CHAPTER I
INTRODUCTION

1.1 Background and rationale of the study

Cholangiocarcinoma (CCA), the malignant tumor arising from bile duct epithelia, is the common cancer and a major public health problem in northeast Thailand where the highest incidence of this cancer in the world has been reported (Vatanasapt *et al.*, 2002). The WHO database indicated a global increase in incidence of CCA (Shaib *et al.*, 2004) and CCA related mortality in Asia and worldwide (Patel, 2001). Liver fluke (*Opisthorchis viverrini*, Ov) infection is the major risk factor in Asia. The strong association of Ov and CCA has been reported in both epidemiological (Elkins *et al.*, 1996; Haswell-Elkins *et al.*, 1994) and experimental studies (Thamavit *et al.*, 1978). Although, the genesis of CCA is still unclear especially the association between Ov infection and tumor development. A previous study of Pinlaor and colleagues has been demonstrated that repeated Ov infection can induce the expression of inducible nitric oxide synthase (iNOS) in the bile duct epithelia and can subsequently cause nitrative and oxidative damage to DNA (Pinlaor *et al.*, 2004). Additionally, Thanan and coworkers (Thanan *et al.*, 2008) reported that the highest level of 8-oxodG, an oxidative DNA lesion, was observed in urine and leukocytes of CCA patients and the higher level of this adduct was observed in Ov-infected patients than in healthy subjects. Dechakhamphu and coworkers (Dechakhamphu *et al.*, 2008) recently demonstrated that urinary lipid peroxidation DNA adducts in Ov-infected subjects were significantly higher than in healthy controls and the adduct levels of the infected group were decreased after 2 months of a single dose of praziquantel treatment. Those particular evidences clearly supported that Ov infection and CCA in both hamster and human model are under chronic inflammatory process resulting in oxidation of DNA bases.

CCA is a slow progression tumor and difficult to diagnose until the disease becomes an advanced or metastatic stage, at which the prognosis is poor. Nowadays, curative surgical resection, radiotherapy and chemotherapy are the treatment options

of CCA patients (Khan *et al.*, 2005). However, the majority of patients undergoing curative resection develops recurrent disease at the surgical site and the overall survival is still poor (Shaib and El-Serag, 2004). Moreover, radiation therapy or current chemotherapy does not appreciably improve long-term survival rates (de Groen *et al.*, 1999). Therefore, CCA treatment remains challenging.

To better understand the molecular mechanism that underlies the carcinogenesis of Ov-associated CCA, Loilome and coworkers (Loilome *et al.*, 2006) had studied gene expression profile during carcinogenesis of Ov and *N*-nitrosodimethylamine (NDMA) induced CCA in hamster model. In this study, hamsters were infected with Ov metacercariae in combination with the intake of a carcinogen, NDMA. Candidate genes associated in CCA development were determined by fluorescence differential display-PCR (FDD-PCR) and verified by reverse northern blot analysis. It was found that 23 transcripts were upregulated whereas only one transcript was down regulated. Among them, protein kinase A regulatory subunit 1 alpha (PRKAR1A) was found to be overexpressed in liver bearing tumor when compared with normal liver and gall bladder epithelia, indicating the association of this gene with CCA development. Our recent study demonstrated that PKA isozyme switching from PRKAR2B/PKAII to be PRKAR1A/PKAI during Ov and NDMA induced hamster CCA development in correlation with transformation and proliferation of bile duct epithelia from normal through preneoplastic to malignant stages (Loilome *et al.*, submitted to J Biomed Science, under revision). Therefore, PKA isozyme switching and PRKAR1A/PKAI pathway might contribute to induction of biliary cell transformation and proliferation in Ov and NDMA-induced cholangiocarcinogenesis.

PRKAR1A is one of the regulatory subunits of protein kinase A type I (PKAI) which plays an important role in numerous cellular processes including the regulation of cell growth, gene transcription, and metabolism (Cho-Chung *et al.*, 1995). PKA is a heterodimer composed of two catalytic subunits (C) bound to a regulatory subunit (R) dimer. Cyclic AMP (cAMP) binds in cooperative manner to two sites on each regulatory subunit. Upon the binding of four molecules of cAMP, the enzyme dissociates into an R subunit dimer with four molecules of cAMP bound and two free active C subunits that can phosphorylate serine and threonine residues on specific

substrate proteins. The regulatory subunits, PRKAR1A or PRKAR2B, determine the isotype of human PKA (type I or type II, respectively) (Krebs, 1972). Human PKA isozyme switching due to the substantial changes in the proportion of the two PKA regulatory subunits, PRKAR1A/PKAI and PRKAR2B/PKAII, occur during oncogenic development, differentiation processes and neoplastic transformation (Cho-Chung, 1990; Tortora and Cho-Chung, 1990). PRKAR2B/PKAII preferentially expresses in normal nonproliferating tissues and in growth-arrested cells, whereas PRKAR1A/PKAI is overexpressed in growth stimulated cells (Cho-Chung *et al.*, 1995; Tortora and Ciardiello, 2002). The PRKAR1A/PKAI is transiently overexpressed in normal cells in response to physiological conditions of cell proliferation and mitogenic signaling , whereas the constitutively overexpressed has been reported in many cancer types both primary tumors and cancer cell lines such as ovarian (McDaid *et al.*, 1999), lung (Young *et al.*, 1995), colon (Bold *et al.*, 1994) and melanoma (Mantovani *et al.*, 2008).

Based on the different roles of PKA isozymes in the initiation and progression of many tumors, several attempts have made to suppress transformed phenotype of cancer cells by inhibiting PRKAR1A/PKAI. A group of site-selective cAMP analogues inhibited cell growth in several cancer cells such as colorectal cancer, breast cancer, lung cancer and thyroid cancer has been reported (Carlson *et al.*, 2000; Gu *et al.*, 2006; Ramage *et al.*, 1995; Robinson-White *et al.*, 2008). Moreover, antisense oligonucleotide (Nesterova and Cho-Chung, 1995; Tortora and Ciardiello, 2000) and also small interfering RNA (siRNA) (Farrow *et al.*, 2003) against PRKAR1A/PKAI could successfully induce growth inhibition in a variety of cancer cell lines both *in vitro* and *in vivo*. Apart from targeting PRKAR1A/PKAI as single, the combination treatments with other drugs have been more considerable in order to increase efficacy of cancer treatment. Using antisense PRKAR1A (Gem231) in combination with the cytotoxic drug, hydroxycamptothecin (HCPT) has shown synergistic effects of tumor cell growth inhibition and apoptosis induction in colon and prostate cancer cell lines (Cho and Cho-Chung, 2003). Treatment with a combination of sulfinosine and 8-Cl-cAMP resulted in synergistic effects on growth inhibition, cell cycle arrest, and induction of apoptosis of human neuroblastoma cell line (Jankovic *et al.*, 2006).

Inhibiting PRKAR1A/PKAI affects cell proliferation in varieties of tumors and cancer cell lines including CCA and is a potential target for cancer treatment, although the mechanism by which PRKAR1A/PKAI contributes to CCA development is still unclear. Therefore, this study aimed to investigate the molecular mechanism by which PRKAR1A/PKAI associated with human CCA development and elucidate whether PRKAR1A/PKAI is suitable to be the potential target for inhibiting CCA cell growth. The new knowledge in molecular mechanism of PRKAR1A/PKAI may give information for effective treatment for Ov- associated CCA in the future.

1.2 Research questions

1.2.1 Could PRKAR1A/PKAI be used as a target for inhibiting CCA cell growth?

1.2.2 What is (are) the molecular mechanism(s) by which PRKAR1A/PKAI regulating CCA cell growth?

1.2.3 Could small molecule inhibitors targeting PKA be used for CCA treatment?

1.2.4 Is there a synergistic and/or additive effect of targeting PRKAR1A/PKAI in combination with protein kinase inhibitors or chemotherapeutic drugs on CCA cell growth inhibition?

1.3 Scope of the study

We hypothesized that PRKAR1A/PKAI is suitable to be the potential target for inhibiting CCA cell growth and there is (are) specific molecular mechanism by which PRKAR1A/PKAI regulating CCA cell development. To prove this hypothesis, the study was performed in human CCA cell lines (Figure 1). Series of experiment were done stepwise as following:

1. To confirm PRKAR1A/PKAI expression in CCA cell lines. PKA subunits expression level in 4 CCA cell lines including KKU-M214, KKU-M156, KKU-OCA17 and KKU100 were determined using western blot analysis.

2. To elucidate whether PRKAR1A/PKAI is suitable to be the potential target for inhibiting CCA cell growth and investigate molecular mechanism by which PRKAR1A/PKAI regulating CCA cell growth, CCA cell lines which express high

PRKAR1A level were selected for RNAi experiment. After that, functional analysis including cell proliferation and apoptosis were analyzed. Moreover, cellular signaling network activated by PRKAR1A/PKAI was investigated using kinase array assay.

3. To study whether small molecule inhibitors targeting PKA can be used for CCA treatment, the effect of PKA inhibitors on CCA cell proliferation was determined.

4. To study the synergistic and/or additive effect of targeting PRKAR1A/PKAI in combination with other drugs including protein kinase inhibitors and chemotherapeutic drugs, the effect of combination treatment on CCA cell proliferation and apoptosis was determined.

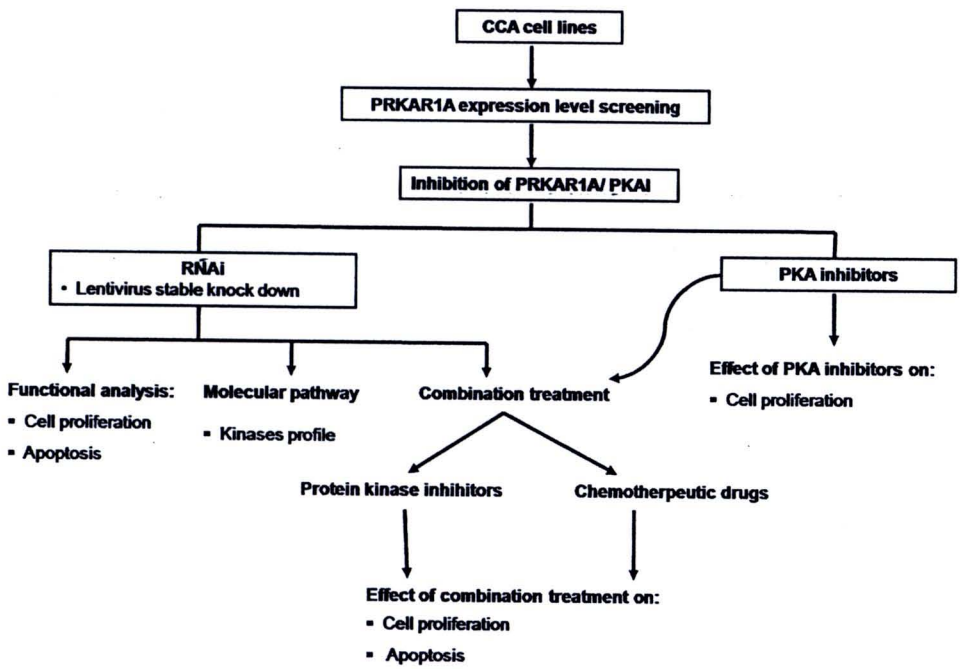

Figure 1.1 The scope of the study to prove the possible mechanism of PRKAR1A/PKAI in cholangiocarcinoma.

1.4 Conceptual framework

Gene expression profiles indicated that overexpression of PRKAR1A in the liver fluke *(Opirthorchis viverrini*, Ov) and *N*-nitrosodimethylamine (NDMA) induced hamster CCA tumors might have contributed to the induction of biliary cell transformation and proliferation in Ov and NDMA-induced progressive

cholangiocarcinogenesis (Loilome et al., 2006). However, the mechanism by which PRKAR1A/PKAI contributes in CCA development is not yet clear. Therefore, the present study was aimed at investigating possible molecular mechanism by which PRKAR1A/PKAI promotes human CCA development and determined if PRKAR1A/PKAI is a suitable target for inhibiting CCA cell growth.

RNA interference of PRKAR1A was used to explore the possible functions of PRKAR1A/PKAI in cell proliferation and apoptosis of CCA cell lines. Moreover, phosphorylation profiles of kinases and their protein substrates activated by PRKAR1A/PKAI were also investigated. In addition, the possibility to targeting PRKAR1A/PKAI as single and in combination with protein kinase inhibitors or chemotherapeutic drugs in order to increase the efficiency of CCA treatment was determined. The conceptual framework of this study is shown as following diagram.

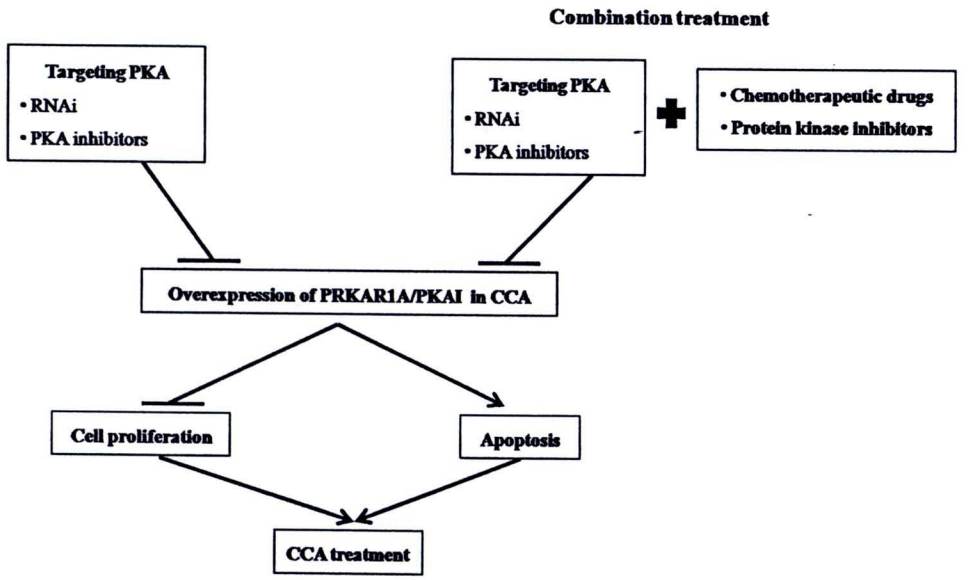

Figure 1.2 The conceptual framework of this study.

1.5 Objectives

1.5.1 To determine whether PRKAR1A/PKAI can be the potential target for CCAcell growth inhibition.

1.5.2 To elucidate the molecular mechanism by which PRKAR1A/PKAI contributes in CCA cell growth.

1.5.3 To determine whether small molecule inhibitors of PKA can be used for CCA treatment.

1.5.4 To determine the synergistic and/or additive effect of combination therapy consisting of shPRKAR1A knockdown or small molecule inhibitors of PKA with chemotherapeutic drugs or protein kinase inhibitors on CCA cell growth inhibition.

According to the scope of study provided in section 1.3 taken together with our experimental results that PRKAR1A/PKAI may play role in CCA development by enhancing cell growth and targeting PRKAR1A/PKAI as single or combination may be benefit for CCA treatment. This thesis is divided into two main parts as:

Part I: PRKAR1A is overexpressed and represents a therapeutic target in human cholangiocarcinoma.

Part II: Antiproliferative effect induced by targeting of PRKAR1A/PKAI in combination with chemotherapeutic drugs or protein kinase inhibitors on human cholangiocarcinoma cell lines.

1.6 Statistical analysis

All results were reported as mean ± SD and the differences between control and test groups were tested using Student's *t* test. Results were considered statistically significant between control and test treatment at the level of $P < 0.05$.

1.7 Location of research conduction

Experimental and analytical processes were performed at (i) at Departments of Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand (ii) Department of Neurosurgery, School of Medicine, Johns Hopkins University, Baltimore, Maryland, USA.

1.8 Anticipated outcomes

The present work may help to explain the possible molecular mechanism by which regulating CCA cell growth especially the role(s) PRKAR1A/PKA and its signaling pathway. This information could help to determine if PRKAR1A/PKA is a suitable target for inhibiting CCA cell growth which may lead to better effectively CCA treatment in the future.