

Many cancer studies revealed that overexpression or suppression of AGR2, in different model systems, can affect cell proliferation, invasion and survival *in vitro*, metastasis and tumors growth *in vivo* (Ramachandran et al., 2008; Wang et al., 2008) Genomic analysis of AGR2-stable cells by cDNA microarray revealed that AGR2 overexpression upregulates the expression of genes involved in cell proliferation, invasion and angiogenesis, (Park et al., 2011) which are very important for tumor progression and metastasis. Conversely, the genes involved in the negative regulation of cell proliferation, adhesion and death are downregulated (Park et. al., 2011). Moreover, the overexpression of AGR2 in grafted cells results in greater propensity to form lung metastases when propagated as xenografts in nude mice showing that AGR2 has an influence on the *in vivo* tumor biology or growth rate *in vitro* and increased the numbers of metastases (Liu et al., 2005). AGR2 involvement in metastasis is also demonstrated in prostate adenocarcinoma cells in which an enhanced invasive behavior of cell-overexpressing AGR2 (Zhang et al., 2010). Subsequently, AGR2 knock down in both non-small cell lung carcinoma and pancreatic cancer cells compromised anchorage independent growth *in vitro* and the growth of xenografted tumors *in vivo* (Zheng et al., 2008). AGR2 expression has also been reported in head and neck squamous cell carcinoma which AGR2 silencing reduced cell proliferation migration and invasion (Zweitzig et al., 2007). Further, detection of AGR2 in excretory systems such as blood serum and urine, suggesting that AGR2 may act in a paracrine or autocrine manner. This pro-metastatic role of AGR2 is broadly accepted. Since its detection in a gene expression screen of cancer cell lines identifying candidate markers for metastasis (Dumartin et al., 2011). AGR2 directly interacts with two transmembrane proteins involved in cell-cell contact or cell-matrix interactions in C4.4A and alpha-dystroglycan (Fletcher et al., 2003). Additionally, ovarian cancer indicates increased plasma concentrations of AGR2 in plasma (Edgell et al., 2010) and cell models of which overexpressing AGR2, demonstrate upregulated gene expression of proteins involved in cell proliferation, invasion, angiogenesis, and suppressed negative regulators of these processes (Park et al., 2011). Moreover, conditioned media from cells silenced in AGR2 have a reduced ability to stimulate proliferation of pancreatic cancer cells

(Ramachandran et al., 2008). In addition, in stress conditions, resembling the tumor microenvironment, for example serum depletion and hypoxia, AGR2 was identified as upregulated in a screen together with other pro-survival, pro-invasive and angiogenic genes (Fletcher et al., 2003). Recently, In 2016 Ma et al. found increased expression of AGR2 affects tumor cells by promoting invasiveness and metastasis through regulation of epithelial-mesenchymal transition. Thus, AGR2 could regulate epithelial–mesenchymal transition (EMT) in development and cancer. It is believed that EMT is a transient state and that the process is reversed once the cells form a metastatic lesion. However, the involvement of AGR2 in EMT needs to be further investigated. The role of AGR2 in metastasis is of significant interest, as successful metastases requires activation of genes that promote cell survival in environments of pathophysiological stress, therefore the necessity to characterize this metastatic marker has emerging clinical significance.

5. The function of AGR2 in metastasis of CCA

AGR2 is mainly expressed in the normal biliary tree in both fetal and adult normal liver. More particularly, the tall epithelial cells covering the large bile ducts as well as gallbladder epithelial cells showed strong AGR2 staining. (Lepreux et al., 2011). Moreover, hepatocellular carcinoma tumors do not show any significant AGR2 staining, whereas both hilar and extrahepatic cholangiocarcinoma reveal positive AGR2 staining (Vivekanandan et al., 2009). In contrast, only 50% of the intrahepatic cholangiocarcinoma analysed display strong AGR2 staining with mucus production (Lepreux et al., 2011). Recent in 2016 Uthaisar et al., have shown reported that the highly metastatic CCA cell line was established from parental cells and explore a gene potentially involved in the metastatic process in CCA cell were investigated using real-time PCR array showed that Anterior gradient 2 (AGR2) gene was highest upregulated among 77 metastatic-associated genes in highly metastatic human CCA cell, as shown in figure 20. In addition, the expression of AGR2 was significantly higher in stage CCA patient tumors than that in the normal bile ducts, as shown in figure 21. Thus, AGR2 might play and important role promote metastasis of CCA cells.

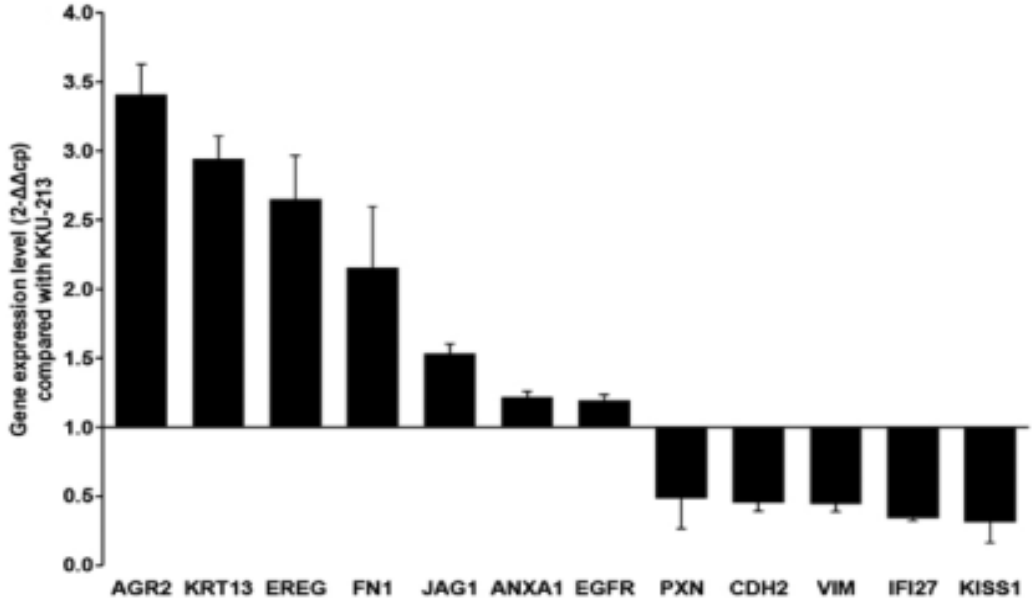


Figure 20 Differential expression of genes in the KKU-213L5 cells. Expression levels of 77 metastatic-associated genes were determined in the KKU-213L5 cells in comparison with the parental KKU-213 cells using real-time PCR.

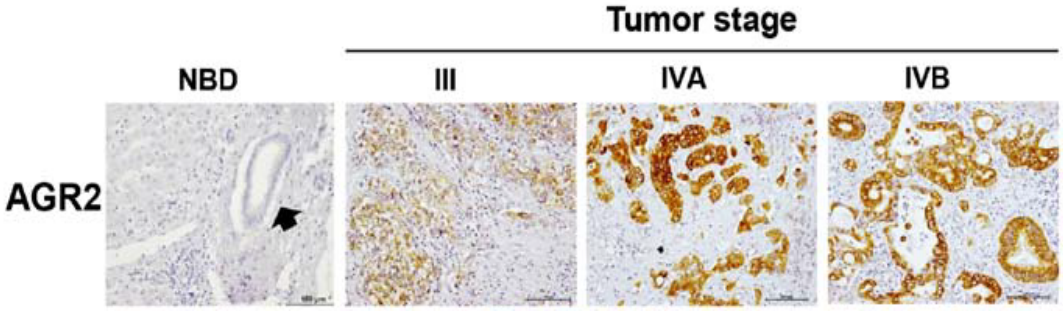


Figure 21 AGR2 expression in CCA patient tissues. Immunohistochemistry of AGR2 was performed in intrahepatic mass-forming CCA patient tissues with different tumor staging.

Source: Uthaisar et al., 2016

CHAPTER III

MATERIALS AND METHODS

Biological materials

1. Parental and high metastatic cholangiocarcinoma cell lines

A highly metastatic cell line namely KKU-213L5 was established from lung metastasized tissue via tail vein injection of parental cell, KKU-213. Briefly, 5 x 10^5 cells of KKU-213 were inoculated into male NOD/scid/Jak3 mice (NOJ mice) by intravenous injection. Approximately 18 days after inoculation, the mice were sacrificed and the tumor in the lung were resected and cultured. This step was repeated five cycles (Uthaisar et al., 2016) as shown in figure 22. KKU-213 and KKU-213L5 cell line were obtained from Professor Dr.Sopit Wongkham, Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Thailand.

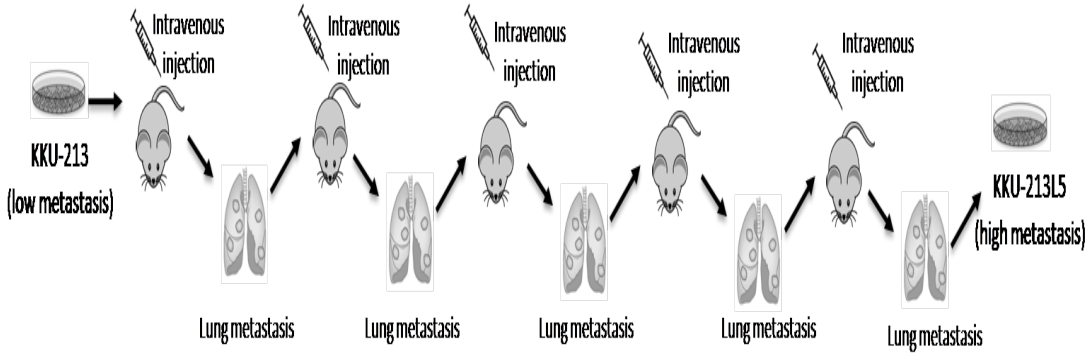


Figure 22 The process for establishment of a high metastatic KKU-213L5 subline.

Source: Modified from Uthaisar et al., 2016

All CCA cell lines were grown in a culture flask or plate with cell adherent manner in DMEM medium supplemented with 10% heated inactivated fetal bovine serum (FBS), 100 unit/mL of penicillin and 100 μg/mL streptomycin. Cells were incubated in 5% CO_2 incubator at 37 °C and subcultured twice a week.

At 80% confluence, cells are detached from culture flask using trypsin/EDTA (0.25% w/v) and processed according to the particular assay.

2. Instrument

Table 6 List of instrument

instrument	Product of
25 cm^3 culture plates	SPL Life Sciences, Korea
96-well culture plates	SPL Life Sciences, Korea
24-well culture plates	SPL Life Sciences, Korea
6-well culture plates	SPL Life Sciences, Korea
60 mm culture plates	SPL Life Sciences, Korea
15 ml centrifuge tube	SPL Life Sciences, Korea
1.5 ml microcentrifuge tube	SPL Life Sciences, Korea
PCR tube	Corning Inc., USA
Autopipet and tips	Corning Inc., USA
CO_2 incubator	Gibco, Grand Island, NY
Laminar flow	Esco Lifesciences
Inverted microscope	LEICA, USA
Hemocytometer	Microbehunter, Austria
Miniprotein electrophoretic set	Bio-Rad, USA
Miniprotein transfer	Bio-Rad, USA
Water bath	Julab, Garmany
Microplate reader	Labsystems, Finland
T100™ Thermal Cycler	Bio-Rad, USA
LightCycler®480 systems	Roche, USA
ImageQuantTM 4000 image analyzer	GE Healthcare, Life Sciences

3. Chemicals and reagents

Chemicals, reagents, commercial kits, and antibodies, are listed in Table 7.

Table 7 List of chemicals and suppliers

Chemical	Product of
Cell culture	
Transwell	Corning Inc., NY
Dulbecco's Modified Eagle Mediun (DMEM)	Gibco, Grand Island, NY
Fetal bovine serum (FBS)	Gibco, Grand Island, NY
MatriGel	Corning Inc., USA
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide : MTT	Bio basic Canada, Inc, CA
Penicilin-Streptomycin	Gibco, Grand Island, NY
Phosphate buffered saline (PBS)	Amresco, USA
Trypan blue	Gibco, Grand Island, NY
Trypsin/EDTA	Gibco, Grand Island, NY
DMSO	Sigma-Aldrich.St. Louis, MO
Crrystal violet	Panreac, E.U.
Gene expression (Real-time PCR)	
Trizol® reagent	Amresco, USA
Chloroform	Lab-Scan, Thailand
Isopropanol	Lab-Scan, Thailand
Ethanol	Merck, Garmany
Tetro cDNA synthesis kit	BIO-65042. Bioline, USA.
LightCycler® 480 SYBR Green I Master	Roche, USA
Primer	Pacific Science, Thailand

Table 7 (Cont.)

Chemical	Product of
Protein expression (Western blot)	
Tris-base	Amresco, USA
NaCl	Ajax finechem, New Zealand
NP-40	Amresco, USA
Sodium deoxycholate	Ajax finechem, New Zealand
EDTA	Ajax finechem, New Zealand
Sodium dodecyl sulfate : SDS	Amresco, USA
DTT	Amresco, USA
Protease inhibitor Cocktails	Amresco, USA
glycerol	Amresco, USA
bromophenol blue	Fluka, China
beta-mercaptoethanol	Gibco, Grand Island, NY
Acrylamide	Amresco, USA
Ammonium peroxidisulphate	Amresco, USA
TEMED	Sigma-Aldrich.St. Louis, MO
Glycine	Amresco, USA
Methanol	Rcilabscan, Thailand
NaCl	Amresco, USA
Tween-20	Amresco, USA
non-fat skim milk	Amresco, USA
mouse anti- AGR2 IgG	Sigma-Aldrich.St. Louis, MO
mouse anti- β-actin IgG	Merck Millipore, USA
goat anti-mouse IgG conjugated-HRP	Invitrogen, USA
Protein ladder	Genedirex, Inc., USA
PVDF Immobilon FL	Millipore, Billerica, MA
Chemiluminescence (ECL)	Bio-rad, USA

Table 7 (Cont.)

Chemical	Product of
Knockdown gene	
Lipofecfamine™ 2000	Invitrogen, Carlsbad, CA
Opti-MEM media	Life Technologies, USA
Silencer® Select siRNA #1	Ambion, USA
Silencer® Select siRNA #2	Invitrogen, Carlsbad, CA
pLKO.1-puro-based short hairpin RNA (shRNA)	Sigma-Aldrich.St. Louis, MO
Zymography	
Gelatin	Amresco, USA
Triton X-100	Iobachemie
$CaCl_2$	Ajax finechem, New Zealand
$ZnCl_2$	Ajax finechem, New Zealand
Coomassie blue R250	Fluka, China

Table 8 List of primer sequences

Primer name	Forward 5'⟶3'	Reverse 5'⟶3'
AGR2	TGAAGAAAGCTCTCAAGTTGCT	AGCCAGTCTTCTCACACTTCTT
β-actin	TCGTGCGTGATTAAGGAG	GAAGGAAGGCTGGAAGAGTG

Methods

1. Gene expression using RT-PCR and real-time PCR assay

1.1 RNA extraction from cell lines

Cell were seed $2x10^5$ cell/well in 6 well plate and incubated 48 h. Cells in 500 µl of TRIzol® were incubated at room temperature for 5 min, 100 µl of chloroform was added, and the tube was shaken vigorously for 15 sec. The reaction was incubated at room temperature for 3 min and centrifuged at 12,000 rpm for 15 min at 4ºC. Pipette the upper phase and transfer to a 1.5 ml sterile microtube. Add 500 µl of isopropanol and mix vigorously. The tube was then incubated at room temperature for 10 min and centrifuged at 12,000 rpm for 10 min at 4ºC. Discard the supernatant and wash the pellet with 1 ml of 70% ethanol then centrifuge at 7,500 rpm for 5 min at 4ºC. The pellet was dried and dissolved in 20 µl of sterile distilled water and stored at -20ºC until use.

1.2 Reverse transcription polymerase chain reaction (RT-PCR)

The total RNA (1 µg) was reverse transcribed in to cDNA using Tetro cDNA Synthesis kit (BIO-65042. Bioline, USA). 1 µl of the cDNA was used in each 25 µl One PCR reaction (Invitrogen, USA). forwarded and reversed primers (Table 8) The PCR protocol consisted of an initial denaturation step of 95^{o}C for 5 min, followed by 30 amplification cycles with 30 s at denaturation 95^{o}C, 30 s at annealing at 58^{o}C and extension 5 min at 72^{o}C. The PCR products were analysed on 2% agarose gel and visualized by gel documentation (GeneDirex, USA and Taiwan). AGR2 expression was normalized against the expression of β-actin.

1.3 Quantitative real-time RT-PCR

The total RNA was isolated from cells with Trizol® reagent and 2 µg of RNA was reverse transcribed in to cDNA using Tetro cDNA Synthesis kit. Quantitative real-time PCR was performed in the LightCycler®480 systems. The reaction mixture (20 µL) contained template DNA, forwarded and reversed primers (Table 8) and Light Cycler®480 SYBR Green I Master. The PCR protocol consisted of an initial denaturation step of 95^{o}C for 5 min, followed by 40 amplification cycles with 30 sec at 95^{o}C, 30 sec at annealing temperature; 60^{o}C and 5 min at 72^{o}C. The expression levels of the target genes were normalized with reference to β-actin. The values were expressed as ($2^{-\Delta cp}$), where Δcp= [cp target gene-

cp internal control and for fold change as ($2^{-\Delta\Delta cp}$), where ΔΔcp= [cp target gene-cp internal control (tested cell)]/[cp target gene-cp internal control (control cells)].

2. Protein expression using western blot analysis

2.1 Cell lysate, protein secretion and protein determination

Cells were washed with PBS and lysed with RIPA buffer. Cell lysate was then centrifuged at 12,000 rpm for 20 min at 4°C. Protein secretions were used free serum media for culture cell 48 h Conditioned media were collected with Amcion®Ultra 3K device and centrifuged at 4,000 rpm for 4 h at 4°C for concentrated protein. Protein concentration was measured by the Bradford method.

2.2 SDS-PAGE and western blot analysis

Proteins were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were treated with 5X SDS-PAGE sample buffers, (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, and 0.1% SDS, freshly supplemented with 1 mM DTT and protease-inhibitor cocktails) and boiled 100 °C for 5 min Protein (40 μg) was loaded into an individual well and separeated with 15% separating gel at a constant current of 120 V at 1.30 h. Until the tracking dye front reached the bottom of the gel then proteins in electrophoresed polyacrylamide gel were transferred onto a polyvinylidene difluoride (PVDF) membranes using transferring buffer. After electro-transfer, the membrane was blocked with 5% (w/v) non-fat skim milk at room temperature for 60 min and then washed 3 times with TBST washing buffer (10 mM Tris-HCl pH8, 150 mM NaCl, 0.1% Tween-20). The membrane was incubated with 1:1000 primary antibody mouse anti-human AGR2 or 1:10,000 anti-β-actin antibody for overnight (16-18 h) at 4°C and washed 3 times with washing buffer then the membrane was incubated in a HRP-linked secondary antibody (1:1,000 for AGR2, 1:20,000 for β-actin) for 1 h at room temperature and then washed 3 times with washing buffer. The immunoreactive bands were detected by chemiluminescence ECL Prime Western Blotting Detection System. The membranes were captured with ImageQuantTM 4000 image analyzer and analyzed using ImageJ™ analysis sortware.

3. Transient knockdown with small interfering RNA (siRNA) transfection

Transient knockdown of AGR2 was performed using two specific sequence of siRNA. First sequence of siRNA targeting AGR2 mRNA of exon 2 was chosen from Ambion's Silencer Select pre-designed siRNA library from the company. The sequence was as follow 5' GGACACAAAGGACUCUCGA-3' antisense 5'-UCGAGAGUCCUUUGUGUCC-3. Second sequence of siRNA targeting AGR2 mRNA of exon 8 was following from the previous work of Li et al., 2014 and was synthesized by Invitrogen, Carlsbad, CA. The sequence was as follow 5'-CUGAUUAGGUUAUGGUUUA-3' antisens 5'-UAAACCAUAACCUAAUCAG-3'. The amount of siRNA was transfected into cells using Lipofectamine^{TM} 2000 and assay time following transfection were optimized. Cells (2 x 10^5 cells) in DMEM media with 10% FBS were plated into a 6-wells culture plate for one day. Then transfections with 75 pmole/mL si-AGR2 or negative control and cell culture in free antibiotic media for 6 h before replacing with complete medium. Cells were incubated at 37°C in a CO_2 incubator for 24- 48 h after transection and used for further analysis.

4. Establishment of stable transfection with short hairpin RNA (shRNA)

Stable knockdown of AGR2 was performed using pLKO.1-puro-based short hairpin RNA (shRNA) expression vectors targeting AGR2 expression, shAGR2 designed as sense sequence 5' CCTTGAGACTTGAAACCAGAA -3' antisense 5'- GGAACTATGAACTTTGGTCTT -3 (figure 23A) or a vector control was not insert shAGR2 (figure 23B), they were also performed by following from Kim et al., 2014 and were designed by Sigma-Aldrich, St. Louis, MO, USA. The amount of shRNA was transfected into cells using Lipofectamine^{TM} 2000 and assay time following transfection were optimized. Cells (2 x 10^5 cells) in DMEM media with 10% FBS were plated into a 6-wells culture plate for one day. Then transfections with 1,000 ng/µl shAGR2 or negative control and cell culture in free antibiotic media for 6 h before replacing with complete medium. Cells were incubated at 37°C in a 5% CO_2 incubator for 48 h. after transection and then, were selected by treatment with puromycin 0.75 µg/mL to establish stable AGR2-knockdown cells. Then selection the positive cell was preformed single cloned dilution and expansion for stable AGR2 knockdown cells or negative control cells.

A **B**

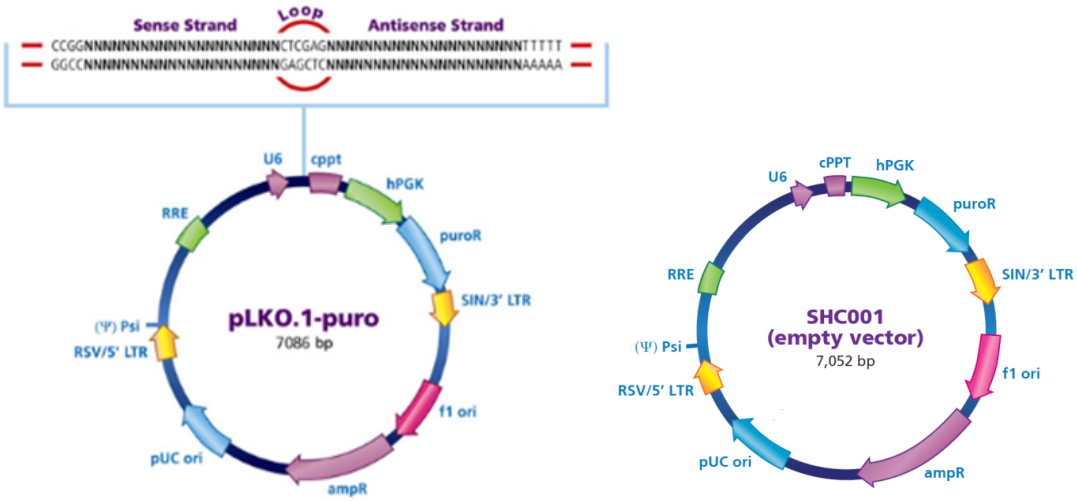


Figure 23 Schematic diagram of pLKO.1-puro-based vector. A. pLKO.1-puro-based vector insert shRNA B. pLKO.1-puro-based vector no insert shRNA was vector control.

Source: https://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/shrna/library-information/vector-map.html

5. Cell proliferation assay

Cells were seed $3x10^3$ cell/well in a 96-well plate for appropriate time (1-5 day). Then 10 µl of 0.5 mg/mL MTT solution was added to each well and at 37°C in a 5% CO_2 for 4 h after MTT formazan was dissolved with DMSO (200 µl/well) and the absorbance measured at 540 nm by a microplate reader (Labsystems, Finland). All experiments were repeated in triplicate.

6. Wound healing assay

Cell lines were seeded in 6-well plates and incubated overnight in DMEM with 10% FBS. Cell monolayers were scratched with a sterile 10 µL pipette tip, washed 2 times with PBS to remove detached cells from the wells. Then incubated further in complete medium and monitored the cell migration to closing wound every 12 h then take pictures and measured the relative migration distance of wound closure.

7. Cell migration and invasion assay

As for migration assay and coated with 0.4 mg/ml Matrigel at overnight for cell invasion assay were performed with 8-μm pore size transwell inserts. 2 x 10^4 of CCA cells were placed onto each upper chamber of the transwell unit and culture in serum-free culture medium. 600 μL of DMEM media with 10% FBS was used as a chemoattractant in the bottom chamber. The cells were allowed to migrated and invade at 37 ^oC and 5% CO_2 for 16 h. Cells were fixed in 4% v/v formaldehyde and stained using 0.5% w/v crystal violet. Cells on the uninvaded side were gently wiped off with a cotton tip applicator. The membranes containing the migrated cells and invaded cells were photographed at a constant magnification (10X) and cell numbers were counted with Image processing and analysis in java software.

8. Gelatin zymography

Zymography was used to analyze the activity of MMP-2 and MMP-9 for digesting gelatin contain on SDS-PAGE. Samples were used condition media without serum of cells culture. Conditioned media were collected with Amcion®Ultra 3K device and centrifuged at 4,000 rpm for 4 h at 4^oC for concentrated protein. The protein obtained in 5X SDS-PAGE loading buffer without β-mercaptoethanol was loaded into an individual well and separated with 7.5% separating gel containing 0.1% gelatin at a constant current of 200V, 2 h at 4 ^oC. After electrophoresis the gels were soaked in renaturing buffer (2.5% v/v Triton-X100 in water) for 30 min, repeated twice times. The gels were developing buffer containing 50mM Tris pH 7.5, 10mM CaCl2, 1mM ZnCl2, 1% v/v Tritox X-100 overnight (16-18 h.) at 37 ^oC. The gels were stained with 0.5% w/v Coomassie blue R250 at 1 h then observe of the proteolytic activity appears as clear bands over a deep blue background

9. Statistical Analysis

Quantitative data were examined in triplicate and expressed as means ± standard deviation (SD). Statistical significance was determined by the unpaired Student's t-test (two tailed) was used for comparison between each group. P-value (*$p < 0.05$ and **$p < 0.01$) was considered statistically significant.

CHAPTER IV

RESULTS

AGR2 expression in metastatic CCA cells

The expression of AGR2 in highly metastatic CCA cells, KKU-213L5 and its parental cells, KKU-213 were examined at the levels of mRNA using RT-PCR as shown in figure 24A and confirmed by real-time PCR as shown in figure 24B. Expression of AGR2 in both intercellular and extracellular fraction were examined at the levels of protein by western blot analysis as shown in figure 25A, B. These results showed that AGR2 mRNA was overexpressed and AGR2 protein was obviously upregulated in both intracellular and extracellular fractions in highly metastatic CCA, KKU-213L5 compared with parental cells, KKU-213.

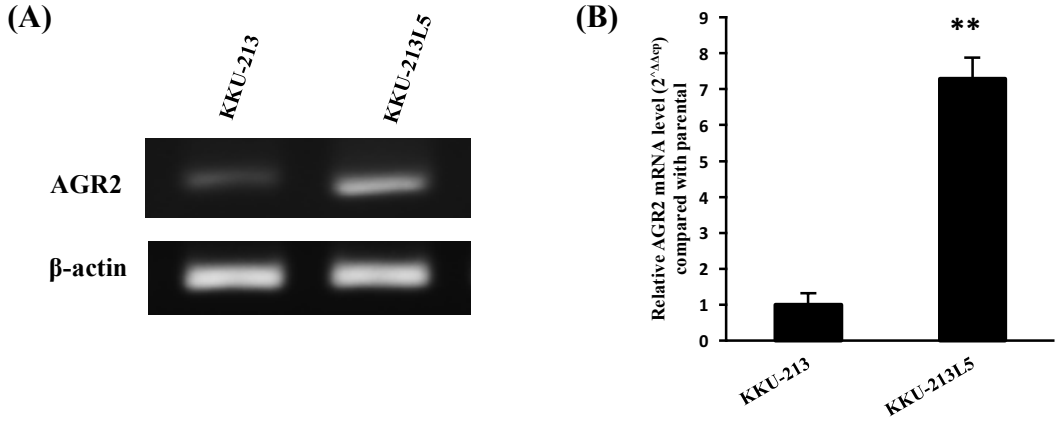


Figure 24 Analysis of AGR2 mRNA expression in CCA lines. The expression of AGR2 mRNA was detected by RT-PCR (A) and real-time PCR (B) in KKU-213 and KKU-213L5 cells. The relative AGR2 expression was normalized with β-actin.

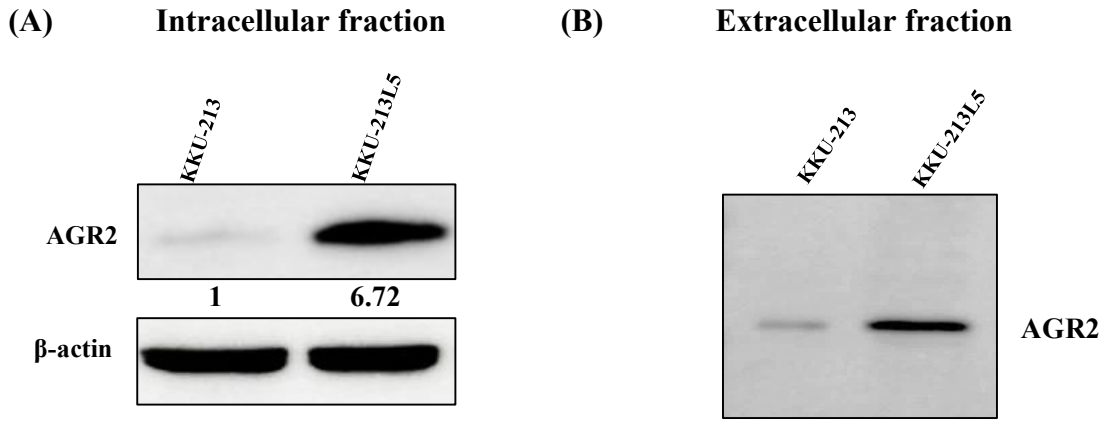


Figure 25 Analysis of AGR2 protein expression in CCA lines. The expression of AGR2 protein was performed by western blot in both intracellular (C) and extracellular fraction (D) of KKU-213 and KKU-213L5 cells. The relative AGR2 expression was normalized with β-actin.

Suppression of AGR2 in highly metastatic CCA cells, KKU-M213L5 using the transient knockdown technique

To examine the functional role of AGR2 in association with metastatic activity of KKU-M213L5, designed two siRNAs were used to knockdown AGR2 expression in KKU-213L5. The amount of si-AGR2 was optimized at 75 pmole/mL transfected at 24 to 96 h and confirmed that both siRNAs targeting AGR2 by western blot analysis. Both si-AGR2 are able to significantly suppress the protein level of AGR2 expression compared with that of the scramble control transfected cells in a time-dependent manner until 96 h as shown in figure 26. However, both si-AGR2 at time 24 h could suppress AGR2 expression to 80% of mRNA level (figure 27A) and decreased protein levels in both intracellular and extracellular fraction (figure 27B), therefore, si- AGR2 at 24 h was selected for use in subsequent studies. β-actin was used as an internal control and demonstrated the specificity of si-AGR2.

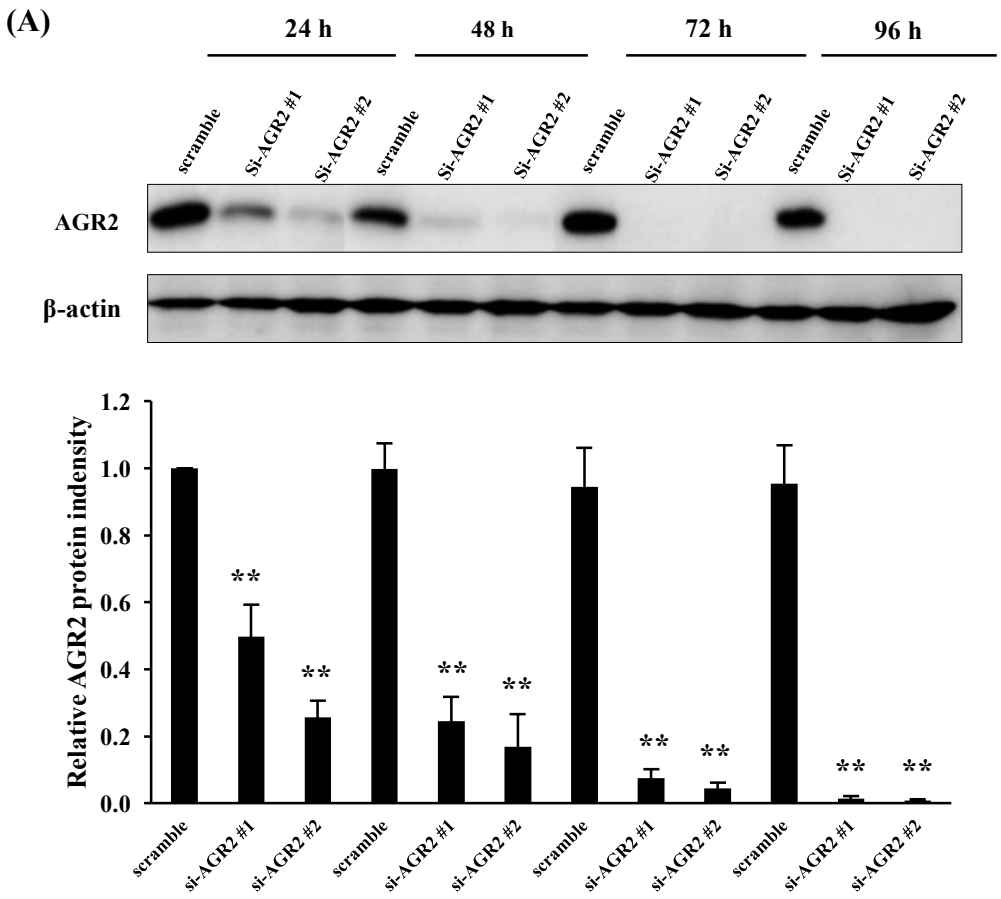


Figure 26 Expression of AGR2 efficiency in a time-dependent manner after transfection siRNA of KKU-213L5 was determined using western blot analysis. The relative AGR2 expression was normalized with β-actin. Student's t-test *p < 0.05, **p < 0.01.

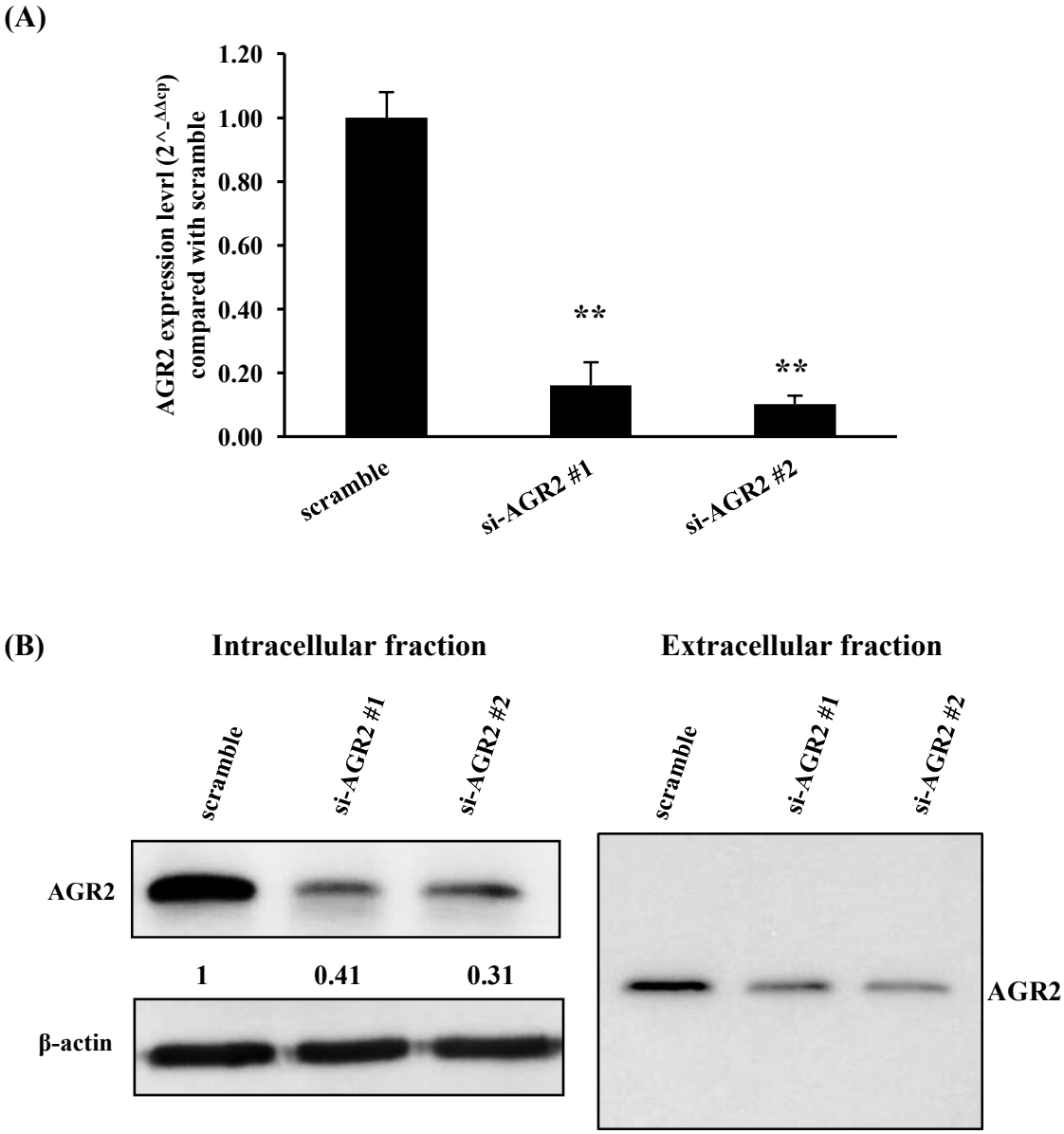


Figure 27 Expression analysis of AGR2 after transfection si-AGR2 at time 24 h AGR2 expression of mRNA level was detected by real-time PCR and (C) protein in both intracellular and extracellular fractions was determined using western blot analysis. The relative AGR2 expression was normalized with β-actin. Result: shown data represent mean ± SD from triplicates. Student's t-test *p < 0.05, **p < 0.01.

Effect of transient knockdown of AGR2 on cell proliferation of highly metastatic CCA

The effects of AGR2 on cell proliferation were first examined. Cells were transfected with 75 pmole/mL si-AGR2 or negative control and were cultured for 24 h then the subculture cells were plated on 96-well plate for 1, 2, 3, 4 and 5 days comparing between scramble control and si-AGR2 KKU-213L5 treated cells. The viable cells in each day were analyzed by MTT assay and measured OD 540 nm. The results are shown, in figure 28. Cell proliferation of si-AGR2 treated cells decreased significantly from day 2, day 3, day 4 and day 5 (*p< 0.05) in both si-AGR2 #1 and si-AGR2 #2 compared to the scramble control cells. These results found that suppression of AGR2 decreased cell proliferation of highly metastatic CCA.

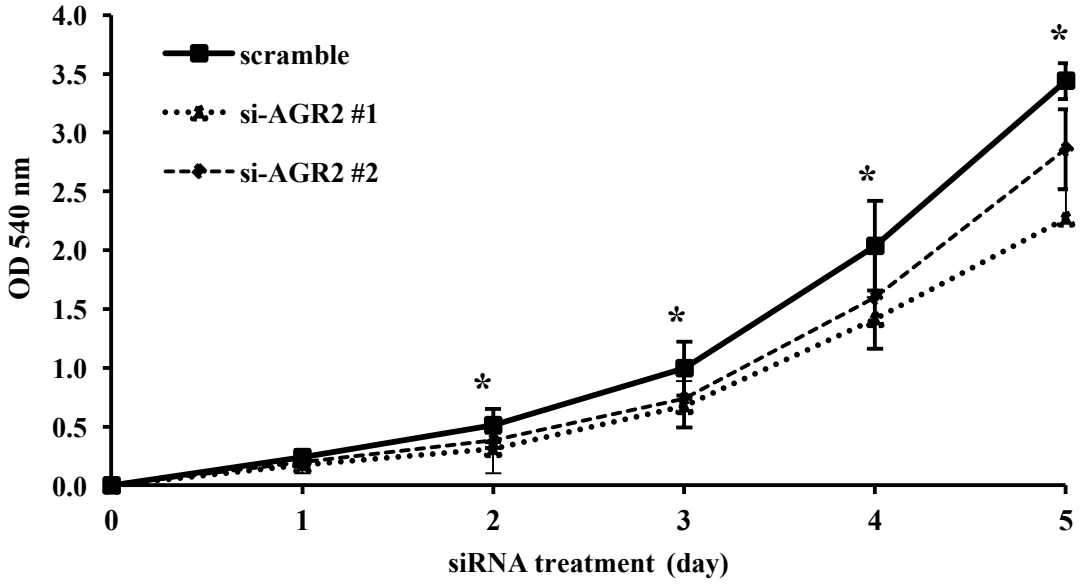


Figure 28 Effect of transient knockdown of AGR2 decreased cell proliferation of KKU-213L5. Expression of AGR2 was suppressed by si-AGR2 and cell growths were determined for 5 days using MTT assay.
The result: shown data represent mean ± SD from triplicates.
Student's t-test *p < 0.05.

Effect of the transient knockdown of AGR2 on cell migration of highly metastatic CCA

The effects of AGR2 on cell migration of KKU-M213L5 were determined by wound healing, reflecting the migration ability of cells and cell-cell interaction. Cells were transfections with 75 pmole/mL si-AGR2 or negative control and incubate for 24 h than the cell monolayers were scratched with a sterile 10 μL pipette tip. After monitoring cell migration to close wounds every 12 h are shown in figure 29A, the scramble controls cells closed wound completely at 24 h while si-AGR2 transfected cells did not in both si-AGR2#1 and si-AGR2#2. The relative migrating distances of wound are shown in figure 29B. These results found that suppression of AGR2 decreased cell migration into the wound closure of highly metastatic CCA.

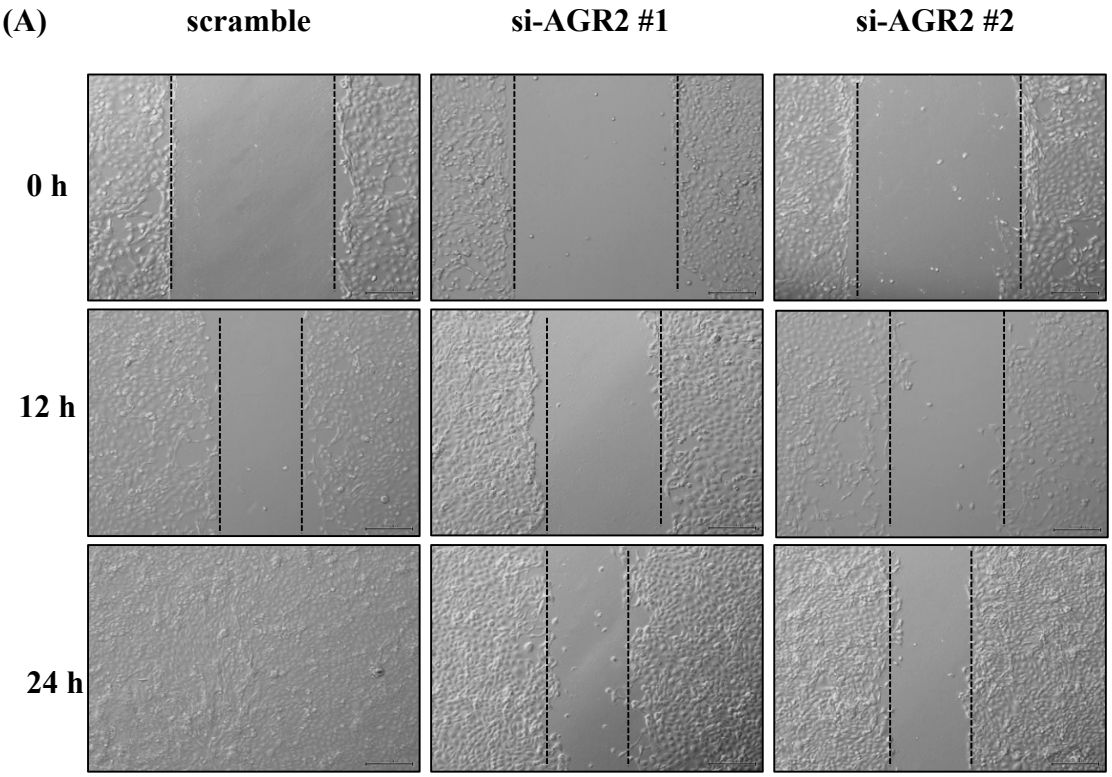


Figure 29 Effect of transient knockdown of AGR2 decreased cell migration of KKU-213L5. (A) Cell migration was determined by wound healing assay at indicated time points. (B) The relative migration distance of wound closure. The result shown data represent mean ± SD from triplicates. Student's t-test *p < 0.05,p < 0.01.**

(B)

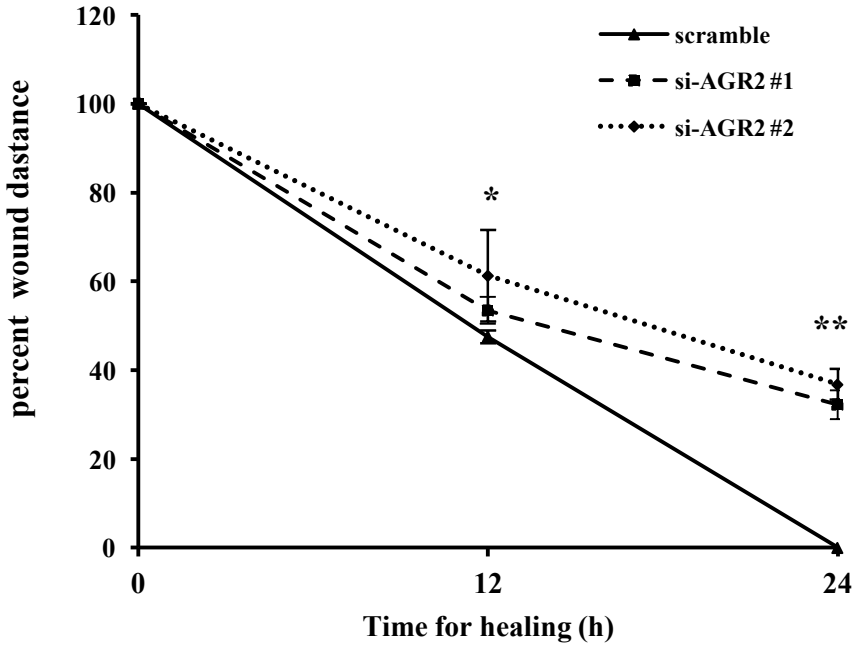


Figure 29 (Cont.)

These results confirmed that AGR2 associated with migration capacities using transwell assay determine cell migration according to chemotactic ability. Cells were transfected with 75 pmole/mL si-AGR2 or scramble control and incubated for 24 h. Cell suspension (2 x 10^4) in serum-free media were added to the upper chamber of the transwell unit and DMEM media with 10% FBS was used as a chemoattractant in the bottom chamber. The cells were allowed to migrate after incubating for 16 h. The results show that knockdown of AGR2 expression using si-AGR2 dramatically reduced the number of migrated cells when compared the scramble controls are shown in figure 30. These results found that the suppression of AGR2 decreased the cell migration of highly metastatic CCA.

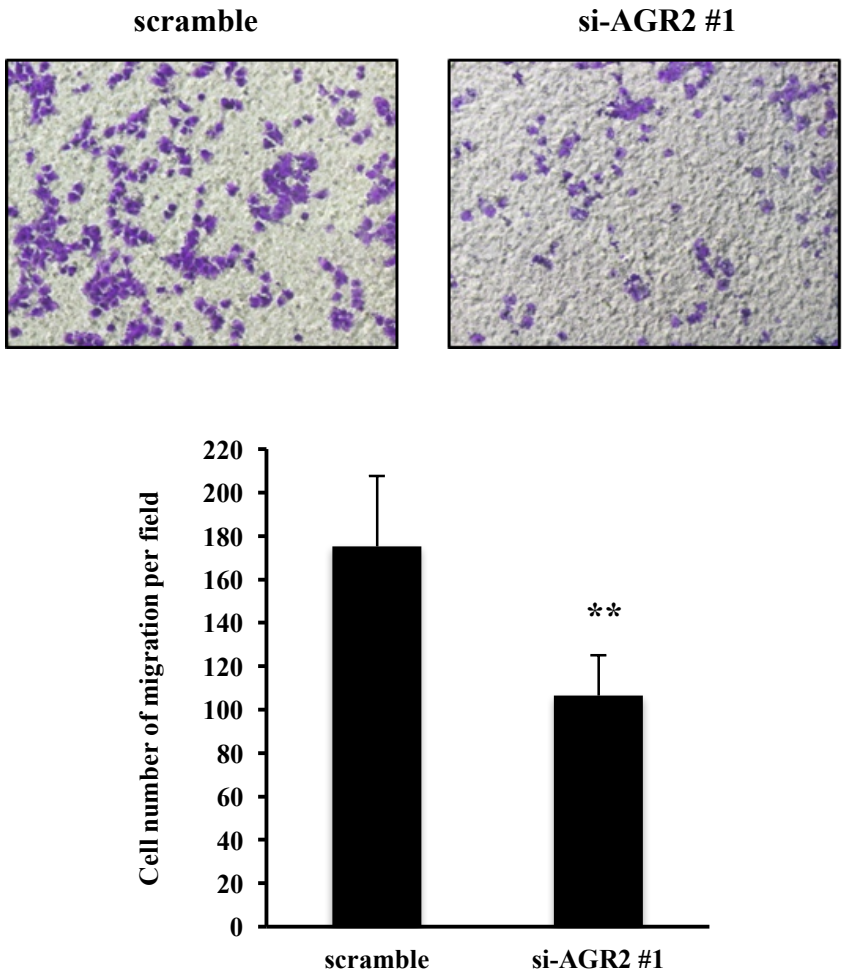


Figure 30 Effect of transient knockdown of AGR2 decreased cell migration of KKU-213L5. The number of migrated cells of KKU-213L5 compared between scramble control and si-AGR2 treated cells. Results are presented as the mean and SD from triplicates. Student's t-test, **p< 0.01.

Effect of transient knockdown of AGR2 on cell invasion of highly metastatic CCA

The effects of AGR2 expression associated with invasion capacities using transwell assay were measuring the ability of cells to invade through Matrigel-coated chambers below according to chemotactic ability. Cell invasion was also performed using transwell chambers in a similar fashion to cell migration, but there is Matrigel coating on the upper transwell compartment. Cells were transfected with 75 pmole/mL si-AGR2 or scramble control and incubated for 24 h. Cells suspension (2×10^4) in serum-free media to determine cell invasion by transwell assay. The cells were allowed to invade after incubating for 16 h. The results showed that knockdown of AGR2 using siRNA in KKU-213L5 cells significantly decreased the number of cells that invaded through Matrigel compared to the scramble control cells as shown in figure 31. These results found that suppression of AGR2 decreased cell invasion of highly metastatic CCA.

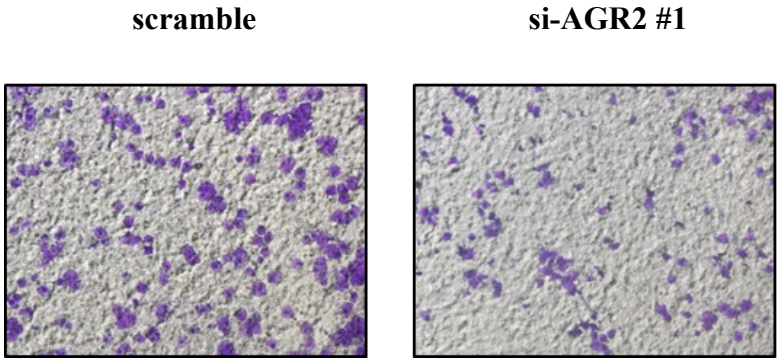


Figure 31 Effect of transient knockdown of AGR2 decreased cell invasion of KKU-213L5. The number of invading cells of KKU-213L5 compared between scramble control and si-AGR2 treated cells. Results are presented as the mean and SD from triplicates. Student's t-test, **p< 0.01.

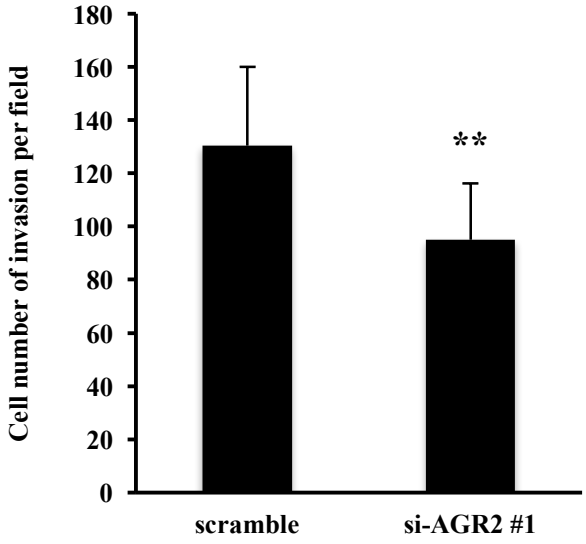


Figure 31 (Cont.)

Effect of transient knockdown of AGR2 on the MMP-2 and MMP-9 secreted level of highly metastatic CCA

MMP-2 and MMP-9 activities are one of metastasis process that successfully promotes cell invasion to degrade and move through the extracellular matrix. To investigate the effect of AGR2 on activation of MMP-2 and MMP-9 secreted in condition medium of KKU-213L, cells were transfected with 75 pmole/mL si-AGR2 or scramble control and incubate for 24 h and subsequently were measured by gelatin zymography. The results showed that suppression of AGR2 did not alter the MMP-2 and MMP-9 activities which were detected the clear bands. As shown in figure 32.

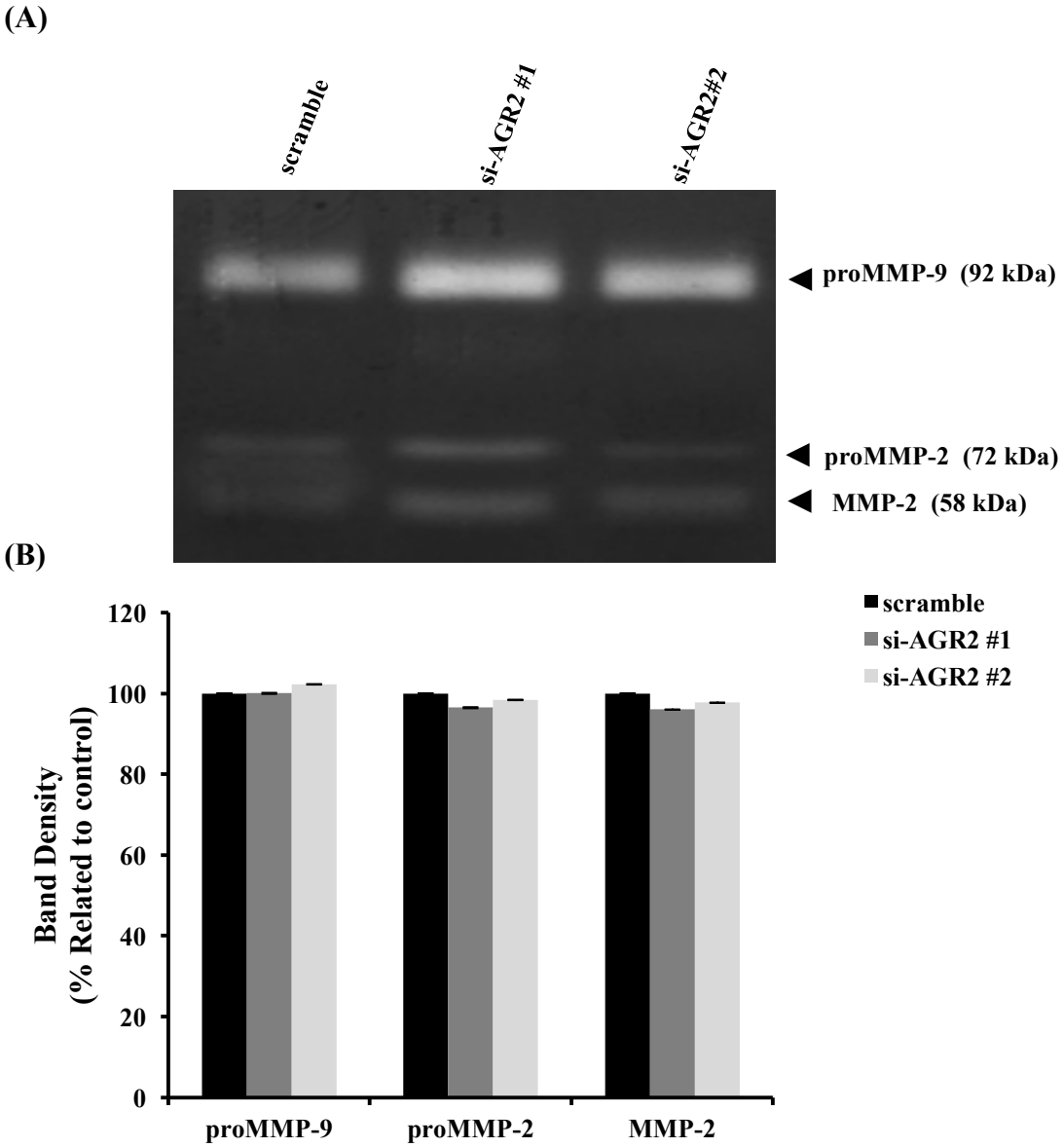


Figure 32 Effect of transient knockdown of AGR2 did not affect MMP-2, MMP- 9 activity of KKU- 213L5. (A) Gelatin zymography of MMP-2 and MMP-9 activities underlying AGR2 suppression in KKU-213L5 are secreted in their latent zymogenic shows clear bands, at 72 kDa (proMMP-2) and 92 kDa (proMMP-9) and the activated forms shows clear bands of 58 kDa for MMP-2. (B) The relative band densities were quantitated by the gel densitometer.

Establishment of stable AGR2 knockdown cells in highly metastatic CCA

At present, the data demonstrates the significant roles of AGR2 promoting cell proliferation, cell migration and cell invasion. These findings suggest that AGR2 could be a novel molecular target for metastatic CCA. To further demonstrate the functional role of AGR2 *in vivo*, the establishment of AGR2 knockdown cells is suitable for this analysis. Therefore, we generated the stable knockdown of AGR2 by silencing its expression with a short hairpin RNA targeting AGR2 in KKU-213L5 cell lines and subsequently investigating the functional role of AGR2 in association with metastatic activity similarly with transient knockdown with small interfering RNA (siRNA). Stable knockdown of AGR2 was performed using pLKO.1-puro-based short hairpin RNA (shRNA) expression vectors targeting AGR2 expression. Transfected KKU-213L5 cells with the shControl and shAGR2 were selected by treatment with 0.75 μg/mL puromycin and the positive cell was performed single cloned dilution following by expansion for stable AGR2-knockdown cells or negative control cells. In this experiment, we obtained 10 clones of stable AGR2-knockdown cells (shAGR2) and 5 clones of their control cells. These cells were subjected to verify the AGR2 expression by western blot analysis. The results showed that short hairpin control cells did not affect AGR2 expression when compared with its parental cell, KKU-213L5 untransfection, as shown in figure 33A. Stable AGR2-knockdown cells investigate AGR2 expression was significantly suppressed of protein level in stable AGR2-knockdown cells, as shown in figure 33B. There data suggested that the stable AGR2 knockdown cells were successful establishment and suitable for the further analysis

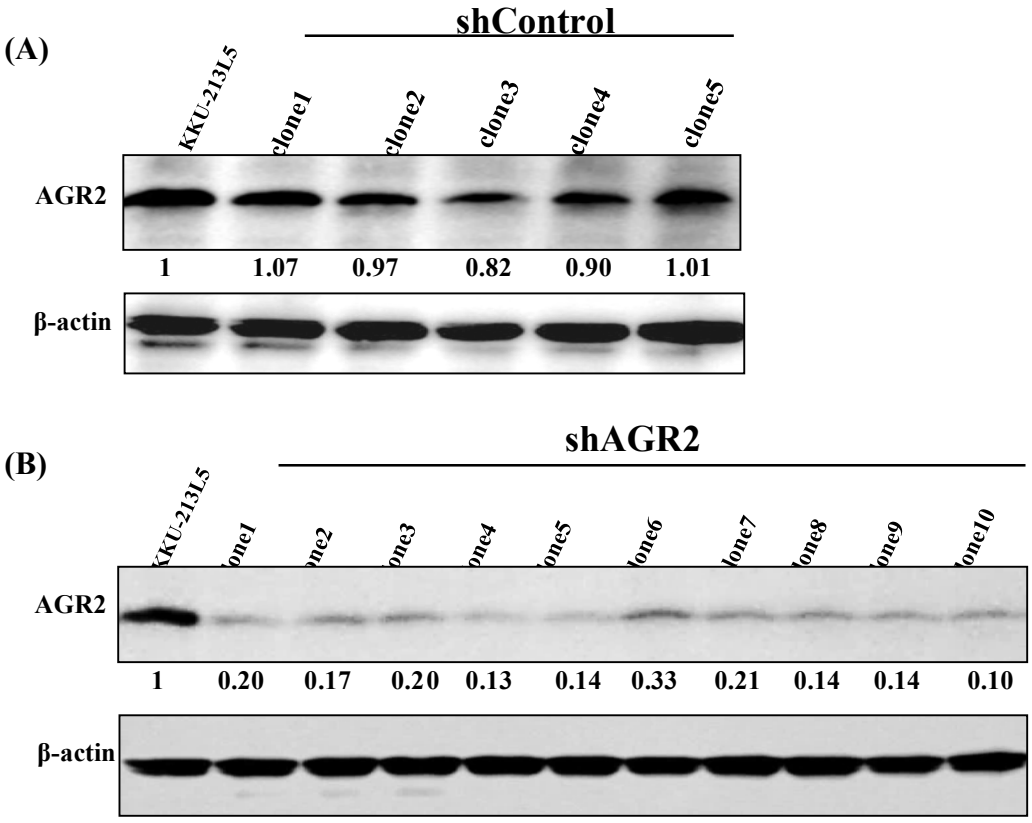


Figure 33 AGR2 expression of stable AGR2 knockdown cells in highly metastatic CCA. Expression analysis of AGR2 in AGR2 knockdown cells was collected and the efficiency of transfection shControl (A) and shAGR2 (B) in KKU-M213L5 cells were determined using western blot analysis. The relative of AGR2 expression was normalized with β-actin.

In this study, our stable AGR2 knockdown cells were validated the metastatic phenotypes of CCA *in vitro.* 2 clones of stable AGR2-knockdown cells (shAGR2-9, shAGR2-10) and 1 clone of short hairpin control cells (shcontrol-1) were selected as a representative of AGR2 knockdown cells and control cells, respectively. In addition, AGR2 mRNA expression levels of the selected cells were determined by real time-PCR. The results showed that AGR2 mRNA expression was almost completely suppressed in both AGR2 knockdown cells compared with their control cells (figure 34A). Moreover, the protein expression levels of AGR2 in both intracellular and extracellular fraction were strongly inhibited in AGR2 knockdown

cells compared with control cells (figure 34B). Next, the metastatic activity was analyzed and related phenotypes of our established stable knockdown cells.

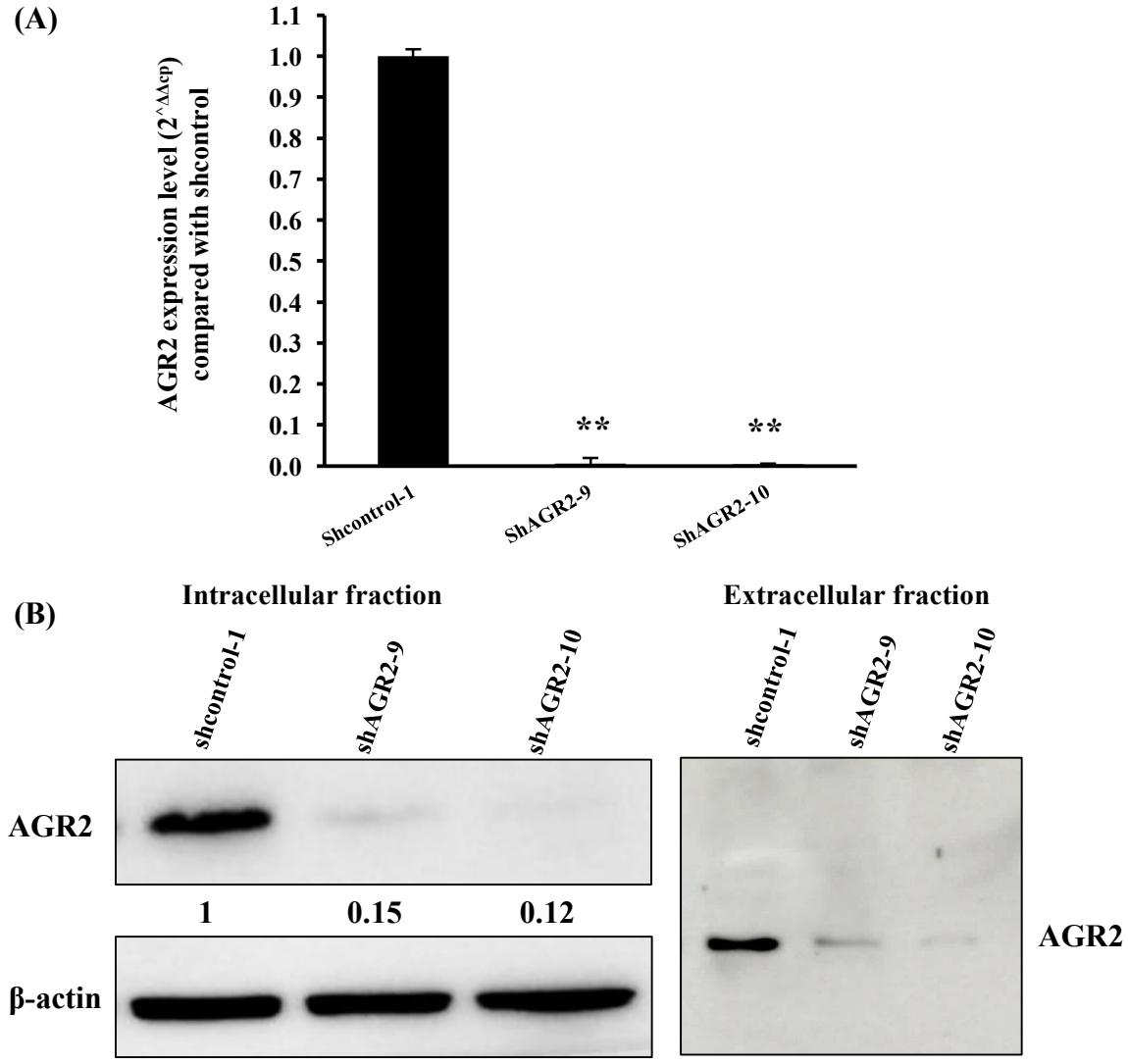


Figure 34 Expression analysis of AGR2 in the selected clones of the stable AGR2 knockdown cells. (A) The AGR2 mRNA expression was determined using real-time PCR, and (B) AGR2 protein expression was determined using western blot in both intracellular and extracellular fractions of the both shAGR2 when compared with short hairpin control cells. The relative of AGR2 expression was normalized with β-actin.

Effect of stable knockdown of AGR2 on cell proliferation of highly metastatic CCA

Stable AGR2-knockdown cells ($3x10^3$) were plated onto 96-well plates for 1, 2, 3, 4 and 5 days, compared between short hairpin control cells. After incubation, viable cells in each day were analyzed by MTT assay. The proliferation of stable AGR2 knockdown cells was decreased significantly from day 3, day 4 and day 5 (*p< 0.05) of shAGR2-9 cells and significantly from day 2, day 3, day 4 and day 5 (**p< 0.01) of shAGR2-10 cells compared to the short hairpin control cells (shcontrol-1). As shown in figure 35. These results found that stable AGR2 knockdown cells decreased cell proliferation of CCA cells.

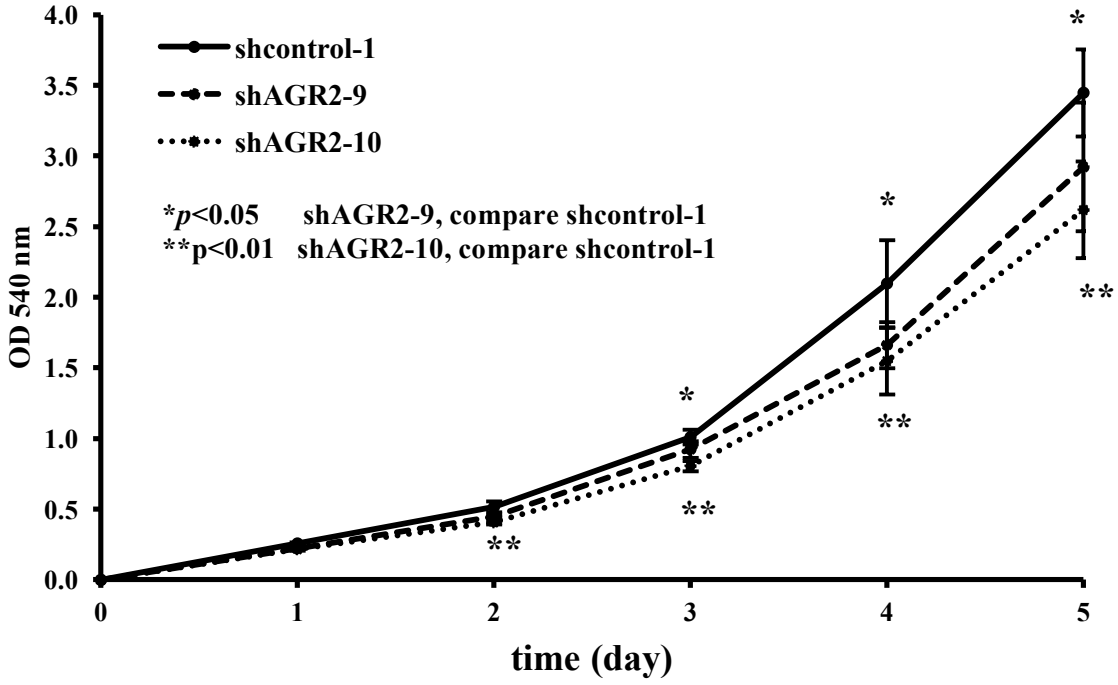


Figure 35 Effect of stable knockdown of AGR2 decreased cell proliferative of KKU-213L5. Expression of AGR2 was suppressed by shAGR2 and cell growths were determined for 5 days using MTT assay. The result shown data represent mean ± SD from triplicates. Student's t-test *p < 0.05, **p< 0.01.

Effect of stable knockdown of AGR2 on cell migration of highly metastatic CCA

Cell migration ability of stable AGR2-knockdown cells were determined by wound healing assay, stable AGR2-knockdown cells were grown as a monolayer in 6-well plates in DMEM medium with 10% FBS for 2 days until 80% confluent than the cells were scratched with a sterile 10 μL pipette tip. Cell migration to close the wound was monitored every 12 h. As shown in figure 36A, the short hairpin control cells (shcontrol-1) closed the wound completely at 24 h. while the stable AGR2-knockdown cells did not in both shAGR2-9 and shAGR2-10. The relative migrating distances of wound are shown in figure 36B.

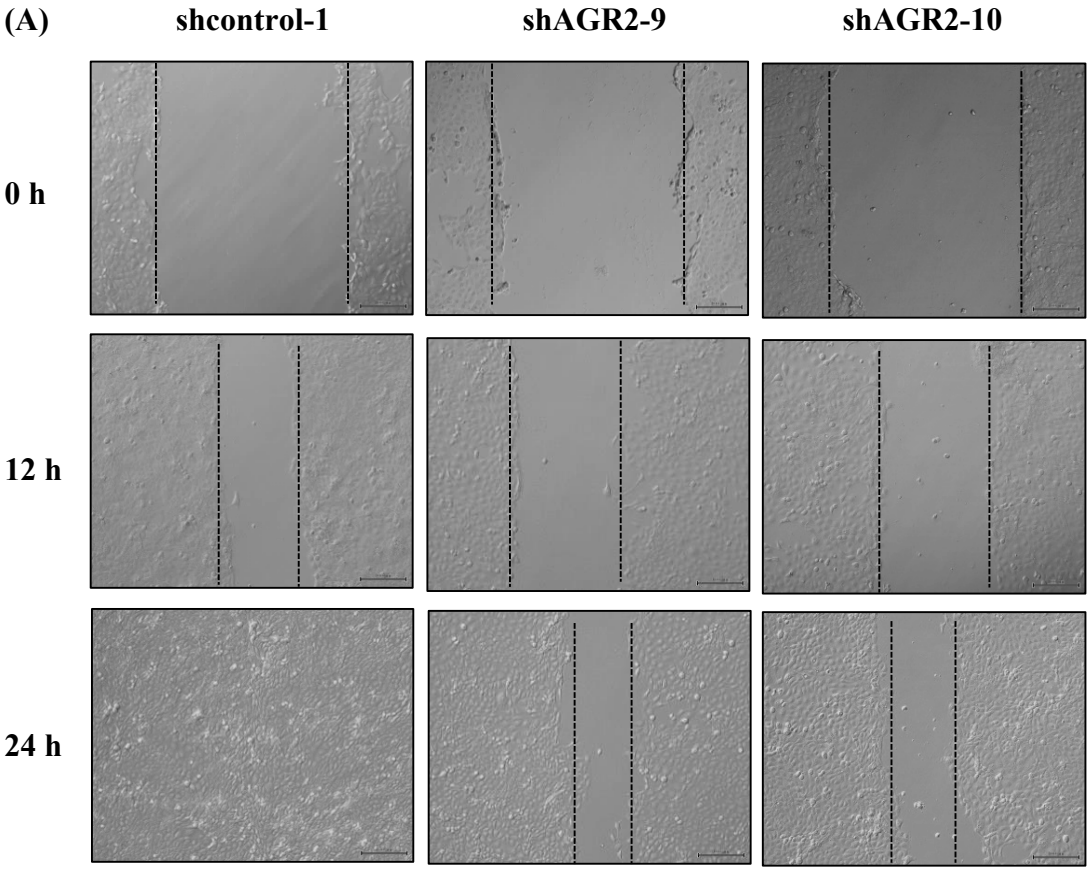


Figure 36 Effect of stable knockdown of AGR2 decreased cell migration of KKU-213L5. (A) Cell migration was determined by wound healing assay at indicated time points. (B) The relative migration distance of wound closure. Results are presented as the mean and SD from triplicates. Student's t-test *p <0.05 **p< 0.01.

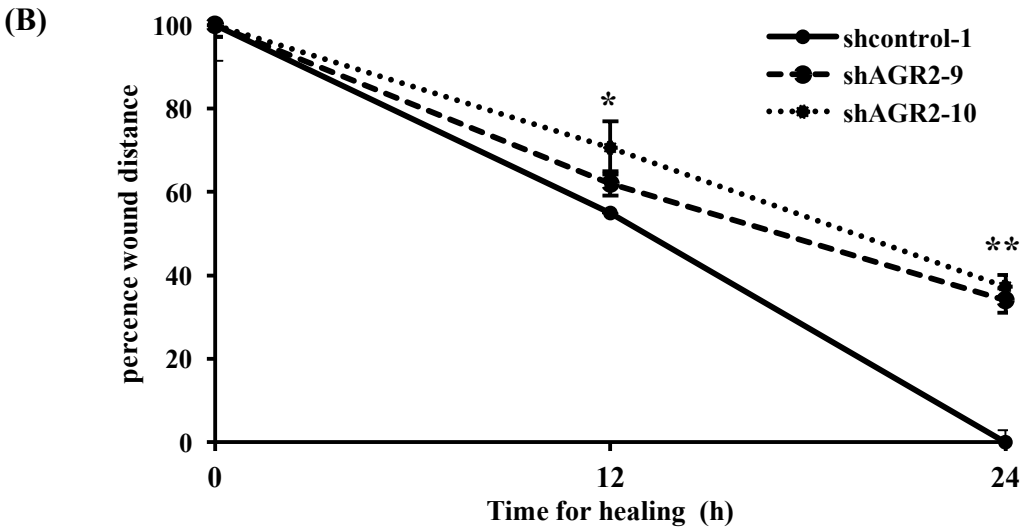


Figure 36 (Cont.)

Similarly, stable knockdown of AGR2 were determined by transwell assay. Cell suspension (2 x 10^4) in transwell chambers and DMEM media with 10% FBS was used as a chemoattractant. The cells were allowed to migrate for incubate 16 h. To show that stable knockdown of AGR2 reduced the number of migrated cells in both shAGR2-9 and shAGR2-10 compared to the short hairpin control cells (shcontrol-1) are shown in figure 37. These results found that stable AGR2 knockdown cells decreased cell migration of CCA cells.

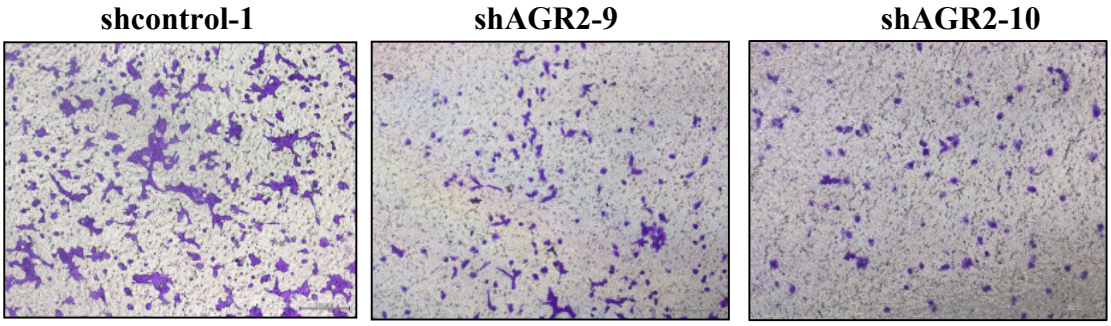


Figure 37 Effect of stable knockdown of AGR2 decreased cell migration of KKU-213L5. The number of migrated cells in stable AGR2-knockdown cells compared to the short hairpin control cells. Results are presented as the mean and SD from triplicates. Student's t-test *p <0.05 **p< 0.01.

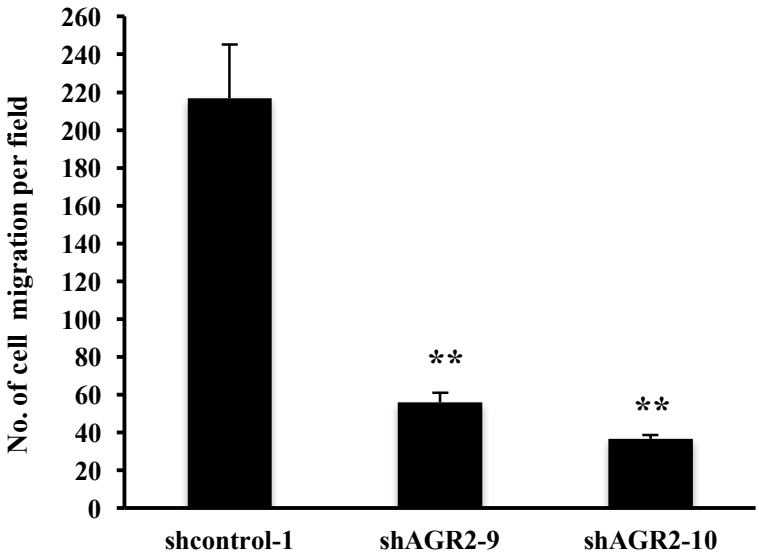


Figure 37 (Cont.)

Effect of stable knockdown of AGR2 on cell invasion of highly metastatic CCA

Cell invasion ability of stable AGR2-knockdown cells were determined by Matrigel-coated transwell assay. Cell suspension (2 x 10^4) in Matrigel-coated transwell chambers and DMEM media with 10% FBS was used as a chemoattractant. The cells were allowed to invade for incubate 16 h. The results show that stable knockdown of AGR2 reduced the number of invading cells through Matrigel in both shAGR2-9 and shAGR2-10 compared to the short hairpin control cells (shcontrol-1) are shown in figure 38. The results found that stable AGR2 knockdown cells decreased cell invasion of CCA cells.

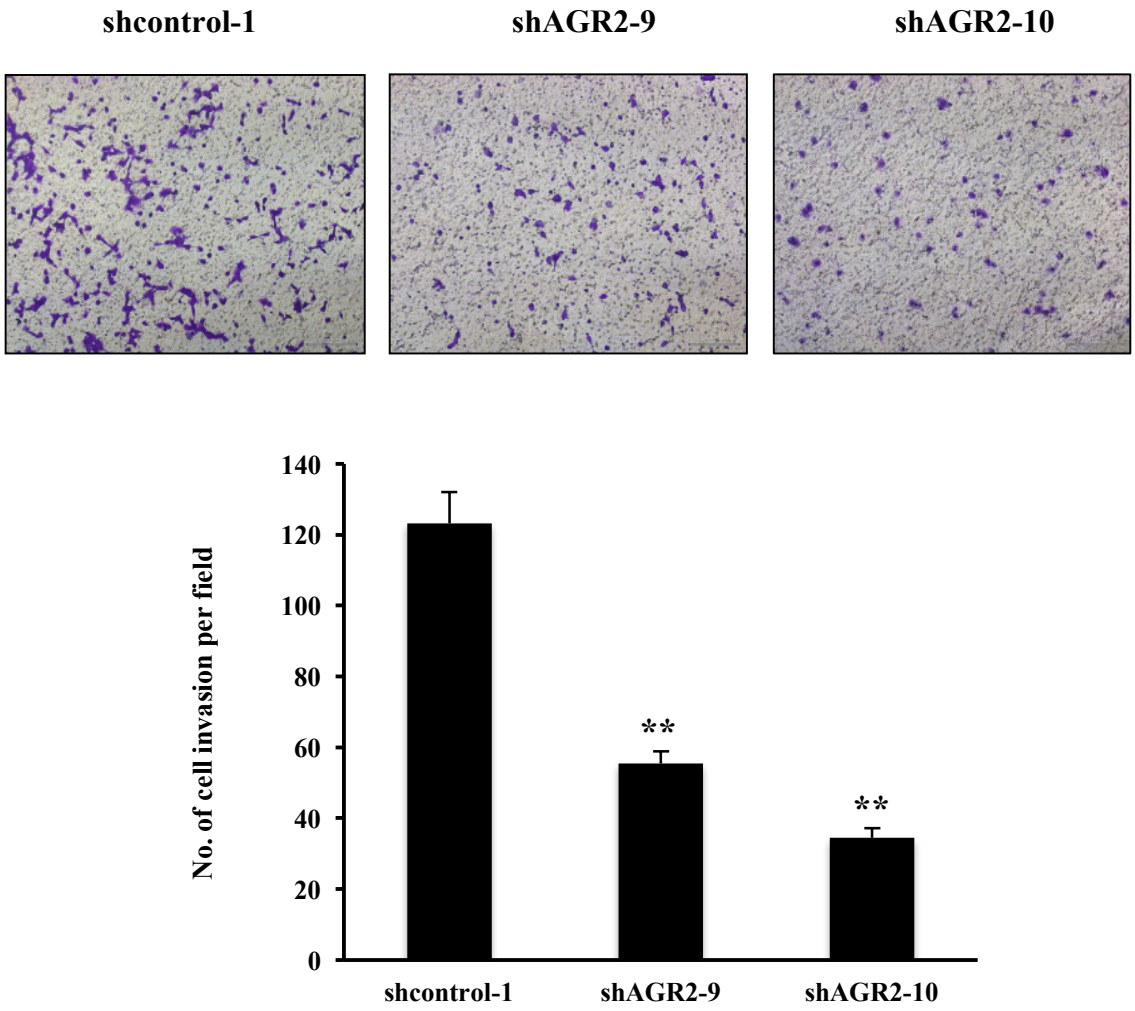


Figure 38 Effect of stable knockdown of AGR2 decreased cell invasion of KKU-213L5. The number of invading cells in stable AGR2-knockdown cells compared to the short hairpin control cells. Results are presented as the mean and SD from triplicates.
Student's t-test **p< 0.01.

Effect of stable knockdown of AGR2 on the MMP-2 and MMP-9 secreted levels of highly metastatic CCA

The effect of AGR2 on the levels of MMP-2 and MMP-9 secreted in the conditioned medium of stable AGR2-knockdown cells and compared to the short hairpin control cells were measured by gelatin zymography. The results show that stable knockdown of AGR2 did not alter the MMP-2 and MMP-9 secreted levels which detected the clear bands when compared to the short hairpin control cells, as shown in figure 39.

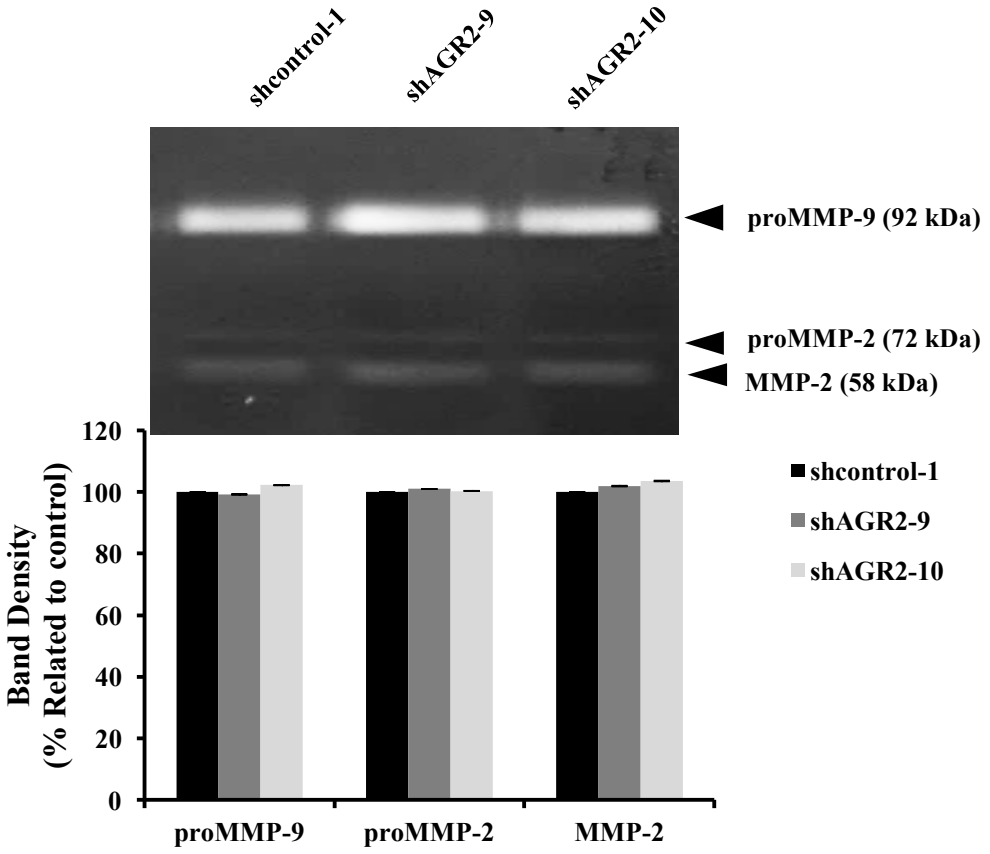


Figure 39 Effect of stable knockdown of AGR2 did not affects to MMP-2, MMP-9 activity of KKU- 213L5. (A) Gelatin zymography of MMP-2 and MMP-9 activities underlying stable AGR2 knockdown cells are secreted in their latent zymogenic shows clear bands, at 72 kDa (proMMP-2) and 92 kDa (proMMP-9) and the activated forms shows a clear band of 58 kDa for MMP-2. (B) The relative band densities were by gel densitometer.

CHAPTER V

DISCUSSION AND CONCLUSION

Discussion

Cholangiocarcinoma, CCA has a very poor prognosis and is extremely aggressive, most patients are diagnosed at metastasis stage, at which chemotherapy and surgery do not provide a better prognosis. Thus, understanding the molecular mechanism of metastasis in CCA might provide an innovative strategy for better treatment of CCA, the highly metastatic CCA cell line model is needed for study leading to a better treatment for CCA. Uthaisar et al., 2016 have reported that the highly metastatic CCA cell line was established from metastatic parental cells and found that the Anterior Gradient 2 (AGR2) gene was highly upregulated and was rated as first in previous studies of highly metastatic CCA compared to the parental cells. The preliminary data shows that AGR2 might be a molecular basis involved in metastasis of CCA. In this study, it was demonstrated that the functioning of AGR2 is involved in CCA metastasis. AGR2 is a member of the protein disulfide isomerase family of endoplasmic reticulum and functions as a molecular chaperone to correct protein folding and control endoplasmic reticulum homeostasis (Persson et al., 2005). The transcription of AGR2 was increased in response to physiological stresses of serum starvation, hypoxia and ER stress (Zweitzig et al., 2007). In addition, the expression of AGR2 is strongly expressed in mucus secreting cells (Obacz et al., 2015) and found that AGR2 upregulation in various cancers including breast cancer (Barraclough et al., 2009), ovarian cancer (Fletcher et al., 2004), lung cancer (Park et al., 2007), liver cancer (Pohler et al., 2004), and prostate cancer (Zhang et al., 2005). Several reports indicated that AGR2 plays an important role to promote cancer metastasis associated with a phenotype cell proliferation, migration and invasion, suggesting the expression of AGR2 may be associated with cancer development and the progression in cancer metastasis. However, the expression and functional role of AGR2 associated with metastatic potential in CCA has not yet been clarified. In the present study, we demonstrated the AGR2 expression and

its functional roles in metastatic CCA models including a highly metastatic CCA cell and its parental cells.

1. Expression of AGR2 in Cholangiocarcinoma cells

Previous studies showed that AGR2 was obviously overexpressed in several cancer metastatic models comparing with their parental cells. Chen et al., 2013 reported the metastatic cell model of head and neck squamous cell carcinoma (HNSCC) by establishing the highly metastatic, HSC-3-5 cells from its parental cells, HSC-3 and investigated gene expression profiles in these cell models by microarray analysis. The result showed that AGR2 expression was a sixteen-fold increase in HSC-3-5 cells compared with HSC-3 cells. In addition, Ma et al., 2017 established adenoid cystic carcinoma (AdCC) cell lines with highly metastatic potential, SACC-LM cells from the lung metastatic focus of SACC-83 parental cells in nude mice. Obviously, the protein and mRNA expression of AGR2 was significantly elevated in SACC-LM cells as compared with the parental cells SACC-83 cells. Here, it was demonstrated the AGR2 expression in highly metastatic CCA, KKU-213L5, which was established from its parental CCA cells, KKU-213 in the mice model. The comparison the AGR2 expression between KKU-213L5 cell line and KKU-213 cell line found that AGR2 was found to be overexpressed in the highly metastatic cells, KKU-M213L5 both of transcript mRNA (figure 24A, B) and protein levels (Figure 25A) of seven-fold when compared with its parental cells, KKU-213. These findings indicated that AGR2 was probably involved in the process of metastasis of CCA cells.

In addition, these findings were the first direct evidence in human CCA cells that AGR2 could be secreted. It was found that the secreted AGR2 was strongly present in the conditioned medium of highly metastatic cells, KKU-213L5 (figure 25B). This result was consistent with previous studies that reported that AGR2 is secreted into the cultured media in variable levels of pancreatic cancer cell lines (Ramachandran et al. 2008, Zhang et al. 2005). Moreover, AGR2 protein has been detected in the serum of mucinous ovarian tumor patients by Western blot and ELISA methods. These results suggested that serum AGR2 could be used as a biomarker for the diagnosis and prediction of mucinous ovarian cancers (Park et al., 2011). Wang et al., 2008 reported that conditioned media from

an esophageal adenocarcinoma cell line expressing higher levels of AGR2 enhanced the migration of cells. Since AGR2 is a secreted protein in CCA, it may generate some of its biological effects by acting in an autocrine or paracrine manner, which may leads to the promotion of CCA metastasis.

Furthermore, the secreted AGR2 was measured in the conditioned medium of both si-AGR2 transfected cells and stable AGR2 knockdown cells. These results found that secreted AGR2 in AGR2 knockdown cells was decreased into extracellular spaces compared to the control cells (figure 25B, 34B). Previously, yeast two-hybrid cloning analysis suggested that AGR2 can interact with the metastasis gene (C4.4a) and with dystroglycan (DAG-1) resulting in induced cell adhesion and migration, which is important for the tumor progression and metastasis (Fletcher et al., 2003). In a recent paper by Guo et al., 2017 suggests that the secreted AGR2 promoted the angiogenesis and fibroblasts, coordinated the tumor cell invasion and metastasis by enhancing the activities of vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2). However, whether these interactions are involved in the effects of AGR2 in cancer remain uncertain. Thus, the exact mechanism of secreted AGR2 performance of CCA its function needs to be further investigated. The results of this study show that AGR2 is overexpressed in CCA with a high potential of metastasis, and it exists in both intracellular and extracellular spaces, suggesting that AGR2 might be molecular based and involved in promoting CCA metastasis. The detailed functional roles of AGR2 regulated CCA metastasis need further investigation.

2. The functional role of AGR2 related with CCA metastasis by using transient knockdown

To study the functional roles of AGR2 associated with metastatic activity in CCA metastasis, it was demonstrated by suppression of AGR2 in KKU-213L5 using the transient knockdown technique specific to AGR2 mRNA. The transient knockdown of AGR2 was performed using two specific sequences of siRNA. The si-AGR2 #1 was chosen from Ambion's Silencer Select pre-designed siRNA library from the company while si-AGR2 #2 was following from previous work of Li et al., 2014 was demonstrated that the knockdown of AGR2 using si-AGR2 #2 in Nasopharyngeal carcinoma cells effectively silenced AGR2 expression for 72 h

post-transfection, and inhibited cell motility and invasion. The data showed that transfection of both si-AGR2 #1 and si-AGR2 #2 suppressed the expression of the AGR2 gene effectively at a time-dependent manner from 24 until 96 h which were verified by western blotting as shown in figure 26A. Therefore, we were selected time for 24 h of transfected cells with both si-AGR2 #1 and si-AGR2 #2 as used in subsequent studies. After transfection of both si-AGR2 #1 and si-AGR2 #2 for 24 h could suppress AGR2 expression to 80% of mRNA level (figure 27A) and obviously decreased protein (Figure 27B) in both intracellular and extracellular fraction compared to the scramble control cells. Thus, transient knockdown with siRNA was efficiently used for the further investigation of the functional role of AGR2 in association with *in vitro* metastatic activity namely cell proliferation, migration, invasion and activation of MMP-2 and MMP-9 in metastatic CCA.

2.1 Effects of AGR2 knockdown on cell proliferation of CCA

In this study, AGR2 was shown to be involved in cell proliferation as knockdown of AGR2 significantly decreased cell proliferation of both AGR2 knockdown cells si-AGR2 transfected cells (figure 28). The results were in accordance with several research projects. They have reported that transient silencing of AGR2 inhibits cell growth, cell-cycle progression and induces cell death of breast cancers via cyclin D1, c-myc, and survivin (Vanderlaag et al., 2010). Knockdown of AGR2 decreased cell viability, promoted cell death and induced cellular senescence in prostate cancer (Hu et al., 2012). In addition, the stable knockdown AGR2 in MPanc-96 pancreatic cancer cell line decreases tumor growth in xenogeneic tumor model (Wang et al., 2008). Taken together, these findings indicate that AGR2 might promote tumor progression of CCA by enhancing cell proliferation for tumor cell circulation and metastasis to a distant organ. However, the underlying mechanism was not investigated in this study.

2.2 Effects of AGR2 knockdown on cell migration and invasion of CCA

Migration and invasion are essential processes for tumor cell circulation and the development of metastatic tumors at secondary sites (Fidler et al., 2003). The functional role of AGR2 in CCA was shown to be involved in cell migration was assessed using wound healing assays, a technique used to study the lateral migration of cell and cell-cell interaction. A scratched wound was introduced

on a confluent monolayer of KKU-213L5 cells transfected with si-AGR2 or scrambled at 24 h (figure 29A and B). The data showed that KKU-213L5 transfected with the scrambled control efficiently migrated into the wound, in contrast cells transfected with si-AGR2 had a reduced migrating ability both at 12 and 24 h. In addition, the confirmation of the AGR2 promoting the cell migration ability was performed by using *in vitro* transwell assays which is an experimental tool used to determine the ability of migratory cells according to chemotaxis. The results showed that KKU-213L5 cells with the depletion of AGR2 by siRNA were significantly decreased in their ability to migrate compared with the control cells (figure 30). These results were consistent with a previous study about the silencing of AGR2 attenuated metastasis-associated phenotypes of migration in various cancers including papillary thyroid carcinoma (Di Maro et al., 2014) and salivary adenoid cystic carcinoma (Ma et al., 2017) However, it has been reported that Zhu et al., 2017 found that AGR2 functions to enhance the wound healing process by promoting cell migration through FAK and JNK pathways.

In addition, the important function of AGR2 involves cell invasion, that is, the ability of cells to invade through extracellular matrix (ECM) for distant dissemination and an essential part of cancer metastasis. The knockdown of AGR2 with si-AGR2 transfected cells were had reduced invasive ability to invade through Matrigel compared with the control by *in vitro* transwell assay (figure 31). The results were consistent with a previous study showing that depletion of AGR2 in AdCC was decreased cell invasion (Ma et al., 2017). Moreover, it was observed that a significant reduction in invasiveness *in vitro* after AGR2 silencing of pancreatic cancer (Ramachandran et al., 2015). On the other hand, forced AGR2 expression resulted in increased cell invasion of papillary thyroid carcinoma (Gennaro et al., 2014) and SNU-478 ampulla of Vater cancer cells (Kim et al., 2014). Taken together, these findings support a functional role for AGR2 in cell migration and invasion, which is important for the tumor progression and metastasis characteristics of CCA.

2.3 Effects of AGR2 knockdown on the MMP-2 and MMP-9 secreted levels of CCA

Cancer cell invasion will be displayed through the stimulation of MMPs activities for degradation of the extracellular matrix, associated with the increase of metastatic potential in many cancer types. MMP-2 and MMP-9 are the major MMPs capable of degrading native collagen type IV, the main constituent of basement membranes (Roomi et al., 2009). Herein, it was demonstrated whether the AGR2 could promote cancer cell invasion through the activation of MMP-2 and MMP-9 secreted for extracellular matrix degradation. This result found that knockdown of AGR2 had no effect with the activation of MMP-2 and MMP-9 in both si-AGR2 transfected cells (figure 32). These findings suggest that AGR2 inducing metastasis might not regulate MMP-2 and MMP-9 activities, which could be secreted for the degradation of the extracellular matrix leading to cell invasion of CCA. Thus, the other pathway of secreted degrading enzymes could be a possible way of AGR2 promoting invasion in CCA cells. Moreover, Dumartin et al., 2011 have been reported that AGR2 promoted cell invasiveness and metastasis through regulation of cathepsin B and D, the lysosomal protease in extracellular matrix degradation resulting in increased invasiveness of prostate cancer. In addition, Xu et al., 2016 has reported that significant expression of AGR2 affects tumor cells by promoting invasion and metastasis through regulation of epithelial-mesenchymal transition (EMT). Thus, the precise molecular mechanisms by which AGR2 regulates cell invasion in CCA need to be elucidated in the future.

3. Establishment of stable AGR2 knockdown cells in highly metastatic CCA

This previous data indicated that AGR2 was overexpressed in highly metastatic CCA cells, KKU-213L5, compared with the parental cells, KKU213. Suppression of AGR2 expression using transient knockdown in KKU-213L5 showed that AGR2 involved in the promotion of metastatic related process including of cell proliferation, cell migration and cell invasion. These finding could be implied that AGR2 might be a novel molecular target for metastatic CCA leading us interesting in the further investigation about AGR2 for supporting our current data. Generation of the stable AGR2 knockdown cells could be more advantage than the transient

knockdown in several ways such as the suitable cells for *in vivo* study and the molecular mechanism of AGR2 associated with CCA. Therefore, this study aimed to establish the stable AGR2 knockdown cells using short hairpin RNA (shRNA) and determine its functional role in metastatic phenotypes *in vitro*. KKU-213L5 cells were transfected with pLKO.1-puro-based short hairpin RNA (shRNA) expression vectors targeting AGR2 and its control vector was also performed by according to Kim et al., 2014. In that work, it was demonstrated that the effects of AGR2 are implicated in tumorigenesis and tumor progression of biliary tract cancer cells (SNU-478 ampulla). Here, the stable AGR2 knockdown cells were established in KKU- 213L5 by selecting for puromycin resistance. The clone 9 and 10 of stable AGR2-knockdown cells were used in subsequent studies as selecting from their suppression efficacy. Both shAGR2-9 and shAGR2-10 effectively silenced AGR2 as well as more than 80% of mRNA level (figure 34A) and decreased protein expression in both intracellular and extracellular fractions (figure 34B) when compared with short hairpin control cells. These data suggested that our stable AGR2 knockdown cells were successfully established and suitable for further analysis. To determine the metastatic phenotypes of the stable AGR2 knockdown cells, cells were subjected to the same experiment as done by the transient knockdown of AGR2. The results found that all the metastatic related characteristics of the stable AGR2 knockdown cells were similar to the results showing by transients knockdown as the significant decreasing of cell proliferation (figure 35), cell migration (figure 36, 37) and cell invasion (figure 38). Moreover, the activities of MMP-2 and MMP-9 in the stable AGR2 knockdown cells still did not change the activities (figure 39). Therefore, the newly established AGR2 knockdown cell could be an advantage tool for further analysis. Accumulating results indicated that AGR2 had a significant role in enhancing metastasis of CCA cells.

Conclusion

The expression of AGR2 was markedly increased at both mRNA and protein levels in highly metastatic CCA, KKU-213L5 compared with the parental cells, KKU-213. Moreover, the secreted AGR2 expression was also increased in highly metastatic cells suggesting the possible role of AGR2 on metastasis of CCA. Furthermore, the suppression of AGR2 expression using knockdown techniques was used to analyze the metastatic phenotypes in CCA cells. The transient and stable knockdown AGR2 in KKU213L5 cells significantly reduced cell proliferation, migration and invasion of KKU-213L5 but did not affect MMP-2 and MMP-9 activity. These findings suggest that AGR2 plays an important role in the metastasis of CCA by promoting cell proliferation, migration and invasion. Taken together, these data suggest that AGR2 may be a reasonable target for the development of future therapies and could be a molecular target for CCA treatment, especially CCA with the tendency of metastasis.

Further works

1. To verify the molecular mechanism which AGR2 plays in the metastatic process of CCA

2. To study *in vivo* metastatic activities of stable AGR2 knockdown cells for tumor progression and metastasis characteristic of CCA

3. To study the potential of AGR2 at the diagnostic and/or prognostic metastasis stages of CCA

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APPENDIX

Table 1 The expression of AGR2 in highly metastatic CCA cells, KKU-213L5 compared with its parental cells, KKU-213 was examined at the levels of mRNA using real time PCR. The results were obtained from tree separate experiment.

		KKU-213		KKU-213L5	
		ATCB	AGR2	ATCB	AGR2
	1	19.15	25.43	18.69	22.88
Cp	2	18.75	25.88	20.22	23.72
	3	18.95	25.66	19.46	23.30
Mean		18.95	25.66	19.46	23.30
SD		0.20	0.23	0.76	0.42

Table 2 The expression of AGR2 in transient knockdown of AGR2 cells compared with its scramble control cells were examined at the levels of mRNA using real time PCR. The results were obtained from tree separate experiment.

		scramble		si-AGR2#1		si-AGR2#2	
		ATCB	AGR2	ATCB	AGR2	ATCB	AGR2
	1	19.45	22.81	19.05	25.27	19.21	26.56
Cp	2	19.69	23.93	19.65	26.31	20.08	26.94
	3	19.57	23.37	19.35	25.79	19.65	26.75
Mean		19.57	23.37	19.35	25.79	19.65	26.75
SD		0.12	0.56	0.30	0.52	0.43	0.19

Table 3 The expression of AGR2 in stable knockdown of AGR2 cells compared with its shControl cells were examined at the levels of mRNA using real time PCR. The results were obtained from tree separate experiment.

		shControl-1		shAGR2-9		shAGR2-10	
		ATCB	AGR2	ATCB	AGR2	ATCB	AGR2
	1	17.70	23.17	17.10	24.63	17.40	25.37
Cp	**2**	17.67	22.87	16.53	24.40	17.00	24.90
	3	17.68	23.02	16.82	24.52	17.20	25.13
Mean		17.68	23.02	16.82	24.52	17.20	25.13
SD		0.02	0.15	0.28	0.12	0.20	0.23

Table 4 The expression of AGR2 in highly metastatic CCA cells, KKU-213L5 compared with its parental cells, KKU-213 was examined at the levels of protein using western blot. The results were obtained from tree separate experiment.

		KKU-123	KKU-213L5	scramble	si-AGR2 #1	si-AGR2#2
Protein density	**1**	1.00	6.64	1.00	0.33	0.29
(related to control)	**2**	1.00	6.86	1.00	0.40	0.37
	3	1.00	6.67	1.00	0.51	0.28
Mean		1.00	6.72	1.00	0.41	0.31
SD		0.00	0.12	0.00	0.09	0.05

		shControl-1	shAGR2-9	shAGR2-10
Protein density	**1**	1.00	0.1	0.12
(related to control)	**2**	1.00	0.21	0.09
	3	1.00	0.14	0.15
Mean		1.00	0.15	0.12
SD		0.00	0.06	0.03

Table 5 Effect of knockdown of AGR2 on cell proliferation were analyzed by MTT assay. The results were obtained from tree separate experiment.

	Day	1	2	3	4	5
	scramble	0.237	0.509	0.995	2.038	3.440
OD 540 nm	**agr2-siRNA #1**	0.172	0.309	0.677	1.416	2.269
	agr2-siRNA #2	0.200	0.383	0.735	1.595	2.858
SD		0.033	0.101	0.169	0.320	0.585
	shControl-1	0.309	0.610	1.220	2.580	3.773
OD 540 nm	**shAGR2-9**	0.219	0.446	0.923	1.660	2.922
	shAGR2-10	0.217	0.406	0.804	1.547	2.619
SD		0.053	0.108	0.214	0.566	0.599

Table 6 Effect of knockdown of AGR2 on cell migration into the wound closure was analyzed by wound healing assay. The results were obtained from tree separate experiment

	Wound distance (% related to control)		
	0 h	**12 h**	**24 h**
scramble	100.00	47.53	0.00
si-AGR2 #1	100.00	53.48	32.26
si-AGR2 #2	100.00	61.28	36.82
SD	0.00	6.90	20.07
shControl-1	100.00	54.90	0.00
shAGR2-9	100.00	62.00	34.00
shAGR2-10	100.00	70.59	37.25
SD	0.00	7.85	20.63

Table 7 Effect of knockdown of AGR2 on cell migration and invasion was analyzed by Transwell assay. Cells were photographed 9 field at a constant magnification (10X) and cell numbers were counted with Image processing and analysis in java software. The results were obtained from tree separate experiment.

	Number of cell migration		Number of cell invasion	
	scramble	**si-AGR2 #1**	**scramble**	**si-AGR2 #1**
1	154	109	82	75
2	169	94	136	104
3	247	154	132	93
4	162	103	156	99
5	155	106	179	115
6	211	96	148	115
7	147	101	121	120
8	170	99	124	60
9	164	99	97	75
Mean	175.35	106.63	130.56	95.11
SD	32.31	18.39	29.48	21.09

Table 7 (Cont.)

	Number of cell migration			Number of cell invasion		
	shcontrol-1	**shAGR2-9**	**shAGR2-10**	**shcontrol-1**	**shAGR2-9**	**shAGR2-10**
1	245	55	39	111	56	34
2	214	53	38	109	56	36
3	257	54	39	130	50	30
4	203	51	38	131	58	33
5	239	58	38	133	52	38
6	171	68	36	124	52	36
7	186	53	34	126	60	34
8	206	53	33	130	59	39
9	232	59	36	118	58	33
Mean	216.89	55.83	36.61	123.22	55.50	34.56
SD	56.93	10.55	4.21	8.89	3.40	2.72

Table 8 Effect of knockdown of AGR2 on the MMP-2 and MMP-9 secreted were measured by gelatin zymography. The relative band densities were quantitated by gel densitometer. The results were obtained from tree separate experiment.

		Band density (% related to control)			Mean	SD
		1	**2**	**3**		
	scramble	100.00	100.00	100.00	100.00	0.00
proMMP-9	**si-AGR2#1**	92.74	107.33	100.03	100.03	7.30
	si-AGR2#2	94.54	109.94	102.24	102.24	7.70
	scramble	100.00	100.00	100.00	100.00	0.00
proMMP-2	**si-AGR2#1**	106.34	86.66	96.50	96.50	9.84
	si-AGR2#2	101.74	94.97	98.36	98.36	3.38
	scramble	100.00	100.00	100.00	100.00	0.00
MMP-2	**si-AGR2#1**	95.42	96.67	96.04	96.04	0.63
	si-AGR2#2	97.34	98.15	97.75	97.75	0.40

Table 8 (Cont.)

		Band density (% related to control)			Mean	SD
		1	2	3		
proMMP-9	**shControl-1**	100.00	100.00	100.00	100.00	0.00
	shAGR2-9	95.60	102.75	99.18	99.18	3.58
	shAGR2-10	101.84	102.76	102.30	102.30	0.46
proMMP-2	**shControl-1**	100.00	100.00	100.00	100.00	0.00
	shAGR2-9	104.50	97.47	100.98	100.98	3.52
	shAGR2-10	94.14	106.51	100.33	100.33	6.19
MMP-2	**shControl-1**	100.00	100.00	100.00	100.00	0.00
	shAGR2-9	92.96	110.98	101.97	101.97	9.01
	shAGR2-10	101.79	105.28	103.53	103.53	1.75