EFFECTS OF SECRETORY LEUKOCYTE PROTEASE INHIBITOR (SLPI) ON TUMORIGENESIS OF CHOLANGIOCARCINOMA

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Chaiwat Chueaiphuk

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ABSTRACT

Cholangiocarcinoma (CCA) which has a high incidence in Thailand, is a chronic inflammation induced-cancer by *Opisthorchis viverrini* (*Ov.*) infection. The lack of symptoms in early stages of CCA progression results in high mortality rate of the patients. Therefore, developing of an early diagnosis method for CCA is very important to improve the outcome for the patients. Secretory leukocyte protease inhibitor (SLPI) is a serine protease inhibitor which responses during inflammatory process. SLPI is an emerging molecular target for cancer therapy, contributes to cancer proliferation, cell survival, tumorigenesis, and metastasis in various cancers. SLPI is highly up-regulated in CCA, and overexpression of SLPI significantly promoted metastatic-related phenotypes. However, the potential role of SLPI serving as an early diagnostic marker for CCA has not been elucidated. In this study, we examined the effect of SLPI on tumorigenesis of CCA. The expression of SLPI in hamster-induced CCA tissues were investigated using immunohistochemistry. We found that SLPI expression was gradually increased H-score index correlated with cholangiocarcinogenesis. Next, interleukin 6 (IL-6), an inflammation cytokine, was treated in immortalized cholangiocyte, MMNK-1 and CCA cell, KKU-213A and determined the SLPI and NF-κB pathway by western blot analysis. The results showed that SLPI expression was significantly up-regulated under inflammation as demonstrated by increasing of NF-κB activation. In addition, IL-6 promoted tumorigenesis by soft agar colony formation assay. We also demonstrated that SLPI

overexpression in MMNK-1 strongly enhanced tumorigenesis compared with control mock cells. Furthermore, overexpression of SLPI increased capability of tumor formation of MMNK-1 tumor xenografts in BALB/cAJcl-nu mice models. From all data suggested that SLPI is induced under inflammation and being as a key molecule to promote tumorigenesis of CCA. In conclusion, our study provides the first evidence about the role of SLPI in carcinogenesis of CCA and highlighting the potential of SLPI as a molecular target for an early diagnosis in CCA.

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CHAPTER I

INTRODUCTION

Rationale for the study

Cholangiocarcinoma (CCA) is a malignant tumor caused by abnormal growth which occurs in the bile duct epithelial area. CCA is relatively rare in western countries but has a high incidence in Southeast Asia, especially in Northeast Thailand, which is a major cause by liver fluke (*Opisthorchis viverrini: Ov.*) infection. The infection can induce an inflammatory response and promote cytokine production such as interleukin 6 (IL-6) to respond in inflammation. The repeated *Ov.* infection can induce overproduction of IL-6 which is entering to chronic inflammation of bile duct that causes a mutation of the genetic material, resulting in abnormal expression of many proteins that regulate cell growth. Then, the tumor forms and develops becoming malignant which causes it to spread. CCA has a high mortality rate due to patients being diagnosed in the late stages of the disease. CCA does not exhibit symptoms in the early stages, therefore the progression of cancer into the metastatic stage is difficult to treat. Most of the patients are often unresponsive to the current treatment so the rate of recurrence is high. Therefore, the development of an early diagnosis for CCA is very important to improve the mortality rate of the patients. Locating the molecular targets associated with CCA at an early stage could be used as a biomarker for diagnosis.

Secretory leukocyte protease inhibitor (SLPI) is a serine protease inhibitor that responses during the inflammatory process. When the body is infected or invaded by foreign materials, the white blood cells will secrete a high level of serine proteases in response to the inflammation. High serine proteases activity can cause tissue damage, so the body produces and secretes SLPI proteins to inhibit this action. Therefore, it can be concluded that the anti-inflammatory SLPI proteins is necessary to maintain the integrity of the surrounding tissue. Previous studies showed that SLPI was found as a major component of the mucous membrane that secretes mucus onto the respiratory,

gastrointestinal epithelium, and reproductive tissues. In addition, dysregulation of SLPI has been reported in a variety of human cancers.

Previous studies showed that SLPI expression was found to be high in the tissue and sera of various patients with lung, ovarian, pancreatic, and stomach cancers. Moreover, expression level of SLPI has been correlated with aggressive cancers including of cell proliferation, cell survival, tumorigenesis, cell migration and cell invasion. The mechanism of SLPI promoting cancers are few and still unclear. However, SLPI could be accelerate the cancer by protease inhibitor activity-dependent or – independent manners. Recent report found that SLPI can modulate transcription factor FoxM1 and turn to activate various phenotypes of cancer. In some cancers, SLPI promotes tumor growth by induce cyclin D1 expression. Recently, SLPI is an emerging molecular target for cancer therapy, contributes to cancer proliferation, cell survival, tumorigenesis, and metastasis in various cancers. Moreover, SLPI is essential molecule for carcinogenesis as demonstrate in lung cancer. Lacking of SLPI in knockout mice could protect the tumor formation in urethane-induced carcinogenesis of lung cancer at the initiation, promotion, and progression stage. Thus, SLPI may be a key molecule that promoting the carcinogenesis of cancer cells.

The recent research in CCA found that expression levels of SLPI in the cellular and secreted forms correlated with aggressive cancer, which were demonstrated in immortalized cholangiocyte (MMNK-1), low metastatic CCA cells (KKU-213A) and high metastatic CCA cells (KKU-213AL5). These results indicated that SLPI might play an important role in promoting the development of CCA through the process of carcinogenesis and metastasis. After establishment of SLPI-overexpressing cell, the effects of SLPI on the metastasis of CCA was further explored. The results demonstrated that overexpression of SLPI in MMNK-1 significantly promoted the *in vitro* characteristics of cancer metastasis, including proliferation, adhesion, migration, invasion, and matrix metalloproteinases activity. These data suggested that SLPI plays an important role, at least in part, for CCA metastasis which is a cancer progression step. However, the role of SLPI in an early stage during CCA carcinogenesis has not been completed yet.

This study aims to investigate the role of SLPI on tumorigenesis of CCA both *in vitro* and *in vivo*. These data will provide further information regarding the SLPI association with CCA development and could be an informative data showing that SLPI has the potential to be a novel biomarker for early diagnosis of cholangiocarcinoma, as well as a targeted therapy for CCA patients.

Objectives of the study

1. To determine the expression of SLPI during carcinogenesis of CCA in the tissue model of CCA-induced hamsters
2. To investigate the expression of SLPI and tumorigenicity under chronic inflammation mimicry by using IL-6 treatment in immortalized cholangiocyte (MMNK-1) and CCA cell (KKU-213A)
3. To investigate the effects of SLPI overexpression in MMNK-1 on tumorigenicity *in vitro* and *in vivo* model

Hypothesis

SLPI expression is high during carcinogenesis of CCA. Inflammation is an early event to induce up-regulation of SLPI. The increasing of SLPI is a key factor that promote the transformation of cholangiocyte to CCA cell. Therefore, SLPI is a novel biomarker of an early stage of CCA.

Scope of the study

The thesis research is designed into 3 parts; Part 1: Study of SLPI expression in the tissues of hamsters at various stages during CCA carcinogenesis (induced CCA by *Opisthorchis viverrini* infection plus NDMA) by immunohistochemical staining. Part 2: Mimicking of chronic inflammation by IL-6 treatment in MMNK-1 and KKU-213A cells, then confirm the cellular inflammatory response by NF-κB pathway activation using the western blot assay. Then, examine SLPI expression level and tumorigenicity by the western blot analysis and soft agar colony formation assay, respectively. Part 3: Determine the effect of SLPI overexpression in SLPI-overexpressing cells on *in vitro* tumorigenicity by soft agar colony formation assay and *in vivo* tumorigenesis study by xenograft assay.

Expected outcome

1. Understanding the role of SLPI in tumorigenesis of CCA *in vitro* and *in vivo*
2. To assess whether SLPI could be used as a biomarker for early detection of CCA in patients

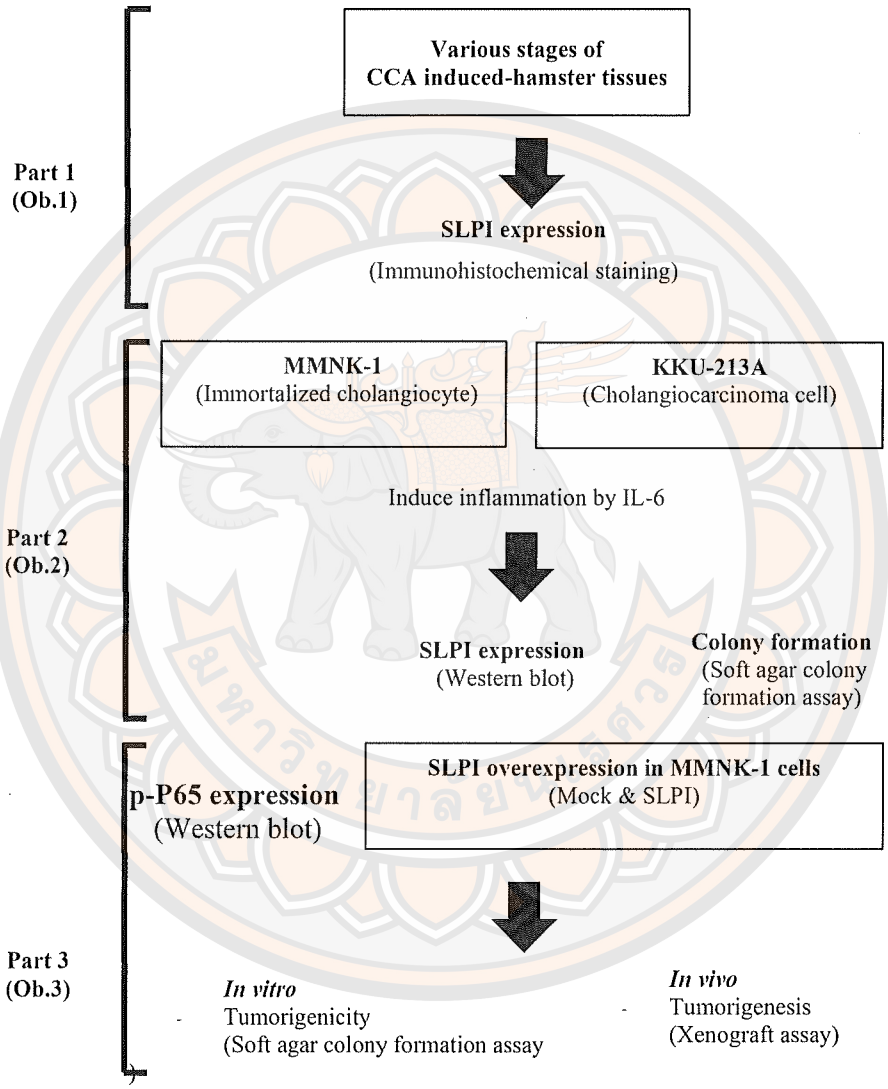


Figure 1 Schematic diagram shown the scope in this study

CHAPTER II

LITERATURE REVIEW

Cholangiocarcinoma (CCA)

Cholangiocarcinoma is a severe type of cancer that occurs in the epithelium of the bile duct constitutes a diverse group in malignancy of the biliary duct tree. CCA are divided into three groups according to the anatomical position, intrahepatic (iCCA), perihilar (pCCA) and distal (dCCA) CCA (Figure 2) (Banales, J. M. et al., 2016). CCA is caused in many areas of the bile duct, both inside and outside the liver, as well as between the bile ducts and the small intestine. The cancerous tumors that occur within the bile duct within the liver are called "Intrahepatic CCA", cancerous tumors in the bile duct outside the liver are called "Extrahepatic CCA", cancer of the bile duct from the liver is called "Perihilar" (Klatskin, G. 1965)

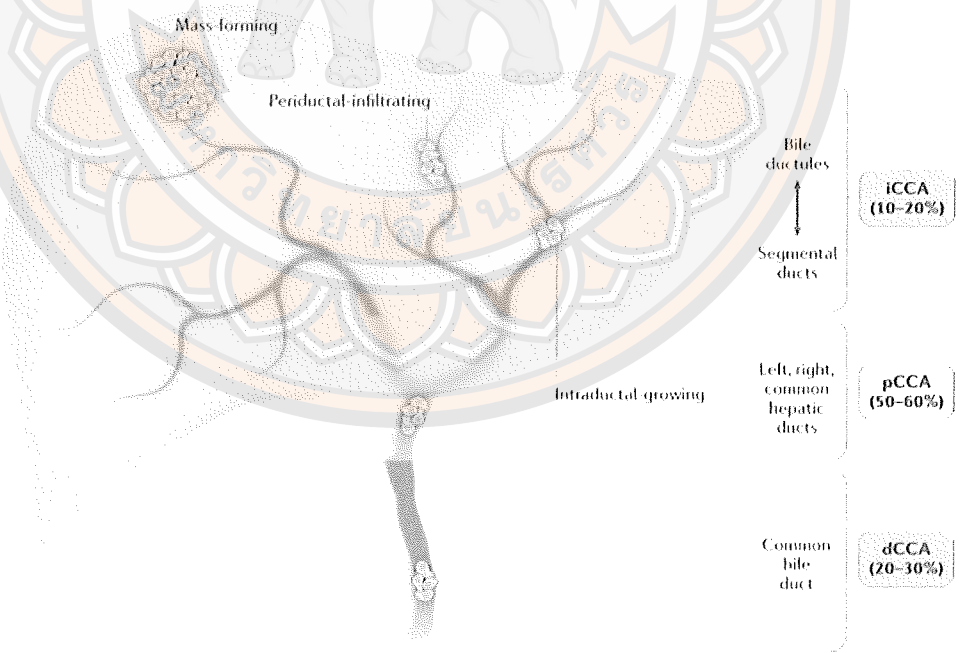


Figure 2 Anatomical classification of cholangiocarcinoma CCA

Source: Banales, JM et al., 2020

It is well known that cholangiocarcinoma is carcinogenic to adrenocortical carcinoma of the bile duct epithelium. But it is not known exactly what type of stem cells are. Even the evidence indicates that stem cells may be Stem cells in the liver (Lee, J. S. et al., 2006). It is believed that cholangiocarcinoma can be caused by several stages of cancer, ranging from a more normal early stage (hyperplasia) to metaplasia and an abnormal growth (dysplasia), resulting in cancer in the end. CCA, a process similar to the process of developing colon cancer and believed that chronic inflammation of the bile duct, gallbladder (Sirica, A. E., 2005) blockage, and bile duct blockage may contribute to the conversion of normal bile duct cells to cancerous platelets (Holzinger, F., et al, 1999).

Cholangiocarcinoma is one of the most common types of canine cancer in the United States. Diagnosis of cholangiocarcinoma in AIDS patients in Thailand is highest among AIDS patients (Vatanasapt, V. et al., 2002). Cholangiocarcinoma is a common cancer in Thailand, especially in the Northeast, which is widespread of high incidence of liver fluke infection. The highest average incidence of cholangiocarcinoma in Khon Kaen is about 84 per 100,000 in males and 36 per 100,000 in females (Bridgewater, J. et al., 2014) (Figure 3).

Figure 3 The worldwide incidence of bile cancer diagnosed by the Cancer Expert Group in 2014

Source: Bridgewater, J. et al., 2014

1. Risk Factors and causes of CCA

Risk factors and causes of cholangiocarcinoma are not known. It is believed that the epithelial cells of the biliary tract are irritated which may be caused by inflammation or due to the presence of gallstones in the liver. As a result, the bile duct cells change from normal and eventually cancer cells. However, the risk factors for cholangiocarcinoma are as follows (Blechacz, B. et al., 2008), there is chronic inflammation of the epithelium of the bile duct inside and outside the liver which be caused by chronic pancreatitis, inflammatory bowel disease or chronic inflammation of the mucous membranes, these is caused by hereditary genetic disorders, such as diseases such as gall bladder dysfunction in the biliary tract, it is caused by Hepatitis B and C infection, there is a gallstone in the liver or gallstones in the gallbladder. Infection with *O. viverrini* causes inflammation and cancer, this is a major risk factor for patients in Thailand and is the cause of bile duct cancer in Thailand.

The main cause of cholangiocarcinoma in Thailand is caused by the infection of liver fluke. The World Health Organization has assigned *O. viverrini* as a carcinogen for CCA (Young, N. D., et al, 2010). Initially, liver fluke infection causes acute inflammation of the large bile duct and portal connective tissue. (Bhamarapravati, N. et al., 1978). The infection can cause many diseases together. Each disease is Jaundice, biliary cirrhosis, biliary tract infection, and gall bladder and bile duct inflammation. Previous study found that the infection of the liver fluke is consistent with the development of bile duct cancer (Figure 4).

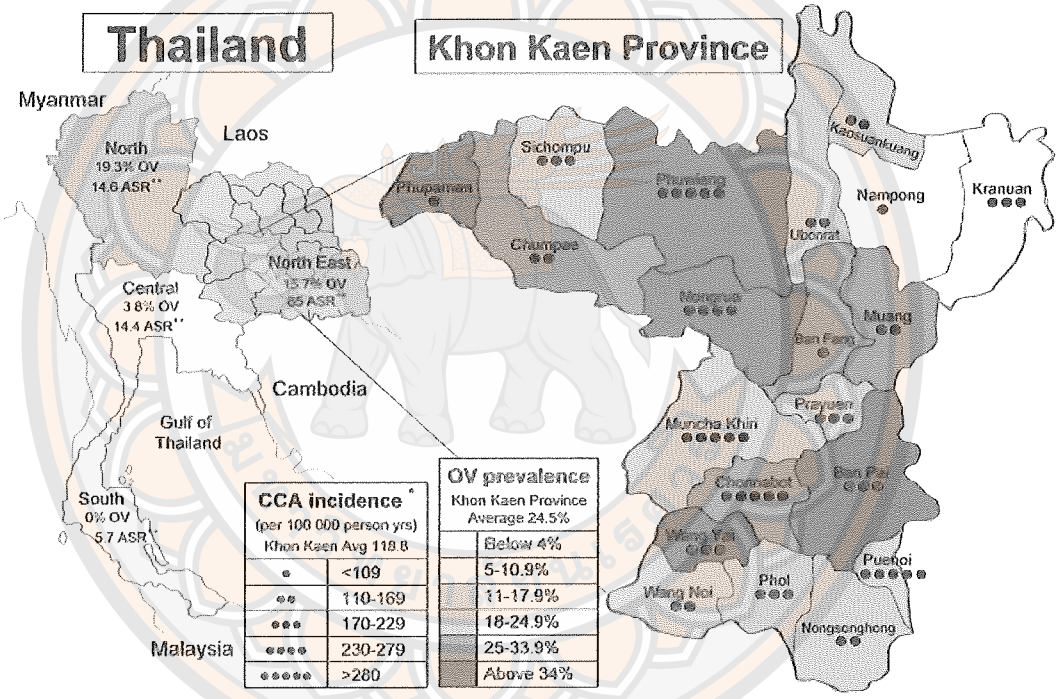


Figure 4 A correlation between the higher incidence of liver fluke infection and the incidence of cholangiocarcinoma incidence in Thailand

Source: Sripa, B. et al., 2007

2. The mechanisms of Cholangiocarcinoma

The results of chronic inflammation are generally recognized on liver fluke–induced CCA (Holzinger, F. et al., 1999; Sirica A. E. 2005). Several mechanisms by which *O. viverrini* infection may promote cholangiocarcinogenesis are summarized

in Figure 5. It is almost certain that a combination of those pathologies defined above (immunopathology, mechanical damage and parasite secretions) terminates in CCA after chronic infection with *O. viverrini*. During a liver fluke infection, inflammation, periductal fibrosis, and proliferative responses, including epithelial hyperplasia, goblet cell metaplasia, and adenomatous hyperplasia, may represent predisposing lesions that enhance susceptibility of DNA to carcinogens (Thamavit, W. et al., 1978). Numerous N-nitroso compounds and their precursors ensue at low levels in fermented food such as preserved mud fish paste, pla-ra, a condiment that is a pervasive component of the cooking of northeastern Thailand and Laos. Indeed, it has been hypothesized that N-nitroso compounds (e.g., nitrosamine) are a primary carcinogen leading to CCA in humans in the northeastern part in Thailand (Migasena, P. et al., 1980). Experimental *O. viverrini* infection in hamsters can induce NO synthase expression by immune effector cells in the inflamed zones surrounding the bile ducts and increased endogenous nitrosation of thiazolidine- 4-carboxylic acid (thioproline) (Oshima et al., 1994). In the case of *O. viverrini* infection, DNA damage is caused in biliary epithelial cells while apoptotic mechanisms are dysregulated, resulting in genetic alterations which may become fixed, leading to malignant transformation (Sripa, B. et al., 2007).

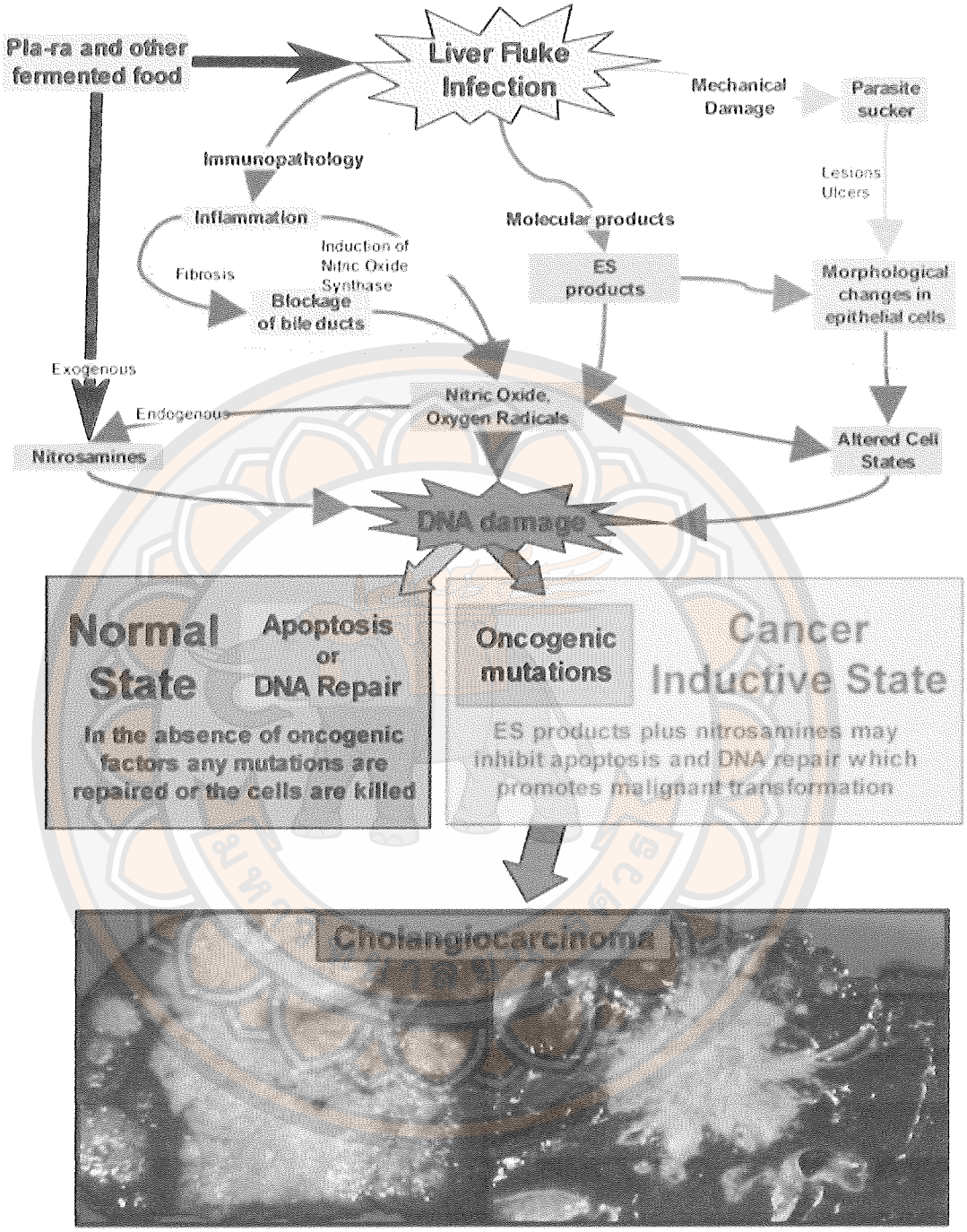


Figure 5 The mechanism of cholangiocarcinoma of the liver resulting from the infection of the liver.

Source: Sripa, B. et al., 2007

Hamster is an animal model for studying the early step of carcinogenesis of CCA, which has been widely used. the infection of *Ov.* or *N-nitrosodimethamine* (NDMA) treatment alone does not induce the formation of CCA in the model, while *Ov.* infection plus NDMA treatment could induce carcinogenesis, leading to the formation of CCA (Thamavit W et al., 1994) (Figure 6). The animal model indicates the association of *Ov.* infection and the development of CCA, combination of *Ov.* infection and supplementation with a carcinogenic NDMA dose of effectively induces CCA in a hamster model (Sawanyawisuth, K. et al., 2011). In the future, the basic knowledge obtained in the hamster model may contribute to the development of new biomarkers for diagnosis, prognosis, therapeutic treatment, and research on the biology of the development and progression of CCA.

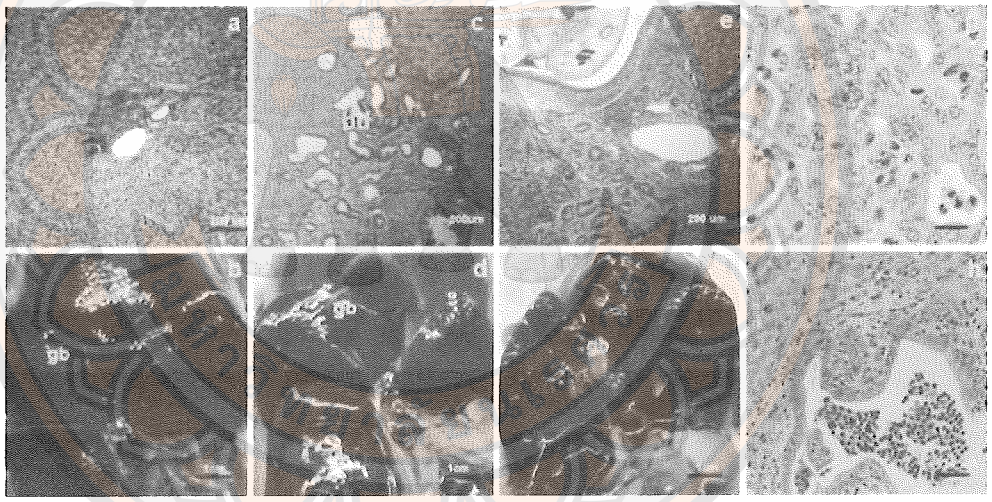


Figure 6 Histopathological changes and gross anatomy of hamster with *N-nitrosodimethylamine* (NDMA) treatment alone at 60 days p.i. (a and b), and *Ov.* infection plus NDMA treatment at 60 days p.i (c and d) and 120 days p.i. (e, f, g and h). Figure g and h showed malignancy dysplastic bile duct and cholangitis. p parasite adult; bd bile duct; gb gallbladder

Source: Boonmars, T. et al., 2011

Primary sclerosing cholangitis (PSC) is a disease caused by abnormalities of the liver, which leads to chronic inflammation of the bile ducts and finally to cirrhosis and kidney failure. (Chalasani, N. et al., 2000). Parasites infect such as Clonorchis sinensis are found in Vietnam and Korea (Choi, B. I. et al., 2004) or Opisthorchis viverrini are found in Thailand and Laos. (Poomphakwaen, K. et al., 2009). The incidence of cholangiocarcinoma in Thailand is high, especially in the Northeast (Sriamporn, S. et al. 2004) (Figure 7).

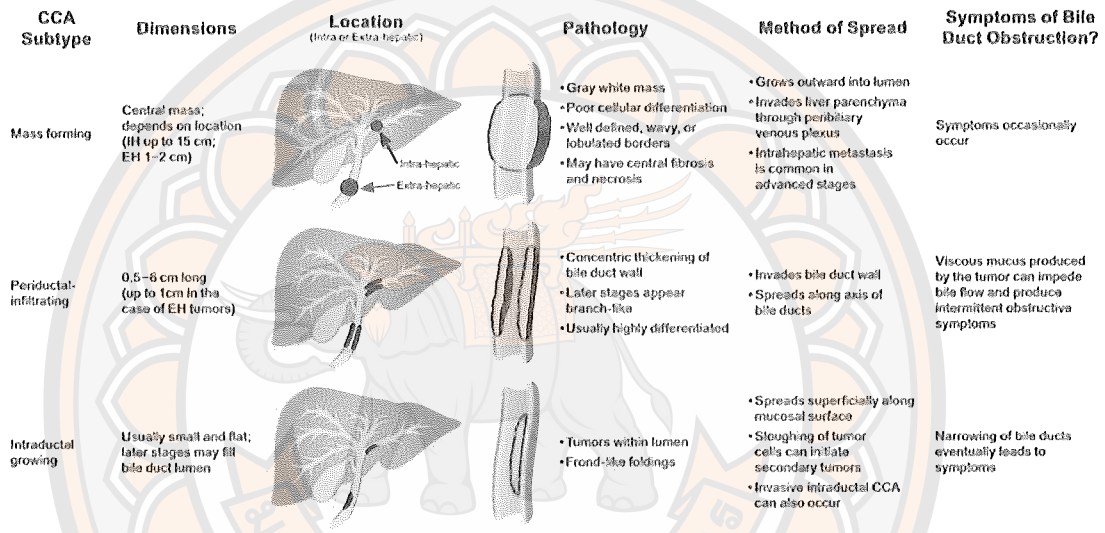


Figure 7 The area of changes in the epithelium of the bile duct

Source: Sripa, B. et al., 2007

Cholangiocarcinoma can be divided into 4 stages according to American Joint Committee on Cancer criteria (AJCC). Stage 1 only one tumor and bile duct cancer is not spread or spread into the blood vessels. Stage 2 has only one tumor as in the first stage. Cholangiocarcinoma is spread to the blood vessels. Cancer may be found in some cases. Stage 3 Cholangiocarcinoma is an invasion of the cholangiocarcinoma into the peritoneal cavity or into the liver or adjacent organs of the liver and gallbladder. Stage 4 Cholangiocarcinoma is the progression of knee disease around the bile ducts or the spread of the disease into the lymph nodes. This may be spread to other organs. There is water in the abdomen and spread to the arteries and finally spread to the lungs.

Although CCA can be divided, there is no evidence of any benefit in predicting the survival rate of cholangiocarcinoma (Zervos, E. E. et al., 2005).

3. Symptoms of Cholangiocarcinoma

Cholangiocarcinoma is not a specific disease, but it has symptoms similar to liver disease, including various types of liver cancer, and early onset cholangiocarcinoma. When bile duct cancer is in severe stage. The most common symptoms of cholangiocarcinoma are: the liver is enlarged, the abdomen is enlarged, the weight is reduced / thinned, and the disease progressively increases, resulting in blockage of the bile duct in the liver resulting in the body such as yellow eye pigmentation around the needle urine, pale stools (jaundice) and in the final stages of the disease. There is often cancer in the abdomen (epiglottis), resulting in tightness, abdominal distention, dyspnea, and eventually death from liver failure and / or respiratory failure (Edge, S. B. et al., 2010). The most common findings of cholangiocarcinoma are the results of the abnormal liver function tests are as follows (Halperin, E., et al, 2013), jaundice (occurs when the bile ducts are blocked by tumors) abdominal pain (30-50%), cars (66%), weight reduction (30-50%), fever (up to 20%) or fecal color or urinary redness, lumps in the liver (14%), colored mucus in gall bladder (6.7%), acute cholecystitis without gallstones (7%)

4. Diagnosis of Cholangiocarcinoma

Diagnosis of cholangiocarcinoma has not been specifically investigated for the diagnosis of cholangiocarcinoma. Although the levels of carcinogenic embryonic antigen and CA19-9 (CA19-9) within the blood (a marker for cancerous bodies) are used for screening, but it's not specific enough. However, these screenings may be useful when used in combination with radiographic examinations to support the pathology seen with cholangiocarcinoma. (Nehls, O. et al., 2004)

Ultrasound examination of the liver and biliary tract is often used in radiography for suspected patients of obstruction of the biliary tract (Saini S, 1997) and the expansion of the bile duct. Sometimes the information is sufficient to diagnose bile duct cancer (Bloom, C. M. et al., 1999). Computerized Tomography (CT) scans may also contribute to the diagnosis of gallbladder cancer (Valls, C. et al., 2000). In some cases, surgical exploration may be necessary to obtain a suitable site for pathological examination and to evaluate the extent of the patient's cancer. Endoscopic Surgery

(laparoscopy) can be used for periodic assessment. In addition, surgery may be the only method of curettage, and may reduce the need for more aggressive surgeries such as laparotomy in some patients (Weber, S., et al, 2002) but it usually success only with initial disease.

5. The treatment of cholangiocarcinoma

Most cholangiocarcinoma are surgically removed by surgery, depending on the stage of the cancer. If cancer is detected in the posterior stage, which is spread to other organs. This treatment may be more likely to return (Czito, B. G. et al., 2006). If no tumor can be removed, all cancers are treatable, and to assess whether surgery can remove all the tumors, surgical evaluation (Su, C., et al, 1996) has led many patients to undergo abdominal surgery. Unless there is a clear indication that the tumor is in a position that cannot be treated by surgery. If cancer can be removed. May be treated with combination chemotherapy or radiotherapy. However, the use of these therapies in combination has not been effective enough to treat long-term cholangiocarcinoma (Sirica, A. E. et al, 2005).

Many patients with cholangiocarcinoma are diagnosed when the disease has spread to other organs that cannot be treated surgically (Vauthey, J. N. et al., 1994). These patients are usually treated with chemotherapy. Palliative chemotherapy may be used in conjunction with radiation therapy. Randomized trials have documented that chemotherapy can improve the quality of life and extend the life expectancy of patients with cholangiocarcinoma who cannot undergo surgery (Glimelius, B. et al., 1996), but no treatment regimens were available as the international standard. Nowadays, there are suggestions to try new ways with other treatments. Chemotherapy is used to treat cholangiocarcinoma using 5-fluorouracil in combination with leucovorin (Choi, C. et al., 2000), gemcitabine alone or gemcitabine in combination with cisplatin (Giuliani, F. et al., 2006) irinotecan (Bhargava, P. et al., 2003) or capecitabine. Several pilot studies indicate that patients with advanced cholangiocarcinoma may benefit from erlotinib tyrosine kinase inhibitors (Philip, P. et al., 2006).

Carcinogenesis

Carcinogenesis or Oncogenesis, or Tumerigenesis, is a cancer process that transforms normal cells into cancerous cells and eventually spreads to cancer cells. This is based on the progression and mutation of the cells in mutated tissues (Loeb, L. A. et al., 2000). Although most tumor cells are mutated, only some cells are involved (Barrett J. C. 1993). Mutations in these cells may result in imbalance in growth. This will increase the growth and decrease of cell death (Luebeck, E. G. et al., 2002). It also includes the optimal conditions for cell growth. All of these are characteristic of cancer cells (Sjöblom, T. et al., 2006). (Figure 8)

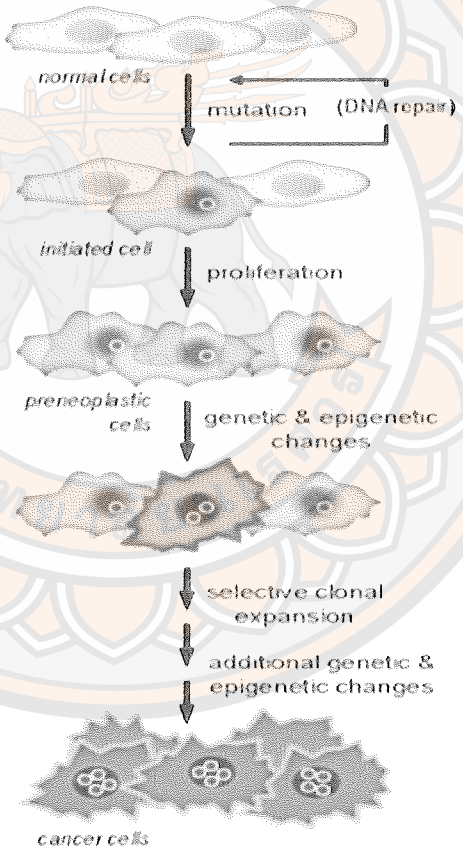


Figure 8 The process of carcinogenesis of cancer cells

Source: William, C. et al., 2011

In cancer, when the DNA is changed, it can start dividing uncontrollably, genes that regulate cell growth must be dysregulated. (Vogelstein, B. et al., 2004), Proto-oncogenes are genes that promote cell growth and mitosis, whereas tumor suppressor genes inhibit cell growth, or temporarily stop cell cycle to carry out DNA repair. A series of several mutations to these genes is required for the transformation of the normal cell into the cancer cell (Brand, K. A. et al., 1997).

Normally, when tissue is damaged, inflammation may occur and the cell stimulates the cytokines and enzymes to express itself around the area to inhibit inflammation (Taniguchi, K. et al., 2015). The secretion of these molecules will result in biochemical changes (Busillo, J. M. et al., 2011), resulting in the change of the phenotype, eventually leading to tissue or cell regeneration (Wang, Y. et al., 2015). This event may inhibit intercellular communication, resulting in unresponsive cell signaling. (Kwon, M. J. et al., 2015) and tumor cells do not respond to the surrounding tissue or cells (Yaniv, M. et al., 2014), resulting in mutated or cancerous cells that develop the tumor masses (Vlahopoulos, S. A. et al., 2015).

The factors that cause cancer are multi-factors. These factors are the causes that may contribute to the carcinogenesis process. DNA damage is considered to be the primary cause of cancer. The naturally occurring DNA damage in one day is on average 60,000 times per cell, per day, due to endogeneous cellular processes. (Bernstein, C. et al., 2013), (Kastan, M. B. et al., 2008). In addition, DNA damage can cause by exposure to exogenous agents (Cunningham, F. H. et al., 2011) such as tobacco or smoke. UV radiation from the sunlight causes the DNA damage and cause some skin cancers (Kanavy, H. E. et al., 2011). Helicobacter pylori infection can cause the gastric cancer due to the high levels of reactive oxygen species that damage the DNA. Damaged or abnormal DNA may be caused by viral infection, resulting in transposition of DNA (Handa, O. et al., 2011). DNA damage can also cause by endogeneous (naturally occurring) agents. Macrophages and neutrophils from the inflammatory responses are the source of reactive oxygen species causing DNA damages that initiate tumorigenesis (Figure 9). In addition to the factors that cause the DNA damage, the abnormalities of DNA repair process are also caused the cancer by DNA damage accumulation (Modrich, P. et al., 2017).

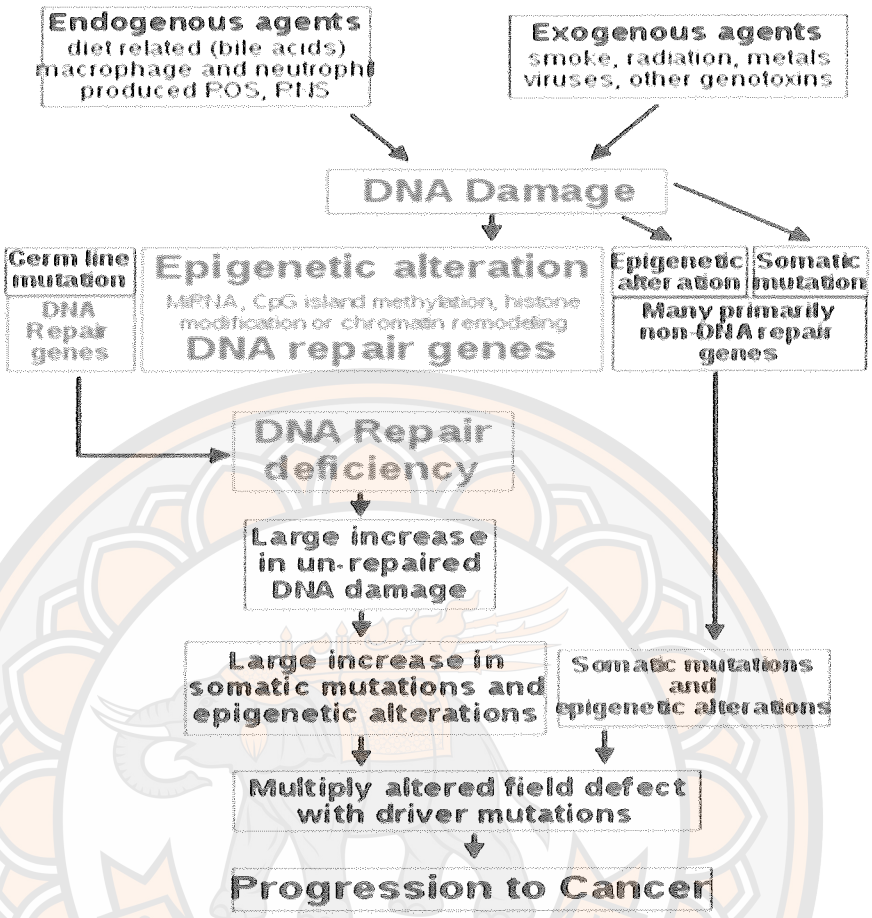


Figure 9 DNA damage and epigenetic defects in DNA repair gene for carcinogenesis

Source: Bernstein, C. et al., 2002

Secretory Leukocyte Protease Inhibitor (SLPI)

Secretory leukocyte protease inhibitor (SLPI) is a glycoprotein moiety that is approximately 11.7 kDa. The position of the SLPI gene is located on the double chromosome 20 q12-q13.2, 107 amino acids. The shape of the SLPI protein is similar to that of a boomerang consisting of four disulphide bonds (Figure 10). SLPI proteins inhibit the serine protease enzymes, elastase, trypsin and cathepsin G. SLPI prevents inflammation with protease. There are implications for the concomitant system (Zani, M. L. et al., 2009). SLPI proteins can be found in saliva, mucosa or other secretions such as mucus, mucous membranes in the lungs (Lin, A. L. et al., 2004), and the

secretion from immune cells such as macrophages and neutrophils (Odaka, C. et al., 2003).

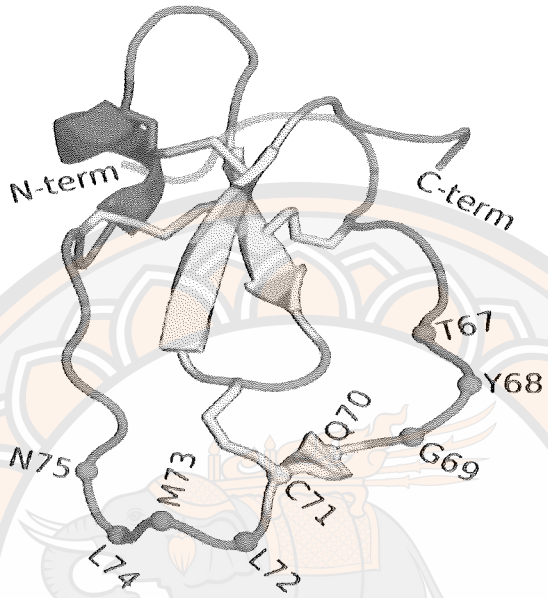


Figure 10 SLPI molecular shape

Source: Monika, M. G. et al., 2016

The structure of SLPI is a cationic secreted protein, boomerang- like shape, non-glycosylated, highly basic-acid stable and single chain serine protease inhibitor. SLPI consist two WAP domains and each domain contain a single WAP motif. The WAP domain is made up from eight cysteine residues that form four characteristic intramolecular disulfide bonds (Figure 11A) (Majchrzak-Gorecka, M. et al., 2016). The structure of WAP II domain (C-terminal) is the protease inhibitor region of SLPI, where it was localized between amino acid residuces 67-74 (Weldon, S., al., 2009). However, the functions of SLPI domain I (N-terminal) is less well understood and predicted that the anti-microbial activity is localized in this domain. The X-ray data (Figure 11B) showed the importance of the residues L72 and Y68 in the primary contact regions for inhibiting (neutrophil elastase) NE by forming hydrogen bonds with NE.

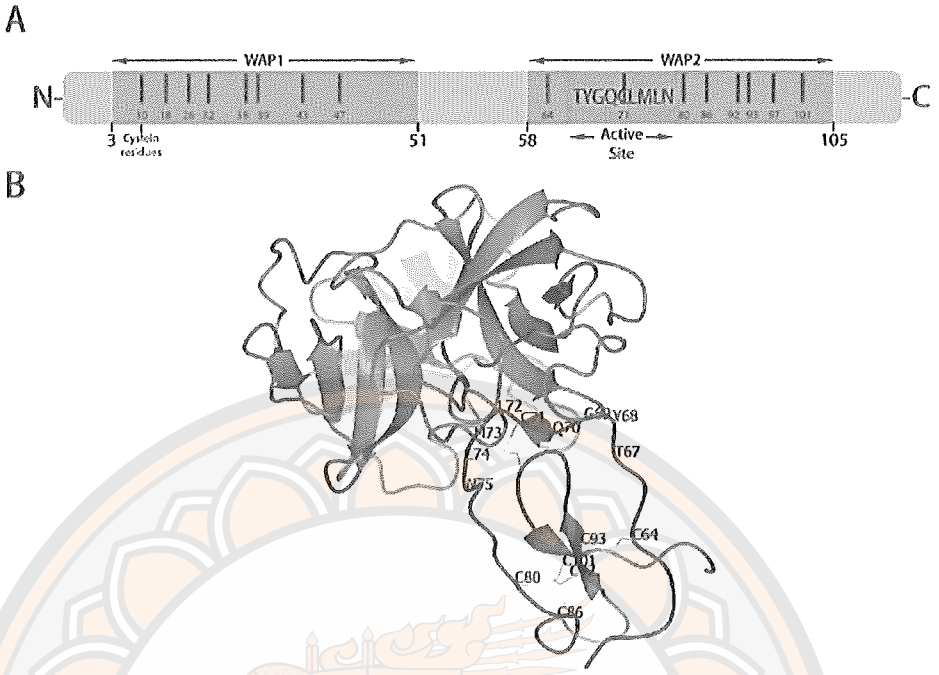


Figure 11 (A) SLPI structure and interaction A) the different domains of SLPI, (B) The interaction of WAP2 domain with neutrophil elastase. Amino acid residues of the active site and disulfide bonds

Source: Tarhini, M. et al., 2018

1. Function of SLPI protein

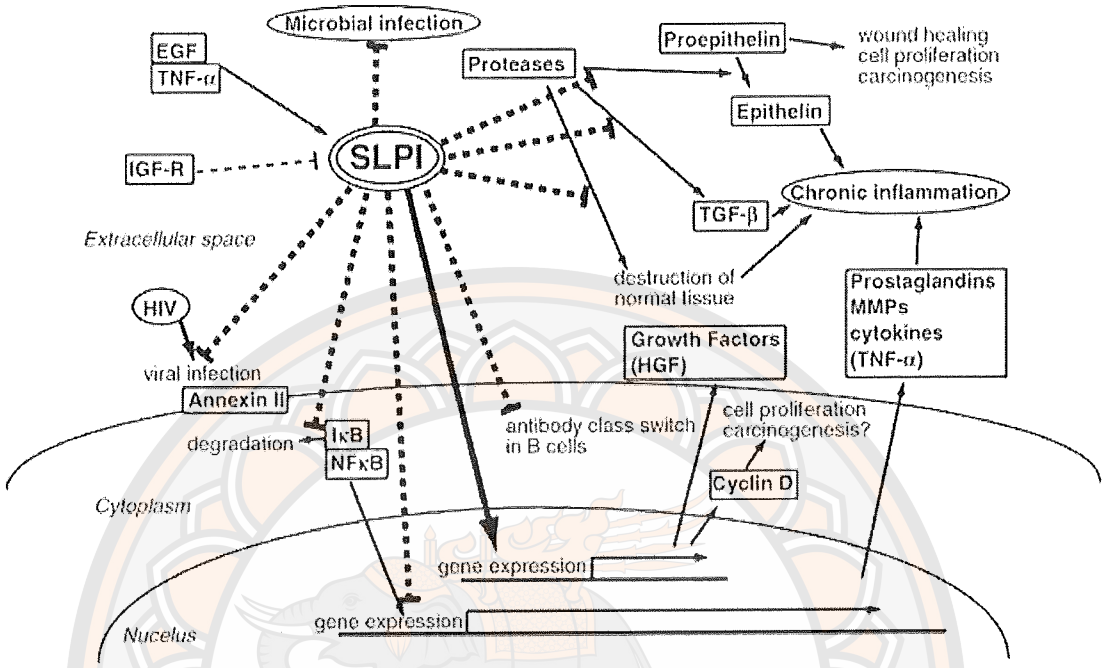


Figure 12 Role and Function of SLPI Protein

Source: Nukiwa T et al., 2008

1.1 Regulation of SLPI expression

SLPI is mostly expressed at both mRNA and protein levels in epithelial cells, including those cells lining the respiratory system, digestive system and reproductive system, as well as those cells of the parotid glands, breast, kidney and skin (Heinzel, R. et al., 1986). This protein is thought to preserve the normal homeostatic and to protect the function of this area from the microorganism invasion (McNeely, T.B. et al., 1995). SLPI is frequently expressed at high micromolar levels at the barriers of body. Moreover, SLPI is transiently or constitutively expressed in myeloid cells, including macrophages, granulocytes, dendritic cells (Odaka, C. et al., 2003).

A wide variety of microbial stimuli have been reported that induce cellular responses, although pattern recognition receptors such as Toll-like receptors (TLRs) have been reported to increase the expression of SLPI, these inflammatory stimuli. These include bacterial cell walls (Grobmyer, S.R. et al., 2000), whole-cell

bacteria (Nishimura, J. et al. 2008), parasites (McCartney-Francis, N. et al., 2014), and viral RNA (Xu, W., et at., 2014)) in response. Against inflammation, cytokine signaling by TNF-α, IL1β, or thymic oral lymphopoetin can induce SLPI expression (Sallenave, J.M. et al., 1994).

On the other hand, the expression of SLPI can be regulated by neutrophils themselves (McKiernan, P.J. et al., 2011). Protease enzymes such as neutrophil elastase can increase the expression of SLPI mRNA in Pleural cells in the lungs *in vitro* But it was accompanied by a decrease in the secretion of SLPI protein (Abbinante-Nissen, J.M. et al., 1993). SLPI in macrophage can be regulated in response to lipopolysaccharide (LPS) as well as cytokines such as IL-6 and IL-10. On the other hand, TNF-α and IL-1β did not appear to have this effect (Jin, F. et al., 1998) in den cells. The SLPI expression was regulated even though the calling receptor (TRL), which was largely dependent on the p38 MAPK route (Vroling, A.B. et al., 2011).

1.2 Inhibition of protease activity

SLPI protein was first discovered as a functional protein, which is responsible for inhibiting the enzyme activity, which is the main function of the SLPI protein. SLPI proteins are composed of two domains, which work together to become active. The anti-protease domain of the SLPI protein is Leu 72 -Met 73 (residues 67-74) on the C-terminal domain (Weldon, S. et al., 2007). Various protease inhibitors such as elastase, cathepsin G, trypsin, chymotrypsin, chymase and tryptase are the target of SLPI protein, but the important target of SLPI is to inhibit neutrophil elastase. Activity of neutrophil elastase is inhibited up to 90% by SLPI proteins in the human bronchial area which is related to high neutrophil elastase content (Abe, T. et al., 1991). (Figure 8)

The most acknowledged role of SLPI can inhibit variety of serine protease enzyme for preserving the local homeostasis in tissue. Neutrophil elastase (NE) has been known that is preferantially inhibited by SLPI and this inhibition can be reversible (Lee, C.H. et al., 1993). In the upper respiratory tract, SLPI accounts for 80-90% of the NE inhibitory capacity (Tegner, H. 1978). This indicates the major role of SLPI in the local control of NE activity. NE is released from the granule of neutrophil and can hydrolyze various protein components of extracellular matrix leading to local tissue injury. According to SLPI acts as a controlling inhibitor of NE, therefore, SLPI

can protect protein degradation by NE. This protection has profound consequences for terminating inflammatory responses (Zhu, J. et al., 2002). Moreover, SLPI can also inhibit the other protease enzymes, including chymotrypsin, cathepsin G and trypsin but the inhibitory effect is quite lesser than NE (Wright, C.D. et al., 1999). In addition, SLPI interfere the protein synthesis of some enzyme including matrix mettaloprotinase-1 and -9 in macrophage (Zhang, Y. et al., 1997).

1.3 Anti-virus capabilities

The ability of antimicrobial or antiviral activity of SLPI proteins is unclear. The cationic nature of WAP domain four-disulfide core protein (WFDC) is based on previous research. ENap-2, a unique cationic polypeptide of WFDC, exhibits antimicrobial activity (Couto, M. A. et al., 1992), (Couto, M. A. et al., 1993). SLPI protein protects against HIV transmission the body, which showed that SLPI proteins are associated with membranes of macrophages of human proteins. Phospholipid, annexin II, is a co-active component of the phosphatidylserine phosphate (Ma, G. et al., 2004) which supports HIV-1 from macrophage (Figure 12). Antimicrobial resistance and HIV resistance of SLPI protein may be due to high concentrations in the mucous membranes of the airways.

1.4 Anti-inflammation

Studies on inflammatory proteins have revealed that SLPI proteins affect the immune system by reducing the activation of NF-κB, a key cytokine of inflammatory cells or tissues (Nakamura, A. et al., 2003). The SLPI protein controls the activity of inflammation at the protein level, not in the mRNA levels of NF-κB to reduce inflammation (Taggart, C. C. et al., 2005). It is well known that TNF-α, monocyte chemoattractant protein-1 (MCP-1) and IL-6 are inflammatory cytokines (Greene, C. M. et al., 2004). SLPI promotes TNF-α and MCP-1 but lower in IL-6 production in macrophages. SLPI protein also promotes the expression of anti-inflammatory cytokines IL-10 and TGF- β by increase the expression (Sano, C. et al., 2000). It was also reported that, after inflammation, cyclin D1 exhibited higher levels. The cyclin D1 may be involved in the proliferation of cell lines and tissue growth after inflammation (Zhang, D. et al., 2002). Thus, SLPI protein acts as a therapeutic agent in many parts, such as acting as anti-protease to submit the wound healing process. In addition, SLPI acts through controlled factors, especially pro-inflammatory by inhibiting the action of

inflammatory cytokines (NF-κB) and reduce the role of cytokines that affect the inflammatory process (IL-6) and induce higher expression of cyclin D1, which is a factor that creates tissue or cells after inflammation.

2. SLPI in cancer

Based on the tissue distribution of SLPI expression, abnormality of epithelial cells on the respiratory, digestive, and reproductive systems could express SLPI transcripts and protein (Bouchard, D. et al., 2006). Expression of SLPI is highly upregulated in these cancer types, such as papillary thyroid, endometrial, pancreatic, uterine cervix, and ovarian cancer, but SLPI is underexpressed in bladder tumors, nasopharyngeal carcinoma, and some breast carcinoma, although the forms of breast carcinoma were correlated with the overexpression of SLPI (Nukiwa, T. et al., 2008). Moreover, the fundamental mechanisms have not been entirely investigated. The report showed that the amplification of chromosome 20q (20q12 to q13.2) encoding the SLPI gene, it caused of increasing SLPI expression on female cancer (Israeli, O. et al., 2005).

A report confirming the altered expression of SLPI protein has been reported in ovarian cancer showing significant expression of SLPI expression in ovarian cancer SKOV3 (Couto, M. A. et al., 1992). Based on the results of the Western blot analysis, it was found to be 60 times higher in expression compared to normal ovarian epithelial cells (McElvaney, N. G. et al., 1992). In addition, the expression of SLPI at mRNA level by real-time PCR, was also show a high expression similarly. It can be concluded that SLPI protein exhibited high levels of ovarian cancer in both protein and mRNA levels, which were highly expressed in all ovarian cancer cells used in the experiment (Nakamura, A. et al., 2003)

The overexpression of SLPI by rhSLPI could increase the proliferation rate in lung cancer cell line, and SLPI could act as a modulator of tumorigenesis base on *in vivo* model (Simpkins, F.A., et al, 2008). The SLPI treatment in KB oral carcinoma cell was increased significantly cell proliferation, and its strongly decreased the cell migration and invasion when treated with SLPI-siRNA. (Guanlin et al., 2011). The SLPI overexpression in AZ521 gastric cancer cells, the transfection or the addition of exogenous SLPI can promoted migration and invasion activity of this cancer *in vitro* (Cheng, W.L., et al, 2008). Moreover, the study in gastric cancer reported that SLPI was increased the MMP-2 and MMP-9 expression via Elk-1 signaling, which is promoting

the metastasis of tumor cells. These results suggest that SLPI acts as a signaling molecule and not as a protease inhibitor in gastric cancer (Choi et al, 2011).

The knockdown of SLPI by siRNA could significantly inhibit the cell proliferation, increased cell apoptosis, and decrease migration and invasion activity of AsPC-1, BxPC-3 and PANC-1 pancreatic ductal adenocarcinoma cells line (Zhang, W. et al., 2015). Silencing of SLPI expression by shRNA in pancreatic cancer cells were significantly reduced cell viability, inhibited the proliferative rate, and induced cell apoptosis (Zuo, J. et al., 2015). In addition, SLPI knockout oral carcinoma cells showed that ΔSLPI cells was decreased cell proliferation, DNA synthesis, and cell migration when compared with WtCa9-22 cells (Mikami et al., 2016).

In addition, SLPI has been reported to be highly elevated in various cancers, including lung cancer, ovarian cancer, and neck cancer, which are associated with severe disease (Dasgupta S et al., 2006). Moreover, SLPI is highly expressed in lung cancer and promote the process of cancer (Cezary, J. et al., 2014). In addition, highly expressed SLPI in ovarian cancer also promotes the onset of cancerous tumors and promotes the spread of cancer to the other organs. (Devoogdt, N. et al., 2009)

The relationship of SLPI and NF-κB activation in urethane-induced carcinogenesis showed that exposure to urethane is associated with NF-κB upregulation and that NF-κB activation is inhibited by IκBα in SLPI-KO mice (SLPI-knockout mice), which suppresses the formation of urethane-induced lung tumors (Figure 13). This data indicate that decrease of SLPI suppressed tumor formation of carcinogenesis in initiation, promotion and progression stage.

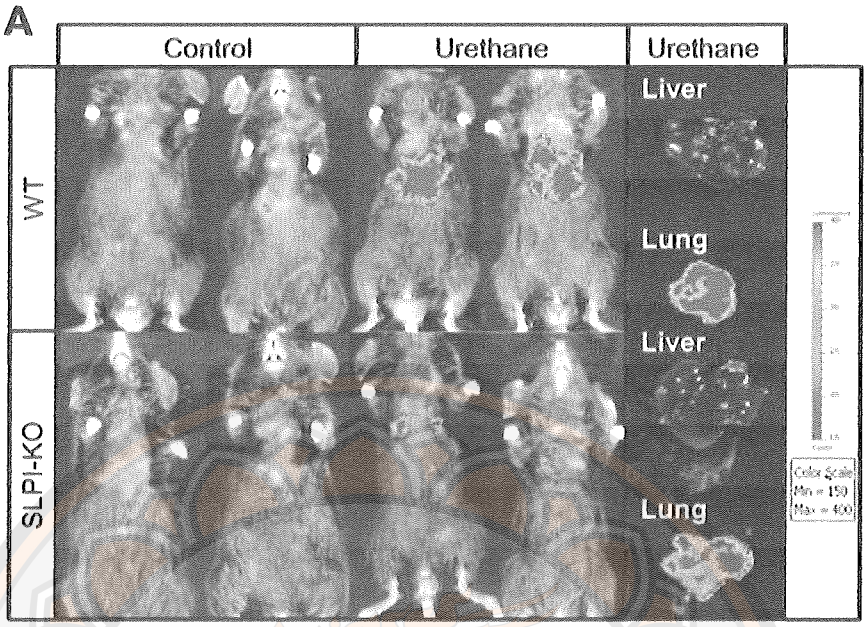


Figure 13 Urethane strongly induces inflammation in WT mice but only moderately in SLPI-KO mice

Source: Jan Treda C et al., 2013

SLPI in cholangiocarcinoma

In 2017, Jeranan J et al. investigated the level of SLPI expression in low metastatic CCA (KKU-213A), high metastatic CCA (KKU-213AL5) and immortalized bile duct cells (MMNK-1). The results showed that the level of cytosolic and secreted SLPI proteins was associated with the metastatic potential (Figure 10). The stable SLPI-overexpressing MMNK-1 cells were demonstrated that SLPI overexpression promoted the metastatic process which increased cell proliferation, cell migration, cell invasion and activity of MMP 2, 9. The results suggested that the enhancement of metastatic activities of cells was due to an increase in SLPI expression. SLPI is plays an important role in CCA progression through the promotion of the characteristics of metastasis. Therefore, SLPI might be a novel biomarker and could be used for targeted therapy of CCA patients, especially in the metastatic stage (Unpublished data). However, the role of SLPI on the initial step of CCA formation need to be further investigated.

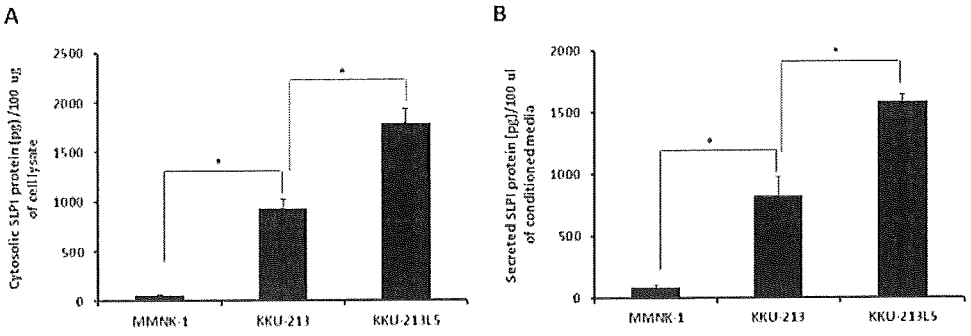


Figure 14 Expression of SLPI on various CCA cells, including MMNK-1 (immortalized cholangiocyte), KKU-213A (low metastatic CCA cells) and KKU-213AL5 (high metastatic CCA cells. The levels of endogenous (A) and secreted (B) SLPI protein of these cells were evaluated by ELISA assay. Values plotted are Means ± S.E.M (n=3). *$P < 0.05$

Source: Jantra, J. et al., 2017

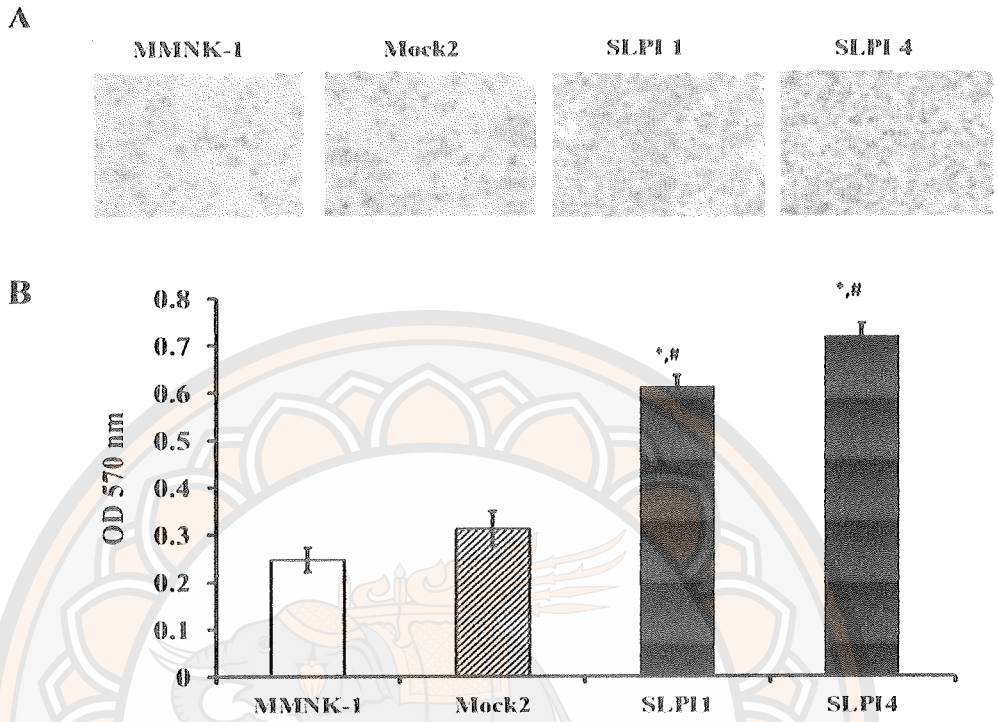


Figure 15 The effect of SLPI-overexpression cells on cell invasion was performed by *in vitro* transwell invasion assay. The pictures were showed the invading cell was stain by crystal violet (A). The plot was showed the invasiveness of 3 cells line (B). Each bar graph represents the mean ± SEM (n=3) and bars with different superscripts are significantly difference compared to MMNK-1: * p< 0.05. compare to Mock2: # p< 0.05

Source: Jantra, J. et al., 2017

CHAPTER III

RESEARCH METHODOLOGY

Materials

1. Biological materials

1.1 Cell models

Immortalized cholangiocyte namely MMNK-1and low metastasis CCA cells namely KKU-213A were provided from Professor Dr.Sopit Wongkham, Departmetment of Biochemistry, Faculty of Medicine, Khon Kean University, Khon Kaen. Mock and SLPI-overexpressing cells were established by Jeranan J et al., 2017 from MMNK-1 cells with pCMV2–SLPI-HA and pCMV2-HA plasmids by lipofectamine 2000 transfection assay. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 100 U antibiotic-antifungal. Cells were incubated at 37° C in a humidified atmosphere with 5% CO_2 (v/v) and 95% air. Cells were subcultured twice a week. At 70-80% confluence, cells were detached from culture flask using trypsin/EDTA (0.25% w/v) and processed according to the particular assay.

1.2 Mice model

Male nude mice (BALB/cAJcl-nu) 6-8-week-old were purchased from Siam Nomura Co.Ltd. and maintained in an isolated clean room held at a regulated temperature (25 ± 2°C) and humidity (approximately 40–50%). The mice will be housed under a 12 h/12 h light/dark cycle and fed ad libitum with rodent diet and water. All protocols were approved and performed according to the guidelines of the Ethics Committee of Naresuan University (Ethics No. 62 01 016). The BALB/cAJcl-nu were device in to two groups for investigation tumorigenicity by xenograft mice assay, Mock (control) n=10 and SLPI-overexpressing cells n=12.

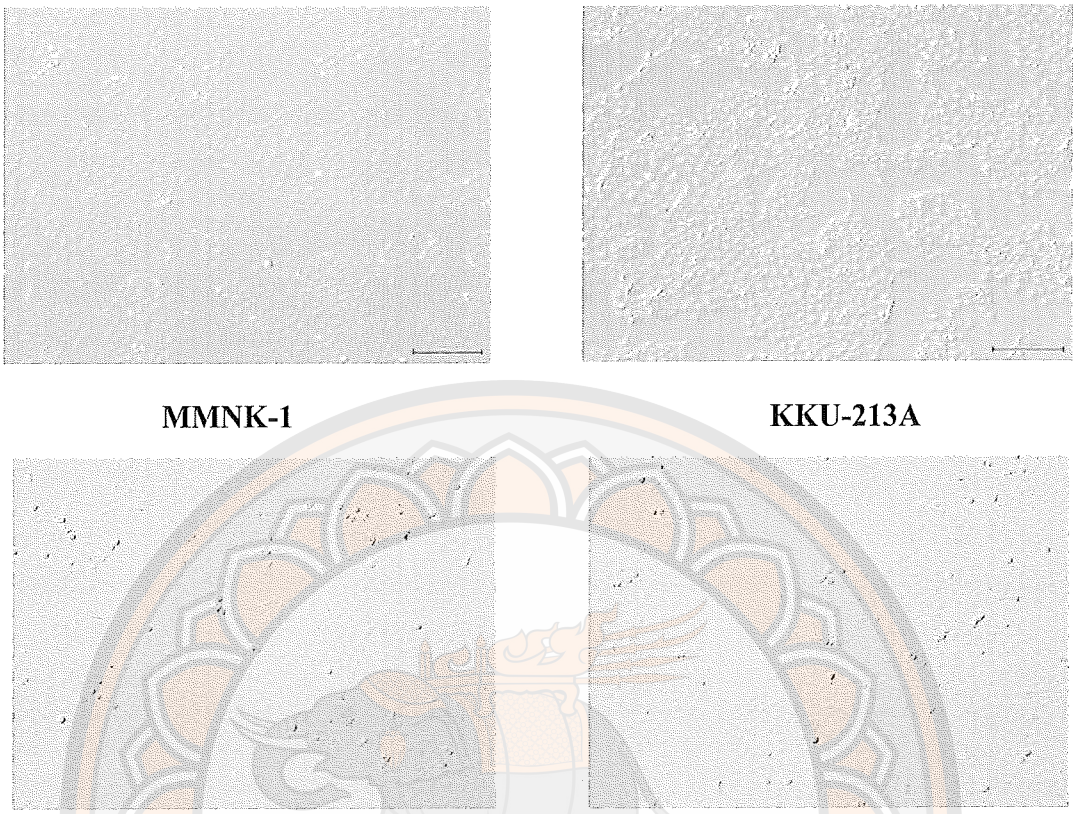


Figure 16 The morphology of the MMNK-1, KKU-213A, Mock, and SLPI-overexpressing cells

1.3 Hamster tissues with the cholangiocarcinogenesis model

Hamster tissues were kindly provided from Professor Dr.Sopit Wongkham, Departmetment of Biochemistry, Faculty of Medicine, Khon Kean University, Khon Kaen, Thailand. The hamsters were treated with 50 *Ov* metacercariae by oral inoculation and/or given with 12.5 ppm of NDMA in water, and sacrificed at 1, 3, and 6 months post-treatment. The livers of five hamsters were collected and subjected for the analysis of histopathology and immunohistochemistry for control group of CCA-induced hamster n=7, 1-month group of CCA-induced hamster n=9, 3-month group of CCA-induced hamster n=4, and 6-month group of CCA-induced hamster n=5.

2. Chemicals and reagents

Chemicals, reagents, commercial kits, and antibodies, are listed in Table 1.

Table 1 List of chemicals and suppliers

Chemicals	Product of
Cell culture	
3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide: MTT	Bio basic Canada, Inc, CA
Crystal violet	Panreac, E.U.
DMSO	Sigma-Aldrich.St. Louis, MO
Dulbecco's Modified Eagle Mediun (DMEM)	Gibco, Grand Island, NY
Fetal bovine serum (FBS)	Gibco, Grand Island, NY
Penicilin-Streptomycin	Gibco, Grand Island, NY
Phosphate buffered saline (PBS)	Amresco, USA
Trypan blue	Gibco, Grand Island, NY
Trypsin/EDTA	Gibco, Grand Island, NY
Agarose	Gibco, Grand Island, NY
Ethanol	Merck Amresco, USA
Protein expression (western blot)	
Acrylamide	Amresco, USA
Ammonium peroxidisulphate	Amresco, USA
Beta-mercaptoethanol	Gibco, GranIsland, NY
Bromophenol blue	Fluka, China
Chemiluminescence (ECL)	Bio-rad, USA
DTT	Amresco, USA
EDTA	Ajax finechem, New Zealand
Glycerol	Amresco, USA
Glycine	Amresco, USA

Table 1 (cont.)

Chemicals	Product of
Methanol	Rcilabscan, Thailand
NaCl	Amresco, USA
Non-fat skim milk	Amresco, USA
NP-40	Amresco, USA
Protease inhibitor cocktails	Amresco, USA
Protein ladder	Genedirex, Inc., USA
PVDF Immobilon FL	Millipore, Billerica, MA
Sodium deoxycholate	Ajax finechem, New Zealand
Sodium dodecyl sulfate: SDS	Amresco, USA
TEMED	Sigma-Aldrich.St. Louis, MO
Tris-base	Amresco, USA
Tween-20	Amresco, USA
Immunohistochemistry	
Xylene	Sigma-Aldrich.St. Louis, MO
Hematoxylin	Sigma-Aldrich.St. Louis, MO
3,3′-Diaminobenzidine tetrahydrochloride hydrate	Sigma-Aldrich.St. Louis, MO
Hydrogen peroxide	Sigma-Aldrich.St. Louis, MO
Ethanol	Merck Amresco, USA
Tween-20	Amresco, USA
Tris-base	Amresco, USA
Methanol	Rcilabscan, Thailand
Phosphate buffered saline (PBS)	Amresco, USA
Antibodies	
Goat anti-human SLPI IgG	Santa Cruz.INC
Mouse anti- β-actin IgG	Merck Millipore, USA
Rabbit anti- p-p65 IgG	Cell Signaling Technology
Rabbit anti- p65 IgG	Cell Signaling Technology

Table 1 (cont.)

Chemicals	Product of
Goat anti mouse IgG conjugated-HRP	Invitrogen, USA
Goat anti-rabbit IgG conjugated-HRP	Invitrogen, USA
Chronic inflammatory inducer	
Interleukin 6 (IL-6)	Sino Biological, Inc.

Methods

1. Immunohistochemical staining

1.1 Principle of immunohistochemistry

Immunohistochemical (IHC) is a technique commonly used to detect protein expression and translation in the context of tissue morphology. It uses antibodies to detect and analyze the expression of proteins while still maintaining the cellular composition and structure of native tissues. IHC specifies the existence and pattern of protein expression in biological samples through the capture of specific antibodies. The precise binding that occurs between antibodies and the epitope enables the detection of highly specific amino acids within the protein by targeting the target domain or broken product (Crosby, K. et al., 2016).

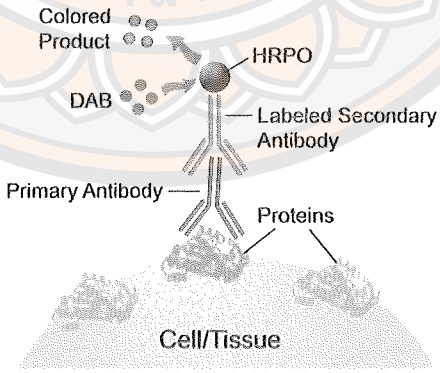


Figure 17 Principle of immunohistochemical staining

Source: Leinco Technologies / https://www.leinco.com/immunohistochemistry/

1.1.1 Protocol of immunohistochemistry

The tissue slides were deparaffinized and passed through a rehydration series, then performed the following washes in xylene twice for 5 minutes each, 100% (v/v) ethanol twice for 5 minutes each, 95% (v/v) ethanol twice for 5 minutes each, 70% (v/v) ethanol twice for 5 minutes each, and DW water twice for 5 minutes each. The antigen was retrieved in tissue slides by autoclave Tris-EDTA buffer pH 9.0 for 3 minutes at 121° C. Next, the tissue slides were blocked of endogenous peroxidase with 0.3% H_2O_2 (v/v) in methanol for 30 minutes at room temperature, then blocked with 1% (w/v) BSA in PBS for 1 hour at room temperature. The immunohistochemical staining were performed using goat anti-human SLPI (1:50), incubated overnight at 4°C. Then, the tissue slides were washed and incubated with secondary antibody agents to HRP conjugated anti-goat IgG for 2 hours at room temperature. Next, the signal of HRP was developed by DAB solution for 2 minutes, then the slides were counter staining with hematoxylin 30 seconds at room temperature. The scoring was assessed semi-quantitatively as negative (no detectable staining or positive staining in <10% of tumor cells); weakly positive (positive staining in 10%-25% of tumor cells); positive (positive staining in 25%-75% of tumor cells), and strongly positive (>75%) by two independent investigators. The scoring was calculated by the H-Score formula (H score = [1 × (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)])

2. Mimicry of *Ov.* infection

In this study, interleukin 6 (IL-6) was used as an inducer of the carcinogenesis of CCA in the early stages caused by liver fluke infection. The previous report of infection with *Ov.* found that *Ov* excretory/secretory products (*Ov*Es) treatment was induced IL-6 production in cholangiocyte which is an inflammatory response (Chaiyadet, S. et al., 2015). MMNK-1 and KKU-213A cells were treatment with 0, 1, 5, and 10 ng/ml of recombinant IL-6 (Sino Biological, Inc) at 12 and 24 hours. Then the treated cells were confirmed inflammatory response by investigation of NF-kB activation signalling pathway.

3. Western blot

3.1 Principle of western blot analysis

western blot is a powerful technique for detection and analysis of proteins. This method based on antigen-antibody interaction, though specific antibody binding to the protein of interest. In this technique a mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis. These results are then transferred to a membrane. The membrane is then incubated with labels antibodies specific to the protein of interest. The unbound antibody is washed off, leaving only the bound antibody to the protein of interest. The bound antibodies are then detected by enhanced chemiluminescence (ECL) substrate solution. As the antibodies only bind to the protein of interest, only one band should be visible. The thickness of the band corresponds to the amount of protein present. The thickness of the band corresponds to the amount of protein present; thus, doing a standard can indicate the amount of protein present (Tahrin, M. et al., 2012)

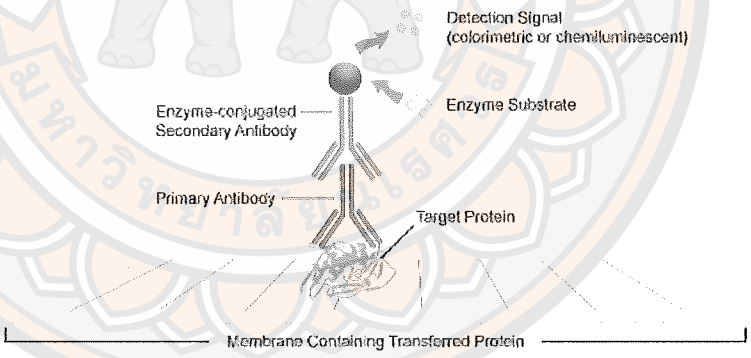


Figure 18 Principle of western blot analysis

Source: http://microbeonline.com/western-blot-technique-principle-procedures-advantages-and-disadvantages/

3.2 Cell lysate, protein secretion, and protein determination

After experiment cells were washed twice with cold-PBS and then lysed with RIPA buffer. Cell lysates were then centrifuged at 13,000 rpm for 20 minutes at

4°C. The supernatant was transferred to a new tube and determined for protein content using Bradford assay.

3.3 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, and boiled for 10 minutes. Protein (30 μg) was loaded into an individual well and separated with 15% separating gel at a constant current of 120 V at 1.30 hours. 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 membranes were blocked with 5% (w/v) non-fat skim milk at room temperature for 1 hour and then washed 3 times with washing buffer. The membrane were incubated with 1:1000 primary antibody Goat anti-human SLPI or NF-kB antibodies (rabbit anti-human p-p65 at 1:500 and rabbit anti-human p65 at 1:1000,) or 1:20,000 anti-β-actin antibody for overnight at 4°C and washed 3 times with washing buffer, then the membrane were incubated in a HRP-linked secondary antibody (1:1000 and 1:20,000) for 1 hour at room temperature and then washed 3 times with washing buffer. The immunoreactive bands was detected by Chemiluminescence ECL Prime Western Blotting Detection System. The membranes were captured with ImageQuant™ 4000 image analyzer and analyzed using ImageJ™ analysis software.

4. Colony formation assay

4.1 Principle of colony formation assay

The soft agar colony formation assay is widely used technique for evaluating cell changes *in vitro*. In this technique, cells were dispersed to culture plates and grown where there are 'feed' cells or conditioned media to provide the necessary growth factors. Anchored growth is the ability of cells that are transformed to grow independently from hard surfaces and are a hallmark of carcinogenesis. The soft agar colony formation assay is a well-established method of describing this ability *in vitro* and is considered one of the most rigorous tests for cancer transformation in cells (Borowicz, S. et al., 2014).

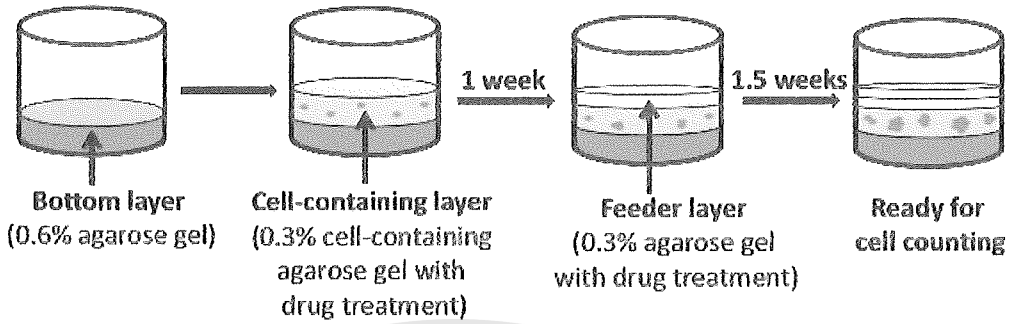


Figure 19 Soft agar colony formation assay diagram

Source: Horibata S et al., 2015

4.2 Preparation of base agar

Firstly, 1% agarose was dissolved in sterile distilled water, the agarose was cooled to 42°C in water bath. Next, 2X DMEM was incubated at 42°C in water bath and supplemented with 20% FBS and antibiotics. Then, 2X DMEM was mixed equal volumes of the agarose to give 0.5% agarose/1X DMEM in final concentration. Next, mixture was added 1 ml into 6-well dish (1 ml/dish), allow 5 minutes to solidify.

4.3 Preparation of top agarose

The $5x10^3$ cells in 2X DMEM were mixed with 0.35% top gel, which were prepared by sterilized 0.7% agarose with distilled water 42°C. After top gel solidified, add 1 ml 1X full media and plates were cultured at 37°C in a humidified atmosphere with 5% CO_2 for 30 days. Cells were stained with 0.05% crystal violet in 20% methanol at room temperature for 30 minutes and the numbers of cell colony were counted.

5. Xenograft assay

5.1 Principle of xenograft assay

Tumor xenografts assay is commonly technical has to used in the preclinical and clinical development of cancer therapeutics, and xenograft models are useful for predictive biomarkers of target modulation *in vivo* study. The tumor cells are transplanted under the skin of nude mice severely compromised immunodeficient so

that the human cells are not rejected, after several weeks, mice bearing a tumor. the volume, size, and weight of tumors was observed.

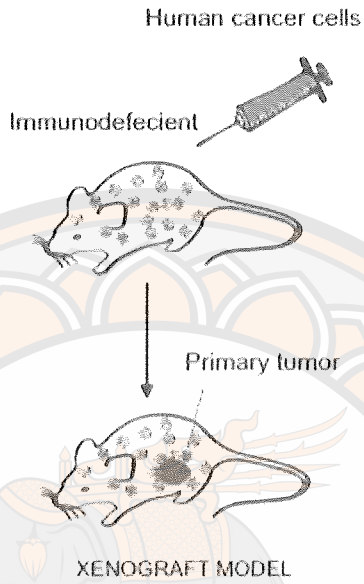


Figure 20 Generation of xenograft tumor models

Source: Horibata, S. et al., 2015

5.2 Preparing cells for transplantation

SLPI and Mock cells were maintained in completed medium at 37 °C in 5% CO_2. After pellet the cells at 60 x g for 5 minutes, the supernatant was removed and resuspended cells homogeneously in PBS. Than, the cell concentration was measured and adjusted to $5x10^7$ cells/ml. Then, 1.5 x 10^6 cells were centrifuged at 60 x g for 5 minutes. The cell pellets were resuspended in 0.15 ml PBS and transferred into a sterile micro centrifuge tube (1.5 ml) for injection.

5.3 Subcutaneous injection of cells

The cell suspension (0.15 ml) were filled in the 1 ml injector, avoiding any bubbles to enter the syringe. The animal was restrained in an upright position by grasping the skin over the shoulders with the thumb and index finger, keeping the fore legs from pushing the syringe away. The dorsal skin was disinfected gently with 70%-ethanol-saturated cotton balls for 2-3 times and gently inserted the needle into the skin

on the dorsal flank to reach the subcutaneous pocket. The survival of nude mice and tumor growth were monitored 2-3 times a week. The tumors were measured the maximum (L) and minimum (W) length of the tumor using a slide caliper. Tumor volume was calculated by the volume formula (Tomayko, & Reynolds, 1989).

$$\mathbf{VT = ½ (L \times W \times W)}$$

L: the maximum length of the tumor

W: the minimum length of the tumor.

When tumor grows to general health 6-8 week, the xenografts were removed from sacrificed nude mice, weight and measure tumor size for tumor volume, fixed in 4% paraformaldehyde and embedded in paraffin, then stored at RT for analysis. Tumor size and weight of different groups are presented as the mean ± SEM, which were adapted for tumor growth curve.

6. Statistical analysis

All values were given as mean ± SEM. Means of groups was compared with the Student's t test (unpaired) or ANOVA test when appropriate. P value <0.05 were considered statistically significant. The data were analyzed using GraphPad Software (San Diego, CA, USA). A P-value of < 0.05 were considered statistically significance. (*p< 0.05, **p< 0.01, ***p<0.001).

CHAPTER IV

RESULTS

The expression of SLPI protein in the tissues of CCA-induced hamster

To examine the hypothesis that SLPI is involved in the CCA progression and the development of the disease. In this study, we compared the expression of SLPI using the IHC method in hamster tissues that has been induced CCA with *Ov.* and NDMA for 1, 3, and 6 months. The results showed that the control group (n=7) had normal bile duct characteristics with very low SLPI expression with a median H score of 22.5 ± 20.3. In the 1-month group of CCA-induced hamster (n=9), most of the bile ducts were hyperplasia/dysplasia (HD) characteristics, found a high expression of SLPI had the median H score was 75.56 ± 42.7. In the 3-month of CCA-induced hamster group (n = 4) found the SLPI expression increases significantly at median H score158.75 ± 85.1 (*P=0.0047*), there are most of the characteristics of bile ducts were HD, also found a little CCA. In the 6-month CCA-induced hamster group (n = 5), found the significantly the highest SLPI expression with a median H score 176.0 ± 81.7 (*P=0.0007*), the most characteristic of the tissues is CCA (Figure 21-22). These data showed that the increased expression of SLPI was positively correlated with CCA development in hamster models. Therefore, it can be concluded that SLPI may be a promoter of the cholangiocarcinoma process. To investigate whether SLPI expression had a significant role in CCA carcinogenesis, we further mimicked the chronic inflammation which is the early step of CCA carcinogenesis in CCA cell lines.

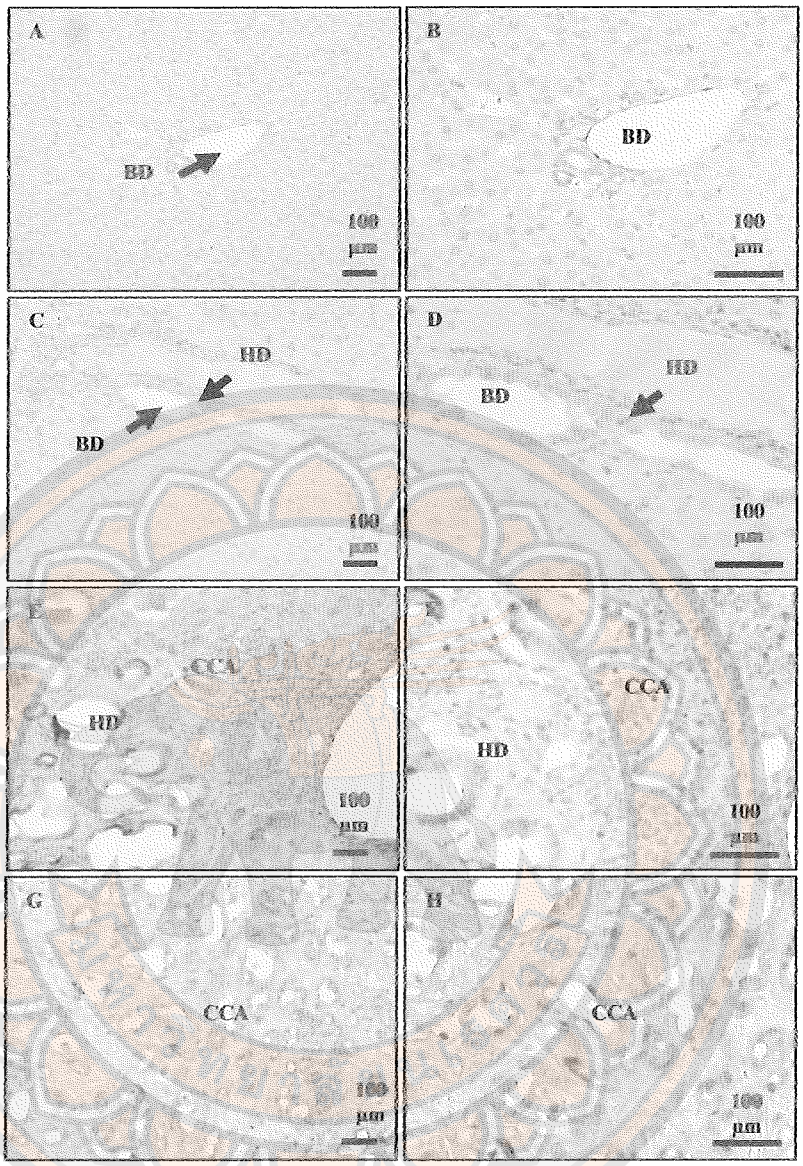


Figure 21 **Histological classification and expression of SLPI protein in the tissue of CCA-induced hamsters compared with the normal bile duct area, the tissue of CCA-induced hamsters was observed under microscopy (10X: A, C, E, and G and 20X: B, D, F, ang H). (A-B): the morphology of CCA-induced hamster tissues in control group, (C-D): the morphology of CCA-induced hamster tissues 1-month groups, (E-F): the morphology of CCA-induced hamster tissues 3-month groups, and (G-H): the morphology of CCA-induced hamster tissues 6-month groups. Hyperplasia/dysplasia (HD) and Bile duct (BD)**

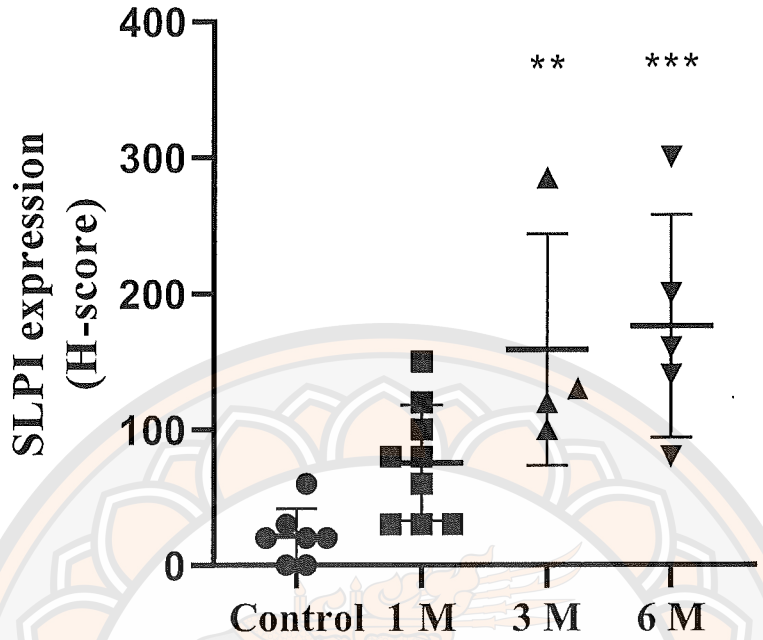


Figure 22 The expression levels of SLPI in CCA-induced hamster tissues 1 M (n=9), 3 M (n=4), and 6 M (n=5) compared with normal hamster tissues (n=7) measured by IHC. The data presented as the mean ± SEM, one way-ANOVA test with Sidak's multiple comparisons **$p < 0.01$ and ***$p < 0.001$

Investigation of the effects of liver fluke infection mimicry on SLPI expression

To examine the hypothesis that SLPI promotes the process of CCA carcinogenesis, we mimicked the carcinogenesis of CCA in the early stages, which are chronic inflammation after repeated infection with *Ov*. The previous report of infection with *Ov*. found that *Ov* excretory/secretory products (*Ov*Es) treatment was induced Interleukin 6 (IL-6) production in cholangiocyte which is a inflammatory response (Chaiyadet S et al., 2015). In this study, IL-6 was used as an inducer of cellular inflammation caused by liver fluke infection.

1. IL-6 stimulates phosphorylation of NF-kB expression in CCA cells.

NF-kB pathway activation is the inflammatory response process of cells. This study used IL-6 to stimulated inflammation caused by liver fluke infection, therefore we confirmed the inflammatory response process by checking NF-kB pathway activation via p65 phosphorylation. After mimicked the inflammation by treatment with IL-6 at 0, 1, 5, and 10 ng/ml in MMNK-1 and KKU-213A cell lines. The results showed that in MMNK-1, the expression level of p65 phosphorylation (P-p65) (Figure 23) had significantly increased by the dose-dependent manner of IL-6 concentration at 12 hours, but slightly increased at 24 hours compared with untreated cells. In KKU-213A, (Figure 24) the expression level of P-p65 had increased expression at 12 and 24 hours compared with untreated cells. These data confirmed that IL-6 can induce inflammation by increasing the expression of P-p65, which is an indicator of cellular inflammatory responses.

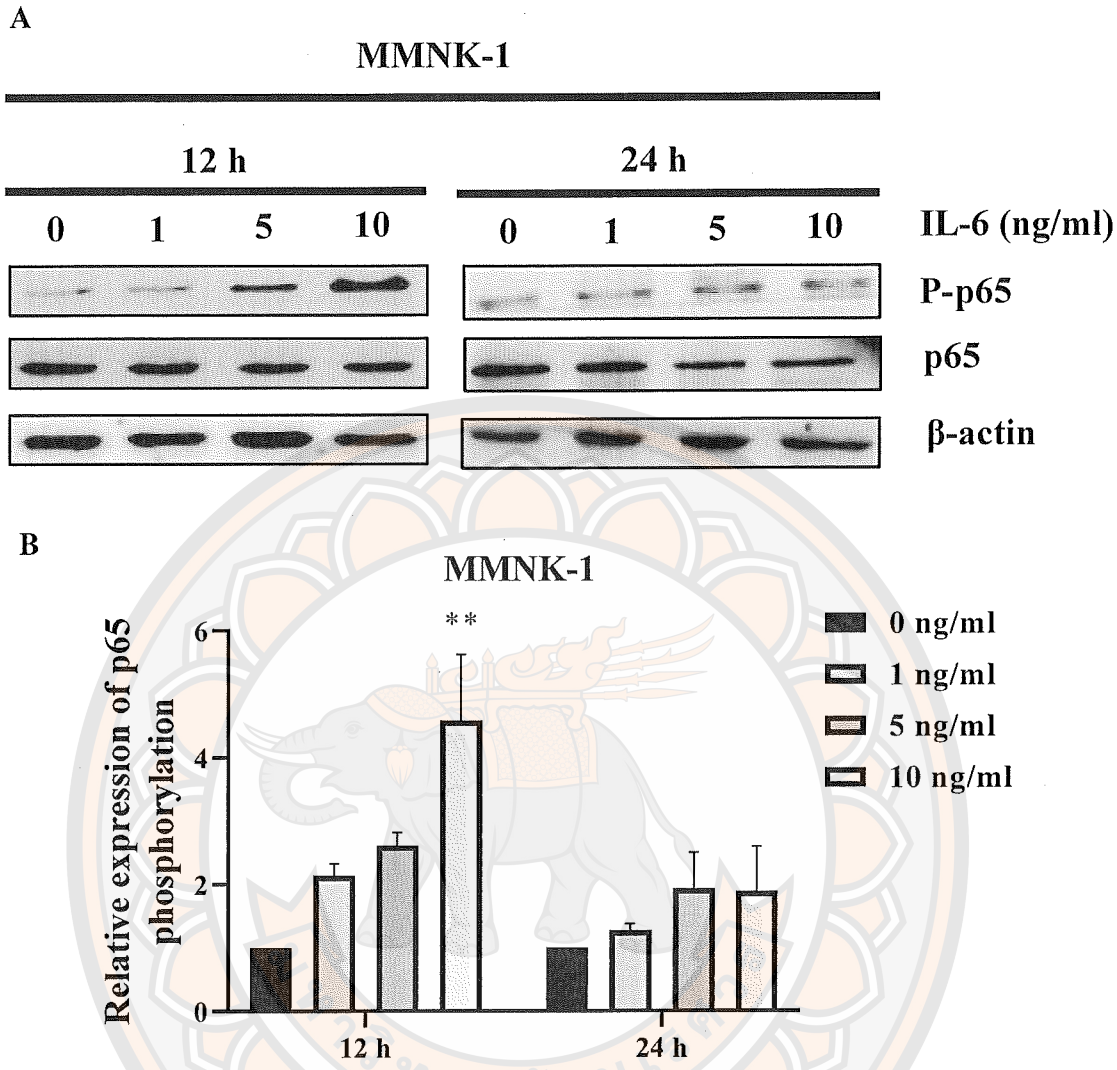


Figure 23 The effects of IL-6 treatment in MMNK-1 cells on p65 phosphorylation expression measured by western blot assay. MMNK-1 were treatment with IL-6 0, 1, 5, and 10 ng/ml at 12 and 24 hours. B: Relative expression of P-p65 was normalized with β-actin. The data presented as the mean ± SEM, one way-ANOVA test with Sidak's multiple comparisons $**p < 0.01$

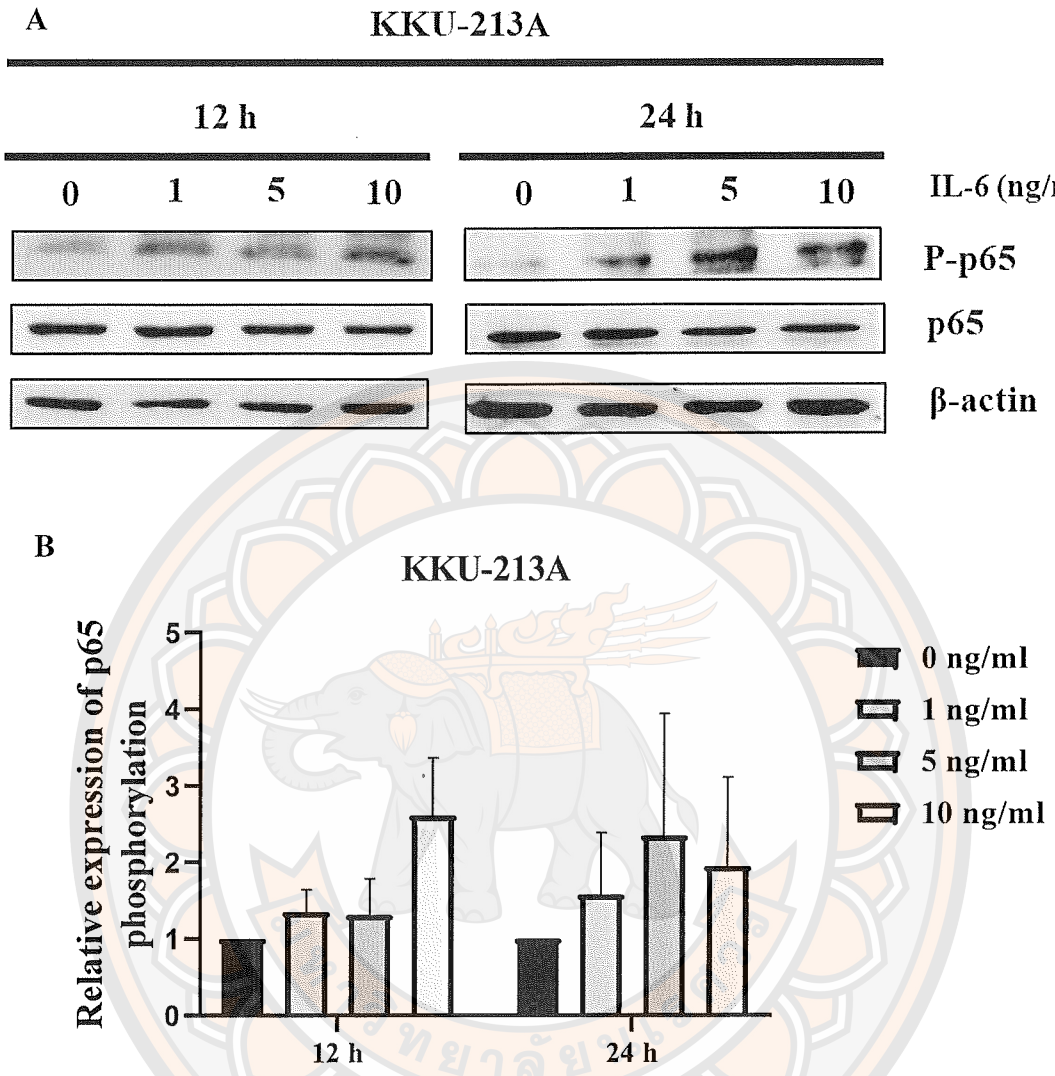


Figure 24 The effects of IL-6 treatment in KKU-213A cells on p-p65 expression measured by western blot assay. KKU-213A were treatment with IL-6 0, 1, 5, and 10 ng/ml at 12 and 24 hours. B: Relative expression of P-p65 was normalized with β-actin. The data presented as the mean ± SEM, one way-ANOVA test with Sidak's multiple comparisons

2. IL-6 stimulates expression of SLPI in CCA cells.

After IL-6 was able to mimicking the inflammation through increased expression of p65 phosphorylation, then we further examined how the expression level of SLPI in responses to IL-6 treatment. In this study, the SLPI expression was monitored by western blot assay. After treatment with IL-6 at 0, 1, 5, and 10 ng/ml in MMNK-1 and KKU-213A cell lines. The results of MMNK-1 cells showed that SLPI expression levels was significant increased by dose-dependent manner (Figure 25) at both 12 and 24 hours of IL-6 treatment. In KKU-213A cells, SLPI expression was tend to be increased by dose-dependent manner at 12 hours (Figure 26) and slightly increased at 24 hours. From these results can be concluded that stimulation with IL-6 resulted in the up-regulation of SLPI expression. Thus, we hypothesize that increasing of SLPI expression in this mimicked inflammation process might be a tumorigenic factor in CCA cell lines.

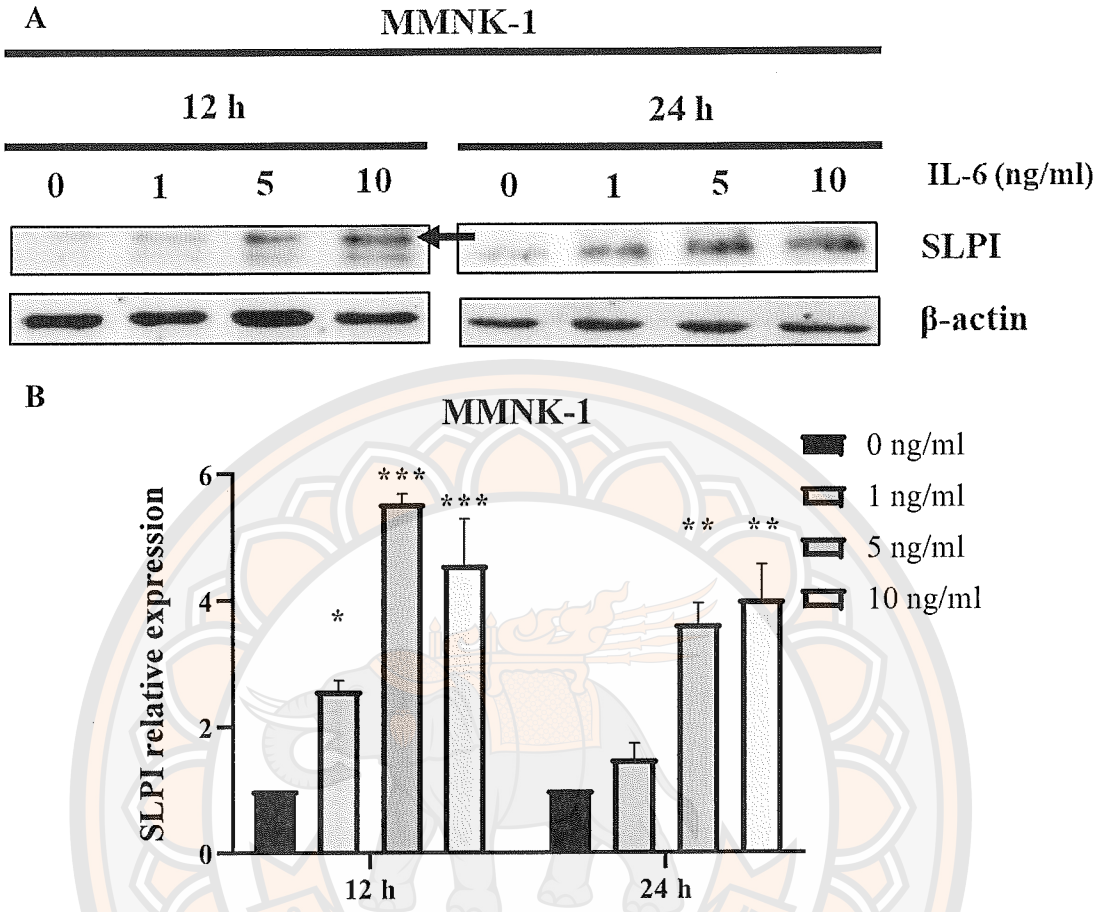


Figure 25 **The expression levels of SLPI after mimicking of chronic inflammation in MMNK-1 cells measured by western blot assay. A: MMNK-1 were treatment with IL-6 0, 1, 5, and 10 ng/ml at 12 and 24 h. B: Relative expression of P-p65 was normalized with β-actin. The data presented as the mean ± SEM, one way-ANOVA test with Sidak's multiple comparisons *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$**

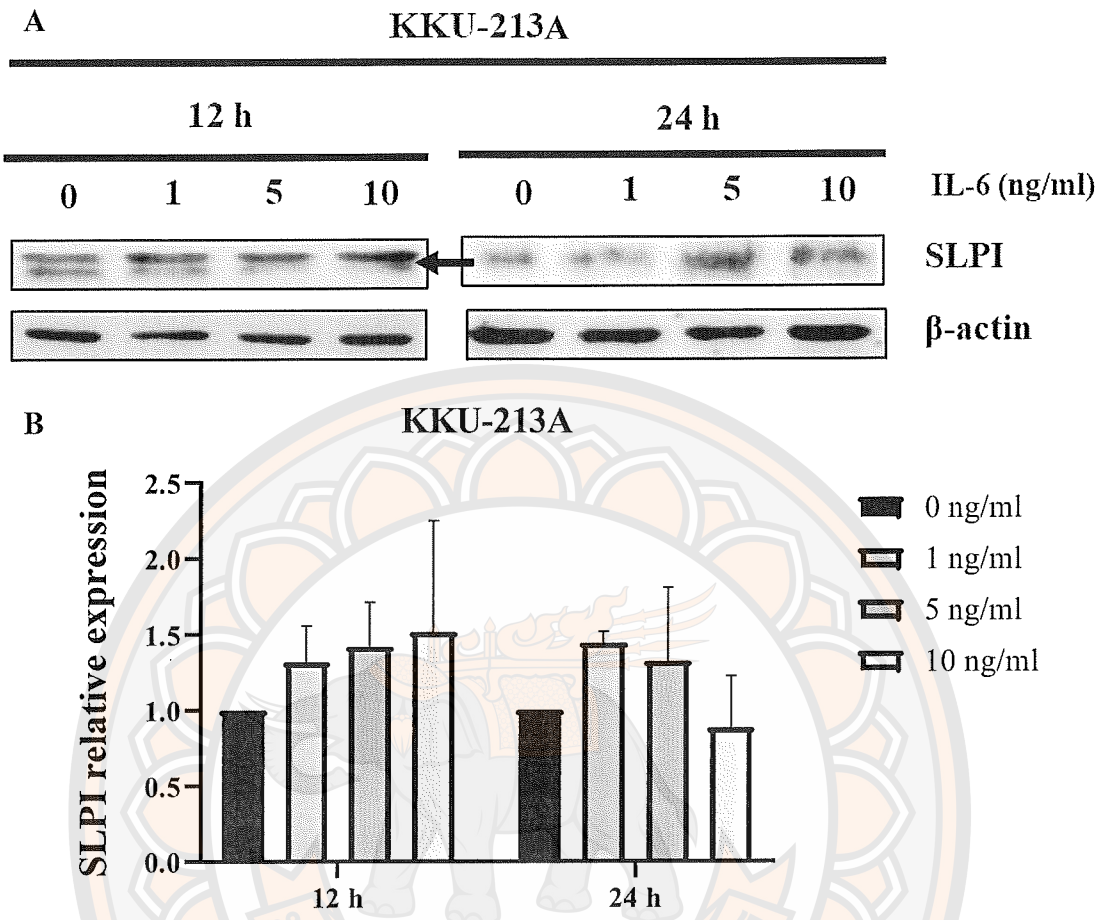


Figure 26 The expression levels of SLPI after mimicking of chronic inflammation in KKU-213A cells measured by western blot assay. A: KKU-213A were treatment with IL-6 0, 1, 5, and 10 ng/ml at 12 and 24 hours. B: Relative expression of P-p65 was normalized with β-actin. The data presented as the mean ± SEM, one way-ANOVA test with Sidak's multiple comparisons

3. IL-6 treatment enhance tumorigenicity of CCA cells.

After inflammatory induction by IL-6 results to increasing of SLPI expression levels. Next, we further examined that these effect is the initial event to induce tumorigenesis of CCA. Tumorigenicity was used to provide a characterization to distinguish between normal and cancer cells. The tumorigenicity can be detected by soft agar colony formation assay. Here, the results found that in treatment with 10 ng/ml of IL-6 groups cells were significantly increased number of colonies in both cell lines MMNK-1 and KKU-213A compared with untreated cells. The number of colonies after IL-6 treatment were increased with 204.3 colony ± 33.74 (p=0.038) and 172.7 colony ± 28.10 (p=0.025) in MMMK-1 and KKU-213A cells, respectively (Figures 27). The results demonstrated that activation with IL-6 can increase the capacity of cells to form colonies suggested that IL-6 is an inflammatory inducer that stimulates the expression of SLPI and results in the process of tumorigenicity. However, to prove that SLPI is the major factor of this process, we used the stable cell lines of SLPI overexpression to examine their tumorigenicity compared with its control mock cell line.

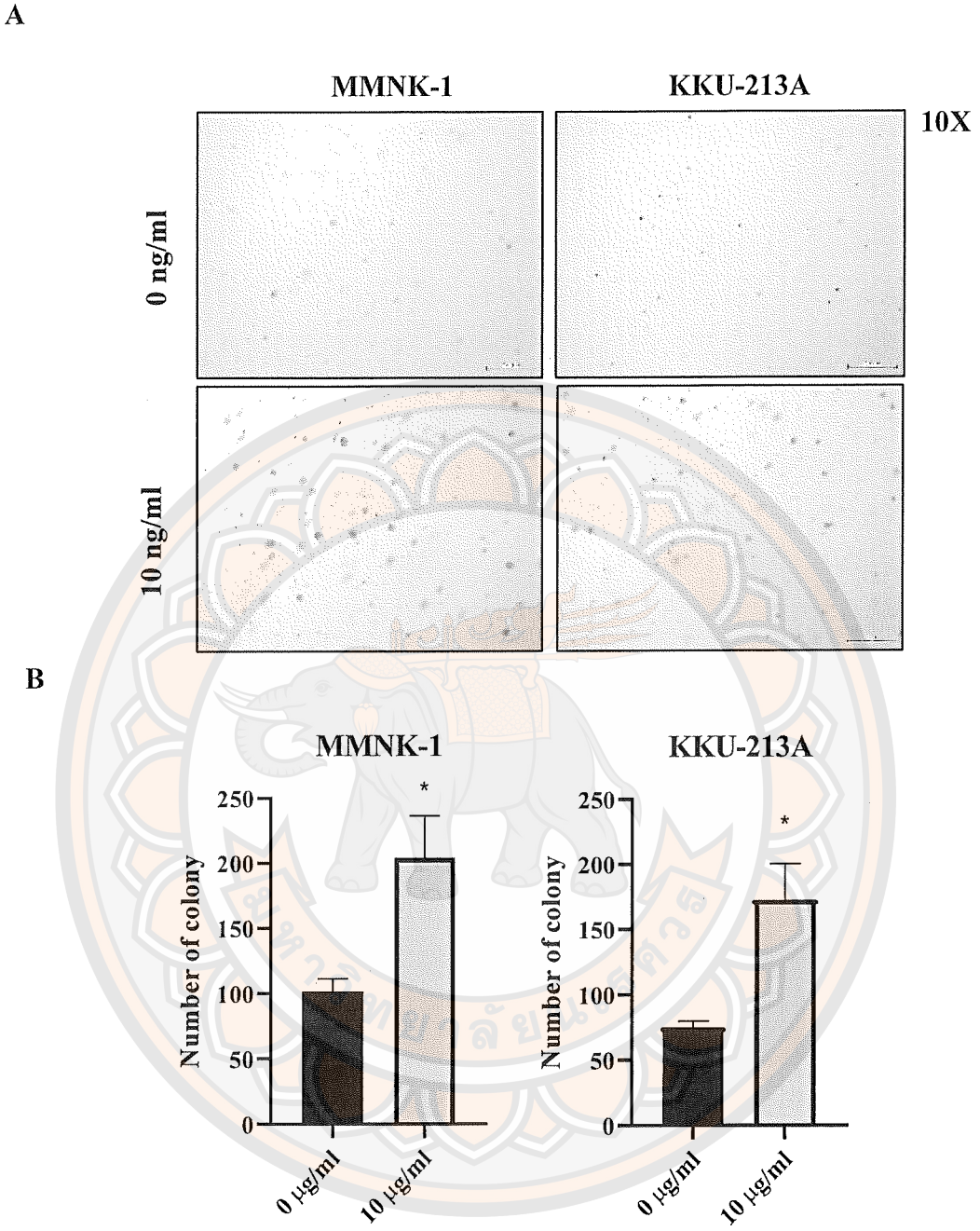


Figure 27 **The effect of IL-6 treatment on tumorigenicity in MMNK-1 and KKU-213A cells. MMNK-1 and KKU-213A cells were treated with IL-6 (0 and 10 ng/ml) for 24 hours and subsequently determined the tumorigenicity by soft agar colony formation assay for 20 days. A: the morphology of colony formation at 30-day culture. B: the colony formation rate compared with untreated cells. The data presented as the mean ± SEM student's unpaired t test** $*p < 0.05$

Evaluation of SLPI-overexpression in cholangiocyte on tumorigenicity

To prove the significant effect of SLPI on promoting the transformation capacity from cholangiocyte to CCA cells as increasing of tumorigenicity, we analyzed the tumorigenicity by soft agar colony formation assay using the model of stable SLPI overexpression in the immortalized cholangiocyte MMNK-1 cell line which previously established in our laboratory. There are two stable cell lines, a SLPI-overexpressing cell (SLPI) and a control cell (Mock).

1. The expression of SLPI in the stable cell lines with overexpression of SLPI

To confirm the expression of SLPI in the SLPI-overexpressing cell model, the protein expression levels of SLPI in Mock, SLPI and the parental MMNK-1 cells were demonstrated by western blot analysis. The result showed that the expression of SLPI in SLPI cells was greater than Mock and MMNK-1 cells (Figures 28) while SLPI expression in Mock and MMNK-1 was almost comparable. These results suggested that our SLPI-overexpressing cell models could be used for the further study.

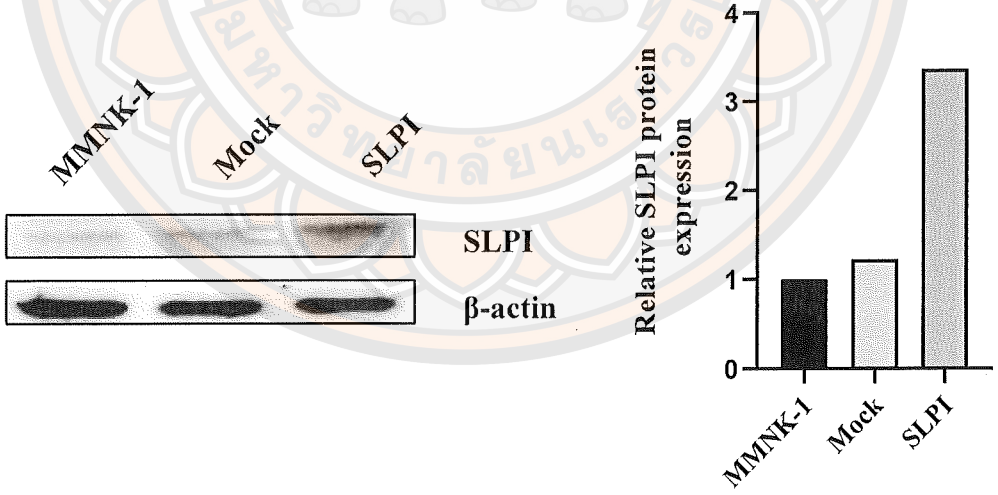


Figure 28 Confirmation of SLPI protein expression level in SLPI overexpressing-cell models (Mock and SLPI) compared with its parental MMNK-1 cells by western blotting. The relative SLPI protein levels were normalized with β-actin

2. The overexpression of SLPI in normal immortalized cells can increase tumorigenicity *in vitro* study.

To explore the effect of SLPI overexpression on tumorigenicity, the SLPI, Mock, and MMNK-1 cells were examined using a soft agar colony formation assay for 20 days. After incubation time, the number of colonies was staining with crystal violet and counted. The results showed that (Figure 29), SLPI overexpressing cells had a significantly increase colony capacity and the number of colonies were increased at 114.0 colony ± 12.0 compared with Mock cells (30.50 colony ± 9.16) and MMNK-1 cells (19.5 colony ± 1.50). These results strongly demonstrated that SLPI has a significant role to accelerate the tumorigenicity or the transformation capacity from cholangiocyte into CCA which occurred in the early step of CCA progression. However, these data were obtained from *in vitro* study. Next, we further examined whether overexpression of SLPI promote tumorigenicity using *in vivo* study.

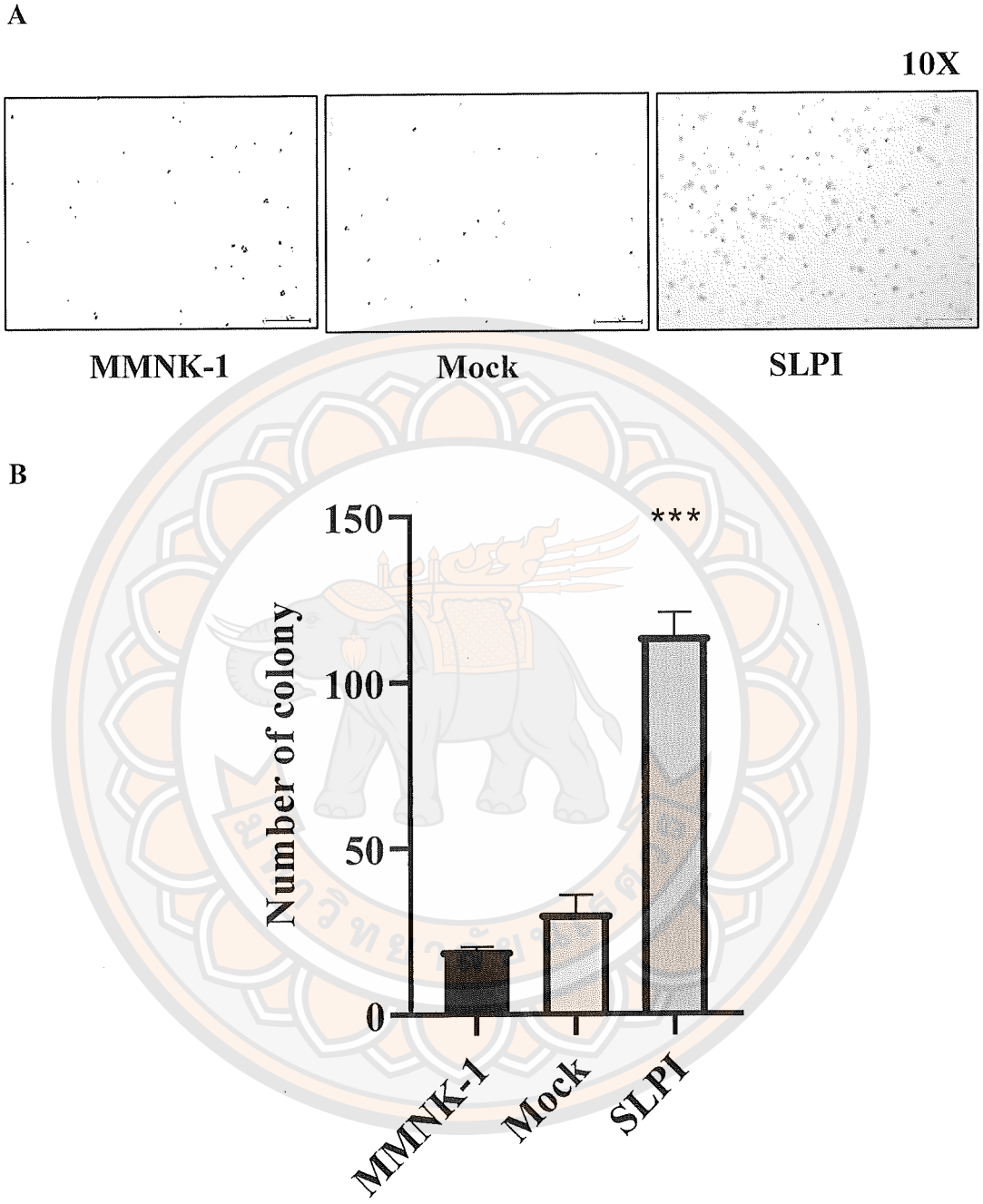


Figure 29 The effects of stable SLPI-overexpressing cells on tumorigenicity. A: Colony formation ability was determined by soft agar colony formation assay for 20 days. B: the relative of colony formation in SLPI-overexpressing cells compare with Mock and MMNK-1 cells. The data presented as the mean ± SEM one way-ANOVA test with Sidak's multiple comparisons $***p < 0.001$

2. The overexpression of SLPI in immortalized cholangiocyte can increase tumorigenicity in mice model.

The tumorigenicity of SLPI-overexpressing cells *in vivo* study was performed by xenograft mice assay, the tumorigenicity cells can be forming a tumor under the skin of animal models. Although the previous report demonstrated that the immortalized cholangiocyte MMNK-1 cell are lacking of tumor formation capacity in mice model after xenograft for 7 months (Maruyama M et al., 2004), we hypothesize that if SLPI is a key molecule to transform the normal to cancer phenotypes, we may observe the tumor formation in this cell line. After subcutaneous injection of SLPI and Mock cells in BALB/cAJcl-nu mice (n=12/each group), the tumor volume was observed every 2 days for 48 days. The results presented the mean of tumor volumes in SLPI and Mock mice group from day 20 to day 48 (Figure 30). We found that the tumor volume of SLPI mice group was larger than Mock mice group. After the mice were sacrificed, two tumor mass formation was observed in Mock group. Surprisingly, eight tumor mass formation was prominent presented in the SLPI mice group (Figure 31A). Moreover, tumor weight of SLPI mice group was exhibited significantly higher than the Mock mice group as 0.011 ± 0.008 g and 0.001 ± 0.004 g, respectively ($P = 0.0049$) (Figure 31B). Therefore, the overexpression of SLPI promoted the tumorigenicity of MMNK-1 cells.

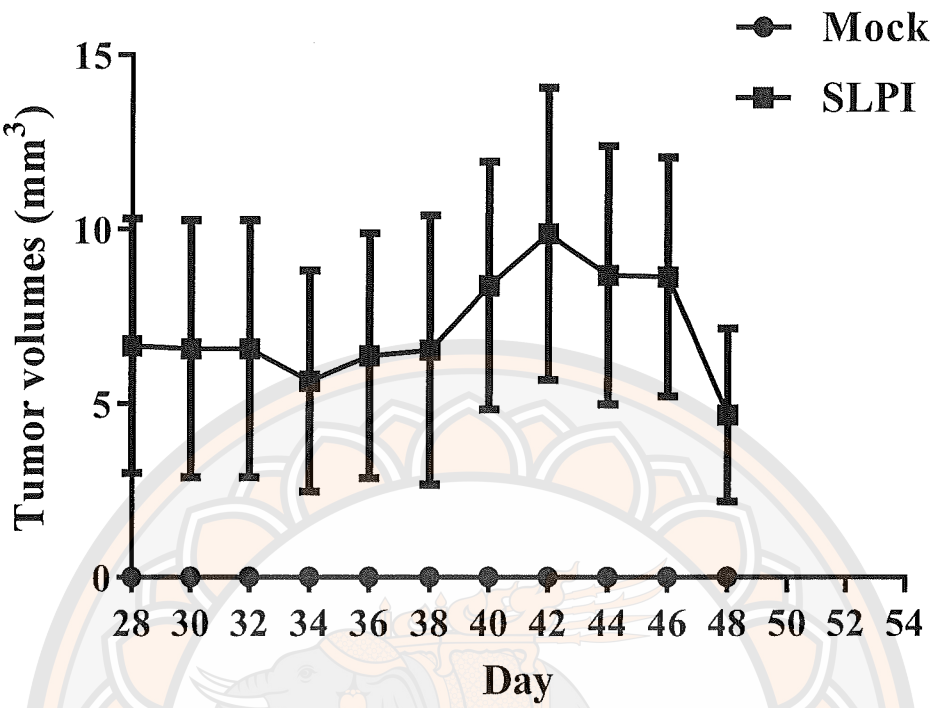
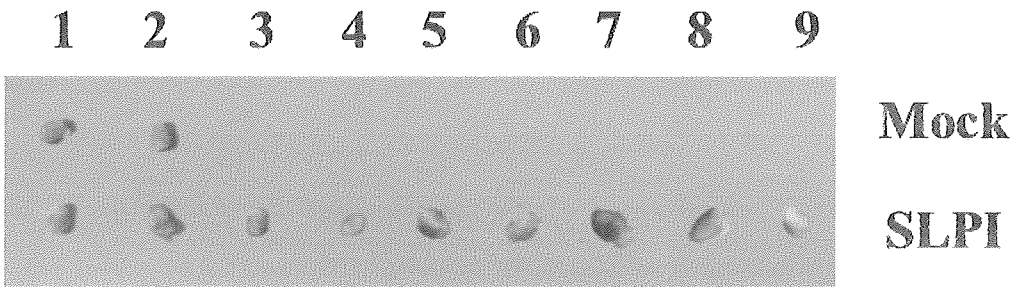


Figure 30 Effects of SLPI-overexpressing cells on tumorigenicity by xenograft mice tumor formation assay. SLPI cells were subcutaneous injection in BALB/cAJcl-nu mice, the tumor volume was measured every 2 days following the first injection, the data are presented as the mean ± SEM of SLPI (n=12) compared with Mock cells (n=10) using student's unpaired t test

A



B

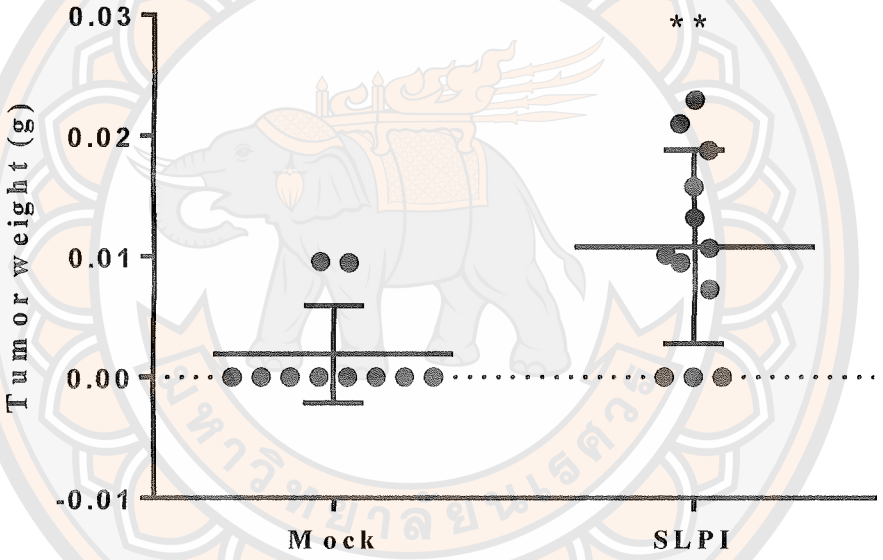


Figure 31 Effects of SLPI-overexpressing cells on tumorigenicity by xenograft mice tumor formation assay. SLPI cells were subcutaneous injection in BALB/cAJcl-nu mice, A: the number of tumors measured 48 days following the first injection, B: the tumor weight of SLPI cells subcutaneous injection groups (n=12) compare with Mock cells injection groups (n=10). The data presented as the mean ± SEM, student's unpaired t test **$p < 0.01$

CHAPTER V

CONCLUSION

Discussion

Cholangiocarcinoma is a malignant tumor with a high metastasis. Since the patient does not exhibit symptom in early stage and there is no specific diagnostic method, it can spread to other important organs of the body results in a high mortality rate of the patients. Therefore, understanding the CCA mechanism may lead to find a novel molecular target for early detection of CCA. It is well known that, the major cause of CCA in Thailand is the repeated liver fluke infections that caused chronic inflammation and lead to carcinogenesis of CCA. Thus, the particular molecules that respond to the inflammatory process and promote the development of cancer may be used as a biological indicator for early diagnosis of CCA. Secretory leukocyte protease inhibitor (SLPI) is an inflammatory response molecule. It is responsible for controlling the balance of protease that may lead to inflammation (Monika et al., 2016). SLPI inhibits serine proteases and many other molecules involved in inflammation. In addition, SLPI also helps to recover the tissue in the inflammation area returned to normal. Although SLPI is primarily responsible for regulating the protease balance, excessive SLPI protein expression affects the pathology of the disease caused by inflammation, for example cancers. Previous reports found that SLPI has high expression in many cancers such as ovarian cancer, liver cancer and, lung cancer (Cheng et al., 2008). In lung cancer, the SLPI play an important role in promoting cancer formation in the early stages (Jan Treda, C. et al., 2013). The recent study of SLPI in CCA found that SLPI expression was correlated with the CCA progression and play an important role in the metastatic process of CCA (Jantra, J. et al., 2017). However, understanding the role of SLPI in an early step is still not known. Here, we demonstrated that SLPI expression was increased during CCA carcinogenesis which were analyzed from the tissues of hamster-induced CCA models. Moreover, SLPI was up-regulated under mimicking the inflammation contributing the tumorigenesis of CCA. In addition, SLPI-overexpressing MMNK-1 cell significantly increased the tumorigenicity *in vitro*

and *in vivo*. Therefore, SLPI plays an important role at the early step of CCA development. These data provided the possibility that SLPI can be used as a biological marker for an early diagnosis of CCA.

1. The expression of SLPI proteins in hamsters induced CCA tissues

Normally, SLPI was found as a product that secreted in body fluids from the cells in the lining of the area, such as epithelial cells under inflammatory response caused by various infections. (Grobmyer, S. R. et al., 2000). SLPI can be found around 26.1 to 65.0 ng/ml in serum, 0.4 to 250 ng/ml in bronchial lavage fluid (Hollander et al. 2007), 0.58 to 2.82 pg/ml) in exhaled breath condensate (Tateosian, N. L. et al. 2012) and 0.3 to 3.2 ug/ml in saliva (Shugars, D. C. et al. 2001). However, the concentration of the SLPI molecules varies according to the age and sex of each person tested. In the present study, SLPI expression was investigated during carcinogenesis which induced by *Ov.* and NDMA. Our data showed that (Figure 21) the SLPI expression was expressed at very low level in hamster normal bile duct area. During cholangiocarcinogenesis, the SLPI expression was up-regulated when the normal tissue becomes to hyperplasia and dysplasia in 1-month groups of CCA-induced hamsters. The previous report has been demonstrated that during the transforming tissues, some inflammatory cytokines such as IL-17, NF-κB, and CD4 were up-regulated, it can be concluded that in 1-month groups of CCA-induced hamster has inflammation states (Wongsena, W. et al., 2017). Moreover, the results in 1-month groups of CCA-induced hamsters has been found the immune cells in HD area that are overlapping with the SLPI expression. It was well known that SLPI has main function to inhibit elastase activity form immune cells (Stetler, G., et al, 1986). These results suggested that increasing of SLPI expression could be induced by an inflammatory process according to their protease inhibitor activity. Thus, SLPI could be detected in an early event of CCA. Interestingly, in 3-month groups of CCA-induced hamster which is the tissue entering to cancerous stage, SLPI was significantly up-regulated compared with the bile duct tissues. These finding was clearly showed that SLPI is not only the inflammatory response molecules, but SLPI levels were sustainable until CCA stage. Therefore, SLPI could be used as a biomarker of an early stage of CCA. In addition, the relationship between SLPI protein expression levels and different stage of hamster induced-CCA was a positive correlation with CCA development. These results are consistent with

previous reports that SLPI expression levels significantly elevated in ovarian cancer patients compared with counterparts with benign cyst patients and healthy women (Tsukishiro, S. et al., 2005). Moreover, SLPI expression f have been demonstrated in different CCA cell lines including of MMNK-1; the immortalized cholangiocyte, KKU213A; the well-differentiated adenocarcinoma cell, KKU213B and KKU213C; the moderately-differentiated adenocarcinoma cell, KKU-055 and KKU-100; the poorly differentiated adenocarcinoma, KKU-213AL5 and KKU-213BL5; the highly metastatic CCA cells. The results found that SLPI expression levels has highly expressed in CCA cells compared with normal cholangiocyte and more increased in highly metastatic CCA cells that correlated with aggressiveness of CCA (figure 32 Chomhom, P. 2017 unpublished data). Therefore, the relationship between SLPI protein expression levels and different stage of hamster induced-CCA was a positive correlation with CCA development. We hypothesize that SLPI is a molecule to response on inflammation in early stage of CCA and tumorigenic factor to promote CCA development. To prove the hypothesis, we need to stimulate inflammatory condition and examine SLPI expression levels in CCA cell lines.

Figure 32 The expression of SLPI in various CCA cell lines determined by western blot assay

Source: Chomhom, P., 2017 (unpublished data)

2. Mimicking the inflammatory condition by IL-6 treatment

It is well known that CCA caused by repeated liver fluke infections undergo chronic inflammation and eventually lead to CCA. Thus, the mimicking early step of cholangiocarcinogenesis, *Ov.* excretory/secretory products (*Ov*Es) was used to stimulation. Our preliminary data found that *Ov*Es treatment in CCA cells couldn't observed the up-regulation of SLPI expression and tumorigenicity as showed in figure 33. This result was due to the limitation of the *Ov*Es efficacy such as the unconstant of protein concentration of *Ov*Es obtaining from Khon Kaen University. Therefore, we decided to change another stimulator involved in *Ov.* infection instead of *Ov*Es. Previous studies have stimulated liver fluke infection by treatment the secretions from liver fluke *Ov*Es into normal bile duct cells compared with CCA cells and other types of cancer cells. They found that in the group of normal bile duct and CCA cells was responded to *Ov*Es by increasing of the production of the IL-6, an inflammatory response molecule and a marker of the chronic inflammatory process (Chaiyadet, S. et al., 2015). The levels of IL-6 are markedly elevated in patients with cholangitis (Van, S. J. et al., 1990). These data implied that the IL-6 is a specific molecule produced by the response from the liver fluke infection of CCA. Therefore, in the present study simulated inflammation caused by liver fluke infection by using IL-6 as a stimulant. To confirm that cellular inflammation is occurring, we investigated the signaling pathway through the activation of NF-κB pathway to ensure the inflammation occurred. NF-κB pathway is a transcription factor that regulates inflammatory responses. NF-κB activity is regulated by a family of IκBs proteins, in their inactivated state, NF-κB complexes composed of p65/p50 are localized in the cytoplasm, in complex with IκB kinase (IKKs). But in the activated pathway, activated IKKb phosphorylates IkBs which is subsequently ubiquitinated and marked for degradation by proteases. The release of NF-κB from the IkB-containing complex will then translocate to the nucleus where it can activate the transcription of its target genes in phosphorylation p65 (Hayden, M. S. et al., 2008), (Karin, M. et al., 2000). Our finding suggests that IL-6 can stimulate the expression of phosphorylation p65 (P-p65) which is an activated form, as a result of an inflammatory response. Consistent with previous report that IL-6 can cause inflammation response through NF-κB activation. IL-6 will cause IkB to transform activated IKKb phosphorylates IkBs, causing NF-κB to change to P-p65 form and

translocate to the nucleus work as a transcription factor activate the target genes resulting in an inflammatory response (Zheng, X. et al., 2015). These results suggest that the mimicking of inflammatory condition was successfully by IL-6 treatment induced NF-κB activation pathway and could be used to study association of SLPI expression levels with inflammatory condition of CCA in early stage.

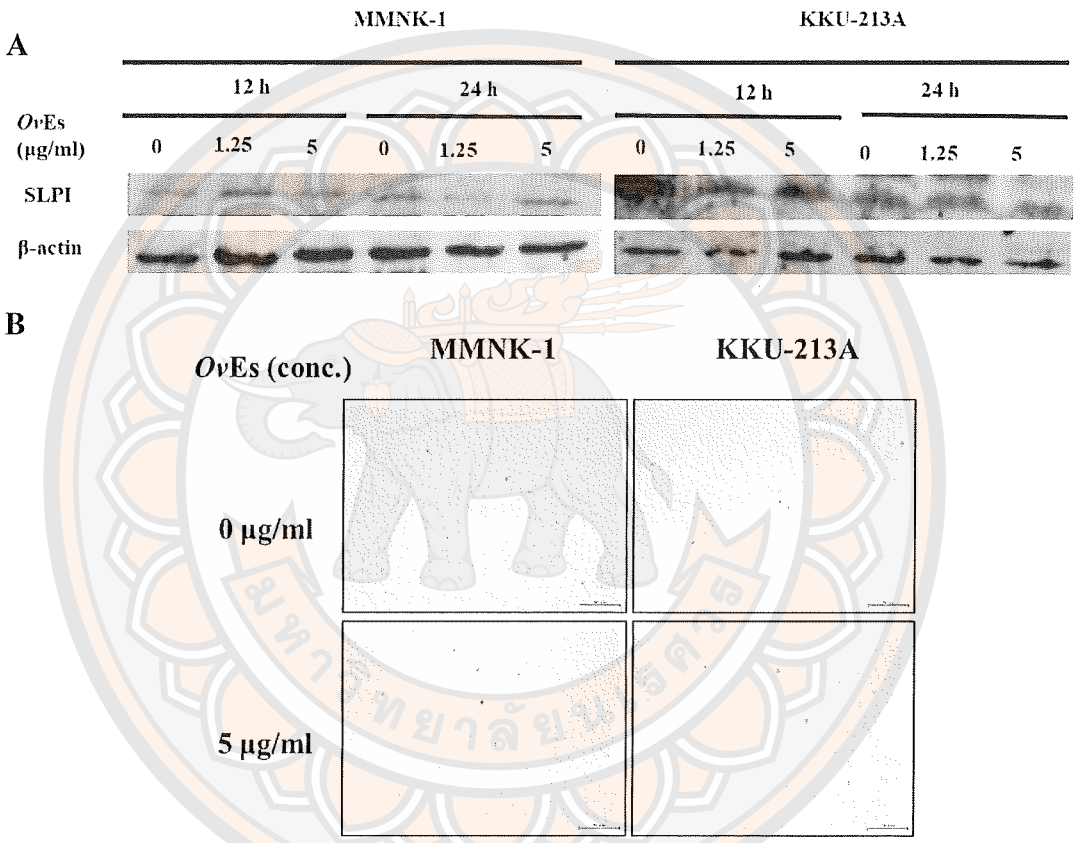


Figure 33 The effects of *Ov*Es treatment in CCA cell lines on SLPI expression and tumorigenicity. A: MMNK-1 and KKU-213A were treatment with 0, 1.25, and 5 μg/ml of *Ov*Es at 12 and 24 hours, investigated SLPI expression by western blot assay. B: MMNK-1 and KKU-213A were treatment with 0 and 5 μg/ml of *Ov*Es at 24 hours, determined tumorgenicity by soft agar colony formation assay

3. IL-6 treatment increased the expression of SLPI in CCA

Secretory leukocyte protease inhibitor is a serine protease inhibitor that response during the inflammatory process. SLPI has been involved in the NF-κB signaling pathway Therefore, the anti-inflammatory SLPI proteins helps to maintain the integrity of the surrounding tissues. In this study, inflammation caused by IL-6 treatment induced NF-κB activation pathway. Our data showed that SLPI expression level increases upon IL-6 treatment. It was also consistent with previously reported in white blood cells, the expression of SLPI increases after treatment with IL-6 (Jin, F. et al., 1998). The higher SLPI expression level is due to the higher NF-κB pathway activation. Previous report found that SLPI was able to inhibit the ubiquitin-proteasome process of IkB degradation, causing NF-κB inactivation and reduced the inflammatory responses (Taggart, C. C. et al., 2002). In other words, increasing NF-κB activation also results in increased SLPI expression in regularity to balance the inflammation process. In addition, after mimicked inflammatory condition of CCA in early-stage by IL-6 treatment, SLPI expression level was significantly increased. Moreover, IL-6 is a molecule that response to liver fluke infections and cause CCA (Chaiyadet, S. et al., 2015). Similar to our previous data of hamsters induced CCA tissues in 1-month groups showed inflammation states caused by *Ov.* infection and highly SLPI expression, the data in this part revealed that SLPI is an inflammatory response molecule from *Ov.* infection, which is the first step of cholangiocarcinogenesis. In addition, our previous data of hamsters induced CCA tissues in 3 and 6-month groups has higher SLPI expression together with CCA development. We hypothesize that SLPI is a tumorigenic factor to promote CCA tumorigenesis.

4. IL-6 treatment promotes *in vitro* tumorigenesis in CCA

The different growth properties of normal cells and cancer cells allow us to distinguish between the two types of cells. One of these is the anchorage dependence, which is a growth property that is necessary for the area of adhesion for normal cell growth. Comparing the growth between normal cells and cancer cells in a semi-solid state, normal cells require the adhesion area to signal the growth, so when cultured in such conditions the cells cannot grow. This is contrast with cancer cells that have an anchorage independence, allowing them to thrive and become colony in a semi-solid condition (Borowicz, S. et al., 2014). In this study, we hypothesized that SLPI

overexpression after the mimicking of inflammation by IL-6 treatment can promote the ability of cells to anchorage independence features or tumorigenicity. Our results reveal that IL-6 treatment significantly increased tumorigenicity of MMNK-1 and KKU-213 but the strongest result was observed in MMNK-1 rather than KKU213 cells. This may due to the endogenous expression level of SLPI in MMNK-1 was very little when compared with the KKU-213 cells (Figure 14). The previous study of SLPI in ovarian cancer cells demonstrated the increased anchorage independence after receiving the higher SLPI (Devoogdt, N. et al., 2009). Therefore, it corresponds to our study that IL-6 treated cells that has been a higher SLPI expression also has higher tumorigenicity. As previously reported the effects of IL-6 that can stimulate tumorigenicity of prostatic epithelial cells, that IL-6 treated cells enhance prostate carcinogenesis *in vitro* by stimulating the growth of transformed cells (Okamoto, M. et al., 1998). From our results, we can conclude that IL-6 induced SLPI expression promoted tumorigenicity in early stage. However, we could not ensure that the SLPI play an important role in tumorigenesis. To prove this hypothesis, we have to determine the effects of SLPI on tumorigenicity by increasing the expression of SLPI only one molecule in MMNK-1 cells using SLPI overexpressing cell models.

5. The effects of SLPI overexpressing cells on tumorigenicity *in vitro* study

SLPI-overexpressing cells were previously established by Jantra J in our laboratory. In this study, the expression level of SLPI in SLPI and Mock cells has been validated. The results confirmed with previous data that SLPI cells were significantly increased compared with Mock cells. Therefore, the cells were suitable for the further investigation about the effect of SLPI on tumorigenicity.

The results showed that SLPI-overexpressing cells has significantly increased tumorigenicity when compared with Mock cells as the increasing of the number of colonies in soft agar colony formation assay. These results clearly demonstrated that SLPI is the essential molecules that functions in tumorigenesis of CCA. From the results in Figure 29, we obtained the data that SLPI-overexpressing cells increased tumorigenesis. This effect was caused by the increasing of SLPI expression for 5-fold when compared with the control (Mock cells). In contrast, the tumorigenicity under inflammation by IL-6 treatment was increased just about 2-fold when compared with the control. Although the expression levels of SLPI under inflammation was much

larger than in the overexpression model. These data suggested that SLPI is a molecule that play an important role in tumorigenesis of CCA. Moreover, it was consistent with previous reported in neck squamous cell carcinoma that upregulation of SLPI in contribute to increase tumorigenicity (Dasgupta, S. et al., 2006), and consistent with previous reported in ovarian cancer cell lines that SLPI-overexpression independently of PI (protease inhibitor) activity increase in tumorigenicity property (Devoogdt, N. et al., 2009). However, in this study, we investigated the effect of SLPI overexpression in the model the immortalized MMNK-1 cells which is similar phenotypes with the normal cell as anchorage dependent growth. Our results demonstrated that after the cells were gained SLPI expression, it can sufficiently transform the phenotype of anchorage dependent to anchorage independent growth as demonstrated by increasing of the number of colonies in soft agar colony formation assay. These results suggested that SLPI is the key molecules of tumorigenesis in CCA. Our finding was the first report to show that increasing of SLPI protein in immortalized cholangiocytes could enough to promote tumorigenesis. To confirm SLPI could enough to transform the phenotypes of normal to cancer. We further investigated the effects of SLPI on tumorigenesis using xenograft mice models.

6. The effects of SLPI overexpressing cells on tumorigenicity *in vitro* study

Xenograft mice tumor growth assay is a method for determining the tumorigenicity in *in vivo* study. This study showed that animals in the SLPI-overexpressing cells injected group has increased tumorigenicity more than the control groups. Our data was extremely surprised, since previous research demonstrated that the tumorigenicity of MMNK-1 cells that are simply immortalized cells. They have the same properties as normal cells and the ability to grow unlimitedly. However, the MMNK-1 cells could not form tumor in animals and other properties similar to cancer cells (Maruyama, M. et al., 2004). Our results showed that SLPI-overexpressing MMNK-1 cells were subcutaneously injected in to the mice and could be able to form the solid tumors after 28 days for 11 tumors while 2 tumors were observed in the group of control Mock cells. These results suggested the role of SLPI in tumorigenesis of CCA. However, there are some different points between our model and previous report that could be affected to the results and should be concerned. Firstly, the different of animal model used in this study and previous study of MMNK-1 on tumorigenicity (Maruyama,

M. et al., 2004), our model used six different severe combined immunodeficiency (SCID) mice while the previous one used BALB/cAJcl-nu mice. The different mice strains can interfere the results. Secondly, although MMNK-1 is not cancer cells, it should be lack of tumorigenesis as cancer cells, but MMNK-1 cells are simply immortalized cells, which properties change to normal cells in the ability to grow unlimitedly. This reason may cause MMNK-1 cells can form a few tumors in control group.

The previous reported in lung cancer that C57BL/6 mice were inhibited SLPI expression as SLPI-KO mice and lung cancer was induced with Urethane. The results showed that the SLPI-KO mice was significantly lower than the control group. These data demonstrated that "SLPI plays an important role from the initial stages of lung carcinogenesis to the progression of lung cancer." (Jan Treda, C. et al., 2013). Our data was consistent with this work that SLPI promotes tumorigenesis. However, in our model was not include the carcinogen for CCA such as *Ov.* or NDMA but we can observe the significant role of SLPI on tumor formation both *in vitro* and *in vivo* studies. Our finding are very strong evidences to demonstrated the critical role of SLPI in tumorigenesis of CCA. Currently, we don't not know yet how SLPI involved in tumorigenesis, but we propose the mechanism of SLPI to promote tumorigenicity. SPLI might be regulated tumor growth via controlling many molecules such as a transcription factors FoxM1 (Lance L. et al., 2018). Previously report found that SLPI can directly bind with the RB molecule which is partner of FoxM1 results to FoxM1 activation and then turn on several target genes associated to tumor growth and metastasis (Kozin, S. V. et al., 2017). In CCA, the IHC analysis of the FoxM1 expression revealed a strong correlated upregulation of FoxM1 (78%) expression at the protein levels in CCA tissues (Intuyod, K. et al., 2018). Moreover, SLPI promoting cell proliferation could be due to cyclin D1 upregulation (Sugimachi et al., 2001). Therefore, we suggesting that SLPI is a molecule that is necessary and important to promote cancer development in animal models.

Perspective experiments

The expression level of the SLPI protein is associated to the severity of CCA, especially in the carcinogenesis process. the study in hamster-induced CCA tissues has provided preliminary results that the expression levels of the SLPI were correlated with the aggressiveness of CCA development. Previous report showed SLPI expression levels in serum could be useful for differentiating benign ovarian cysts from malignancies and to improve the specificity of diagnosis (Tsukishiro, S. et al., 2005). Hence, we should demonstrate the serum SLPI expression levels in patients prior entering CCA (the liver fluke infection stage, chronic inflammation stage, stage I, and, II) and CCA metastasis (stage III and IV) compared with normal people, each population group has an expression level of serum SLPI protein by ELISA. Another, carbohydrate antigen 19-9 (CA19-9) is a recent tumor marker of CCA (Perkins, G. et al., 2003), it is also a tumor marker many types of gastrointestinal cancer, such as colorectal cancer and hepatocellular cancer (Goonetilleke, K. S. et al., 2007), which is not specific marker to early CCA. Hence, investigation the relationship of CA19-9 and SLPI expression can be distinguish between CCA and many types of gastrointestinal cancer. These results may support the SLPI could be used as novel biomarker for early diagnosis and can reduce mortality in CCA patients.

Moreover, the mechanisms of SLPI are also associated with inflammatory processes, which is the first process of CCA developing caused by repeated infections from *Ov.* Our results showed the SLPI protein is a molecule that response to inflammation and promotes the tumorigenesis of CCA. Therefore, inhibiting the expression and activity of SLPI proteins may be also inhibit the carcinogenesis and metastasis of CCA. For example, miRNA525 suppress prostate cancer metastasis via regulate SLPI expression (Yang, Z. et al., 2020). However, the SLPI is still an important molecule in the inflammatory response process of the body. Inhibition of SLPI activity may not have a positive effect on the body. Since SLPI is a molecule that can control inflammation in the body to a balanced state. Another, inhibition of SLPI activity in other domain are not WAP II domain, which is PI domain of SLPI has a function to inhibit protease activity form inflammatory process (Weldon, S. et al., 2009). it also another way for suppressed cancer that correlate with inflammatory response process of SLPI activity. Therefore, controlling the expression level of SLPI protein to have the

appropriate expression may be a good solution to use as a biological marker. Finally, the study an in-depth mechanism and the expression level of SLPI in other diseases needs to be used to provide additional information to confirm that SLPI molecules can be used as biological indicators for detection can be diagnosed in the early stages of CCA patients

Conclusion

In conclusion, SLPI has high expression due to the development of CCA, with high expression in early stage compared with normal bile duct cells, and more highly expressed in the CCA, and showed the highest expression in the malignant CCA with statistically significant. These data indicate that SLPI is related to carcinogenesis of CCA in the hamster-induced CCA model. The role of the SLPI *in vitro* study found that SLPI is a molecule that response to the inflammation stage, which is the first step in the development of CCA, and the high expression of SLPI in response to the process also promotes tumorigenicity. In addition, the overexpression of SLPI in the immortalized cholangiocyte contributes to the tumorigenicity in both *in vitro* and *in vivo* study. Therefore, SLPI plays an important role in promoting the development of CCA. These findings suggest that SLPI is a tumorigenic factor that drives the transforming phenotypes of cholangiocyte toward CCA and could be used as a novel target for early diagnosis.

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APPENDIX

Table 2 The expression levels of SLPI protein in hamster-induced CCA tissues by immunohistochemistry

CCA-induced in hamster (*Ov.*+ *NDMA*)	SLPI expression levels (H score.)									Mean	SEM.
	1	2	3	4	5	6	7	8	9		
Control	0	20	0	60	20	20	20	-	-	22.50	20.30
1 M	80	150	30	80	30	100	120	60	30	75.56	42.75
3 M	130	120	285	100	-	-	-	-	-	158.75	85.08
6 M	140	80	160	300	200	-	-	-	-	176	81.73

Table 3 The levels of p-p65 expression after mimicked chronic inflammation with IL-6 treatment in MMNK-1 and KKU-213A cells by western blot assay. The results were obtained from three separate experiments, each done in triplicates

Cells	Times	Interleukin 6 (0 ng/ml)			Mean	SEM.
		1	2	3		
MMNK-1	12 h	1.00	1.00	1.00	1.00	0.00
	24 h	1.00	1.00	1.00	1.00	0.00
KKU-213A	12 h	1.00	1.00	1.00	1.00	0.00
	24 h	1.00	1.00	1.00	1.00	0.00
Cells	**Times**	**Interleukin 6 (1 ng/ml)**			**Mean**	**SEM.**
		1	**2**	**3**		
MMNK-1	12 h	2.39	1.77	2.27	2.14	0.33
	24 h	1.17	1.47	1.18	1.27	0.17
KKU-213A	12 h	0.78	1.71	1.56	1.35	0.50
	24 h	3.17	0.56	1.00	1.58	1.40
Cells	**Times**	**Interleukin 6 (5 ng/ml)**			**Mean**	**SEM.**
		1	**2**	**3**		
MMNK-1	12 h	2.59	2.99	2.27	2.62	0.36
	24 h	1.50	3.05	1.26	1.93	0.97
KKU-213A	12 h	1.03	0.68	2.24	1.31	0.82
	24 h	5.51	0.46	1.08	2.35	2.75
Cells	**Times**	**Interleukin 6 (10 ng/ml)**			**Mean**	**SEM.**
		1	**2**	**3**		
MMNK-1	12 h	4.87	6.23	2.66	4.59	1.80
	24 h	1.42	3.27	0.98	1.89	1.21
KKU-213A	12 h	1.14	3.16	3.55	2.62	1.30
	24 h	4.24	0.51	1.10	1.95	2.00

Table 4 The levels of SLPI expression after mimicked chronic inflammation with IL-6 treatment in MMNK-1 and KKU-213 cells by western blot assay. The results were obtained from three separate experiments, each done in triplicates

Cells	Times	Interleukin 6 (0 ng/ml)			Mean	SEM.
		1	2	3		
MMNK-1	12 h	1.00	1.00	1.00	1.00	0.00
	24 h	1.00	1.00	1.00	1.00	0.00
KKU-213A	12 h	1.00	1.00	1.00	1.00	0.00
	24 h	1.00	1.00	1.00	1.00	0.00
Cells	**Times**	**Interleukin 6 (1 ng/ml)**			**Mean**	**SEM.**
		1	**2**	**3**		
MMNK-1	12 h	2.89	2.39	2.46	2.58	0.22
	24 h	1.97	1.24	1.26	1.49	0.34
KKU-213A	12 h	0.96	1.23	1.77	1.32	0.33
	24 h	1.36	1.60	1.38	1.44	0.11
Cells	**Times**	**Interleukin 6 (5 ng/ml)**			**Mean**	**SEM.**
		1	**2**	**3**		
MMNK-1	12 h	5.47	5.82	5.33	5.54	0.20
	24 h	3.50	3.14	4.24	3.63	0.46
KKU-213A	12 h	1.05	2.00	1.21	1.42	0.41
	24 h	1.33	2.16	0.49	1.32	0.68
Cells	**Times**	**Interleukin 6 (10 ng/ml)**			**Mean**	**SEM.**
		1	**2**	**3**		
MMNK-1	12 h	4.42	3.40	5.88	4.56	1.02
	24 h	3.10	3.94	5.43	4.16	0.97
KKU-213A	12 h	0.44	2.91	1.20	1.52	1.03
	24 h	1.11	1.33	0.21	0.88	0.48

Table 5 The number of colonies and colony formation rate after mimicked chronic inflammation with IL-6 by soft agar colony formation assay. The results were obtained from three separate experiments, each done in triplicates

Cells	Interleukin 6 (0 ng/ml) Number of colonies			Mean	SEM
	1	2	3		
MMNK-1	85.67	99.33	119.67	101.56	17.11
KKU-213A	83.33	74.33	67.67	75.11	83.33
Cells	**Interleukin 6 (10 ng/ml) Number of colonies**			**Mean**	**SEM**
	1	2	3		
MMNK-1	191.00	156.33	265.67	204.33	33.74
KKU-213A	148.33	228.00	141.67	148.33	28.10
Cells	**Interleukin 6 (0 ng/ml) Colony formation rate (%)**			**Mean**	**SEM**
	1	2	3		
MMNK-1	100.00	100.00	100.00	100.00	0.00
KKU-213A	100.00	100.00	100.00	100.00	0.00
Cells	**Interleukin 6 (10 ng/ml) Colony formation rate (%)**			**Mean**	**SEM**
	1	2	3		
MMNK-1	222.96	157.38	222.01	200.78	37.59
KKU-213A	178.00	306.73	209.36	231.36	67.12

Table 6 The number of colonies and colony formation rate on SLPI-overexpressing cells by soft agar colony formation assay. The results were obtained from three separate experiments, each done in triplicates

Cells	Number of colonies			Mean	SEM
	1	2	3		
MMNK-1	21.00	18.00	19.50	19.50	1.50
Mock	21.33	39.67	30.50	30.50	9.17
SLPI	126.00	102.00	114.00	114.00	12.00
Cells	**Colony formation rate (%)**			Mean	SEM
	1	2	3		
MMNK-1	100.00	100.00	100.00	100.00	0.00
Mock	101.59	120.37	129.02	116.99	14.02
SLPI	600.00	566.67	552.94	573.20	24.20

Table 7 Weight of Mock cells injection in BALB/cAJcl-nu mice by xenograft mice tumor formation assay

Mock cells injection	weight (g)								Mean	SEM.
Day	1	2	3	4	5	6	7	8		
2	23.02	23.93	22.57	22.68	24.22	22.72	24.13	-	20.41	0.74
4	24.03	25.52	22.37	23.20	24.66	23.26	24.40	-	20.93	1.06
6	26.72	24.32	23.07	24.51	25.34	23.63	24.56	-	21.52	1.18
8	27.46	24.73	23.61	25.04	25.80	24.01	24.73	-	21.92	1.27
10	28.48	24.94	24.07	25.08	26.35	24.60	25.11	-	22.33	1.48
12	28.64	24.74	24.60	25.31	25.63	24.68	25.15	-	22.34	1.42
14	28.20	25.37	25.30	25.54	26.29	24.56	25.78	-	22.63	1.16
16	27.09	25.28	25.33	24.83	27.43	25.86	28.03	-	22.98	1.24
18	27.01	25.12	25.19	24.40	27.05	25.82	28.30	-	22.86	1.37
20	27.48	25.29	24.52	25.54	27.02	25.46	28.23	-	22.94	1.36
22	27.60	25.16	24.22	25.96	26.30	25.61	28.28	-	22.89	1.40
24	28.45	25.24	24.88	26.55	26.20	26.09	27.15	-	23.07	1.20
26	28.64	25.84	25.17	26.55	26.61	26.86	27.33	-	23.38	1.10
28	28.36	25.08	25.03	26.70	26.60	26.73	26.48	-	23.12	1.13
30	27.84	25.18	24.28	26.66	26.18	25.88	25.86	-	22.74	1.12
32	26.91	24.91	24.38	26.68	27.36	26.78	24.82	-	22.73	1.22
34	27.42	25.58	25.21	27.05	27.67	26.73	26.40	-	23.26	0.92
36	26.98	25.47	25.32	26.95	27.79	26.25	26.71	-	23.18	0.88
38	27.26	25.50	24.07	27.42	27.59	25.69	28.37	-	23.24	1.51
40	27.90	25.36	25.14	27.14	27.68	26.00	27.80	-	23.38	1.19
42	28/64	26.20	26.12	28.08	27.38	26.04	27.81	-	23.09	0.93
44	27.96	25.72	25.00	27.49	27.55	26.00	27.44	-	23.40	1.14
46	28.54	26.35	25.34	27.67	27.88	26.75	28.04	-	23.82	1.12
48	28.76	25.78	25.67	28.14	28.24	26.46	27.57	-	23.83	1.25

Table 8 Weight of Mock cells injection in BALB/cAJcl-nu mice by xenograft mice tumor formation assay

SLPI cells injection	weight (g)								Mean	SEM.
Day	1	2	3	4	5	6	7	8		
2	20.16	24.22	23.22	23.17	24.27	24.34	23.89	24.06	23.42	1.39
4	20.86	26.01	24.48	24.95	23.20	23.50	23.14	22.96	23.64	1.54
6	23.18	28.72	26.12	26.41	25.22	25.50	24.64	24.05	25.48	1.69
8	23.61	29.09	26.66	26.59	26.63	26.13	25.62	25.39	26.22	1.54
10	23.70	30.08	27.63	27.73	27.76	27.62	25.71	26.50	27.09	1.86
12	24.53	29.55	27.53	27.24	27.64	27.20	25.48	25.32	26.81	1.61
14	24.59	29.56	27.78	27.94	26.11	26.22	25.76	25.21	26.65	1.65
16	24.82	29.44	28.62	28.00	27.11	23.78	27.32	26.03	26.89	1.91
18	24.49	28.88	28.54	27.37	27.17	22.85	26.63	25.82	26.47	2.03
20	24.54	28.97	28.75	27.30	27.22	23.64	26.75	26.65	26.73	1.85
22	24.82	29.44	28.65	27.93	27.05	27.24	26.38	26.49	27.25	1.44
24	24.84	29.55	29.28	27.94	27.17	24.25	26.62	26.64	27.04	1.89
26	25.43	29.88	29.77	28.74	27.30	24.29	26.41	26.54	27.30	2.03
28	25.28	29.55	30.36	27.58	27.53	24.07	26.66	26.51	27.19	2.07
30	25.02	29.19	29.33	26.90	27.39	26.71	25.98	25.89	27.05	1.54
32	26.28	30.48	29.89	28.37	27.53	26.25	27.54	27.20	27.94	1.56
34	26.38	30.65	30.49	27.83	28.10	25.97	26.78	27.12	27.92	1.78
36	26.18	30.42	30.73	28.36	27.81	24.46	27.28	26.79	27.75	2.10
38	26.41	30.21	30.49	28.38	27.71	26.76	26.95	27.21	28.02	1.56
40	26.59	30.13	30.76	28.41	27.67	26.31	27.43	27.23	28.07	1.61
42	26.45	30.22	30.50	28.32	27.60	26.63	27.45	27.14	28.04	1.55
44	26.81	30.21	31.03	28.56	28.94	26.78	27.65	27.53	28.44	1.56
46	27.01	30.19	30.83	28.54	29.22	25.49	27.89	27.64	28.35	1.73
48	27.53	30.33	30.97	28.72	29.01	26.12	28.02	27.88	28.57	1.56

Table 9 Tumor volumes of Mock cells injection in BALB/cAJcl-nu mice by xenograft mice tumor formation assay

Mock cells injection	Tumor volumes (mm^3)										Mean	SEM.
Day	1	2	3	4	5	6	7	8	9	10		
28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
34	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
38	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
40	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
42	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
44	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
46	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
48	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Table 10 The tumor volumes of SLPI-overexpressing cells injection in BALB/cAJcl-nu mice by xenograft mice tumor formation assay

SLPI cells injection	Tumor volumes (mm^3)												Mean	SEM.
Day	1	2	3	4	5	6	7	8	9	10	11	12		
28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	42.59	25.33	32.00	7.69	6.66
30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	45.56	29.66	23.33	7.58	6.57
32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	45.56	23.33	29.66	7.58	6.57
34	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	39.75	25.33	19.65	6.52	5.65
36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	37.04	21.44	37.04	7.35	6.37
38	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	34.46	14.90	48.67	7.54	6.54
40	23.98	14.11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	37.04	13.50	37.04	9.67	8.38
42	14.90	23.98	0.00	0.00	0.00	0.00	0.00	0.00	0.00	51.91	25.33	32.00	11.39	9.87

Table 10 (Cont.)

SLPI cells injection	Tumor volumes (mm^3)												Mean	SEM.
44	27.44	19.65	0.00	0.00	0.00	0.00	0.00	0.00	0.00	45.56	12.19	25.33	10.01	8.68
46	27.44	17.97	0.00	0.00	0.00	0.00	0.00	0.00	0.00	37.04	19.65	27.44	9.96	8.64
48	23.33	23.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	23.33	5.38	4.67

Table 11 The tumor weight of Mock and SLPI-overexpressing cells injection in BALB/cAJcl-nu mice by xenograft mice tumor formation assay

Cells type injection	Tumor weight (g)												Mean	SEM.
	1	2	3	4	5	6	7	8	9	10	11	12		
Mock	0.01	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-	-	0.002	0.004
SLPI	0.01	0.02	0.01	0.01	0.02	0.02	0.02	0.01	0.01	0.00	0.00	0.00	0.013	0.008