รายงานวิจัยฉบับสมบูรณ์

การค้นหากลไกการดื้อยาเคมีบำบัดของเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีในหลอดทดลอง

Investigation of the mechanistic effect of chemotherapeutic drug resistance on human cholangiocarcinoma cell lines *in vitro*

โดย

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ผู้วิจัย

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สังกัด

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มะเร็งท่อน้ำดีเป็นโรคมะเร็งที่เป็นปัญหาสำคัญของประชากรไทยอีสาน แต่เป็นมะเร็งที่พบน้อยในภูมิภาคอื่นๆ ทั่วโลก การรักษาโรคโดยวิธีเคมีบำบัดให้ผลไม่ดีเนื่องจากขาดองค์ความรู้พื้นฐาน การศึกษากลไกการตอบสนองต่อยาเคมีบำบัดโดยใช้เซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีจึงเป็นวิธีการที่สามารถช่วยในการรักษาผู้ป่วยมะเร็งท่อน้ำดี ผู้วิจัยได้ทำการวัดระดับความไวต่อยาเคมีบำบัดของเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีจำนวน 5 ชนิด โดยวิธี MTS assay ได้ผลดังนี้ KKU-M055 ไวต่อยาเคมีบำบัดจำนวน 6 ชนิด ได้แก่ cisplatin, etoposide, oxaliplatin, irinotecan, paclitaxel และ doxorubicin แต่ดื้อต่อยา 5-FU ในระดับปานกลาง (IC_{50}=46 μM) KKU-100 เป็นเซลล์เพาะเลี้ยงที่ดื้อต่อยาเคมีบำบัดทั้ง 7 ชนิด โดยดื้อยา 5-FU (IC_{50}=1,020 μM) และ etoposide (IC_{50}=416 μM) ในระดับที่สูงมาก KKU-OCA17 ไวต่อยา 5-FU มากที่สุด (IC_{50}=3.46 μM) KKU-M055 เป็นเซลล์มะเร็งที่ไวต่อยาเคมีบำบัดทั้ง 6 ชนิด แต่ดื้อต่อ 5-FU ในระดับปานกลาง สำหรับ KKU-M156 และ KKU-M214 มีการตอบสนองต่อยาเคมีบำบัดทั้ง 7 ชนิดดังกล่าวได้ในระดับปานกลาง ได้ตรวจสอบการตายแบบ apoptosis ของเซลล์เพาะเลี้ยงทั้ง 5 ชนิด โดยยา 5-FU ได้ผลที่สอดคล้องกับระดับความไวของยา (IC_{50}) ด้วยค่า r=0.775 การศึกษาโดยใช้ยาบำบัด 2 ชนิดร่วมกัน พบว่า cisplatin และ paclitaxel สามารถเสริมฤทธิ์ของ 5-FU โดยลดค่า IC_{50} ในเซลล์ KKU-100 ได้อย่างมีนัยสำคัญ ($p < 0.05$)

ได้ทำตรวจวัดระดับการแสดงออกของจีนที่เกี่ยวข้องกับการดื้อยาเคมีบำบัดด้วยวีธี semiquantitative RT-PCR ได้ผลคือ เซลล์เพาะเลี้ยงทั้ง 5 ชนิดมีระดับการแสดงออกจีน TS, DPD, GST-π, MRP-1. -2, และ –3 ในระดับมากน้อยต่างกัน และ พบว่า การแสดงออกของจีน MRP3 มีความสัมพันธ์กับระดับความไวของยา doxorubicin (r = 0.943) และ etoposide (r = 0.946) อย่างมีนัยสำคัญ เมื่อใช้ verapamil ซึ่งเป็นตัวยับยั้งการทำงานของโปรตีน MRP สามารถเพิ่มความไวของยา doxorubicin ได้ ไม่พบความสัมพันธ์ระหว่างการแสดงออกของจีน TS, DPD และ ระดับความไวต่อยา 5-FU ในเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี ดังนั้นในอนาคต การศึกษาโดยการกระตุ้นให้เซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีดื้อต่อยา 5-FU อาจสามารถบ่งชี้ถึงกลไกการดื้อยา 5-FU อย่างจำเพาะได้

คำหลัก : เซลล์เพาะเลี้ยงมะเร็งท่อน้ำดี ยาเคมีบำบัด ความไวต่อยา การดื้อยา การแสดงออกของจีน

ABSTRACT

Project Code : MRG4580010

Project Title : Investigation of the mechanistic effect of chemotherapeutic drug resistance on human cholangiocarcinoma cell lines *in vitro*

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Project Period : 1st July 2002 to 30th June 2004

Cholangiocarcinoma (CHCA) is a rare cancer, which causes a major problem in Thai-Esarn population. Treatment by chemotherapy is not the curative way due to lacking of the basic knowledge. This project aimed to examine the inhibitory effect of chemotherapeutic drugs in human intrahepatic CHCA cell lines and the intrinsic drug resistance in CHCA cell lines was determined. Five human CHCA cell lines were subjected to *in vitro* drug sensitivity assay against a panel of chemotherapeutic drugs in various modes of action. The assay on CHCA cell lines to 7 chemotherapeutic drugs was determined by cytotoxic test using the MTS assay. Results demonstrated that KKU-M055 was the most sensitive CHCA cell line to 6 drugs including cisplatin, etoposide, oxaliplatin, irinotecan, paclitaxel and doxorubicin but it was moderately resistance to 5-FU (IC_{50}=46 μM). In contrast, KKU-100 was the most resistant cell line to 5-FU (1,020 μM) and etoposide (IC_{50}=416 μM) and it had high degree of resistance to other 5 drugs. KKU-OCA17 was the most sensitive cells to 5-FU (IC_{50}=3.46 μM) compared to other cell lines. KKU-M055 was the most sensitive cells to most types of drug. KKU-M055 however was not the most resistant cells to 5-FU. KKU-M156 and KKU-M214 showed moderate resistance to such 7 drugs. Histopathological types of CHCA cell lines did not show any significant relationship with drug sensitivities. Apoptotic level and IC_{50} of 5-FU had a moderate association (r=0.775) observed for KKU-100. Drug combination of 5-FU combined with either cisplatin or paclitaxel can reduce IC_{50} of 5-FU dramatically (p < 0.05).

Multidrug resistance in cancer confers resistance to multiple drugs that may be frequently encountered during chemotherapy of CHCA. Results from semi-quantitative RT-PCR revealed that genes of TS, DPD, GST-π, MRP-1. -2, and –3 were expressed in all CHCA cell lines albeit at different levels. The expression of MRP3 is significantly correlated with resistance to doxorubicin (r=0.943) and etoposide (r=0.946). Results confirmed that verapamil, the inhibitor of MRP proteins was markedly reduced IC_{50} of doxorubicin, indicating the reverse drug resistant effect. No correlation was observed between the intrinsic expression of TS and DPD in five CHCA cell lines with the cytotoxic effect of 5-FU. Further investigations of TS and DPD gene expression under 5-FU drug exposure need to be performed.

Key words : Cholangiocarcinoma cell lines, CHCA, *in vitro* drug sensitivity, multidrug resistance, semi-quantitative RT-PCR

OUTPUT จากโครงการวิจัยที่ได้รับทุนจาก สกอ. และ สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

Tepsiri N, Jaturat L, Tassaneeyakul W, Sripa B, Namwat W, Wongkham S, Bhudisawasdi V. Drug sensitivity and drug resistance profiles of human intrahepatic cholangiocarcinoma cell lines. Submitted to Cancer Chemotherapy Pharmacology (Manuscript preparation to be submitted to Cancer Chemotherpy Pharmacology).

2. การนำผลงานวิจัยไปใช้ประโยชน์

- เชิงวิชาการ มีการพัฒนาการเรียนการสอน โดยสามารถนำเทคโนโลยีการเพาะเลี้ยงเซลล์มาใช้ในการเรียนการสอน ทั้งระดับปริญญาตรีและบัณฑิตศึกษาของคณะแพทยศาสตร์
- มีการสร้างนักวิจัยใหม่ และได้ความร่วมมือระหว่างนักวิจัยต่างสาขา ได้แก่ เภสัชวิทยา พยาธิวิทยา และ ศัลยศาสตร์
- มีการนำเอาความรู้จากงานวิจัยนี้ไปอ้างอิงในการรักษาผู้ป่วยมะเร็งท่อน้ำดี

3. การเสนอผลงานในที่ประชุมวิชาการ

มีการนำผลงานวิจัยนี้ไปเสนอในที่ประชุมวิชาการจำนวน 4 ครั้งได้แก่

3.1 **Tepsiri N**, Sripa B, Sookprasert A, Somkuna K, and Bhudisawasdi V (2002). The *in vitro* synergistic effect of the chemotherapeutic drug combination on the cholangiocarcinoma cell line. Abstract in: *Proceedings of the 9th Annual Meeting Toxicology Society of Thailand*, P-4.

3.2 **Tepsiri N.** Investigation of the mechanistic effect of chemotherapeutic drug resistance on human cholangiocarcinoma (CHCA) cell lines. บทคัดย่อใน: การประชุม “นักวิจัยรุ่นใหม่…พบ…เมธีวิจัยอาวุโส สกว..” ณ โรงแรมเฟลิกซ์ ริเวอร์แคว จ.กาญจนบุรี วันที่ 9-11 ม.ค. 2547.

3.3 Chaturat L, **Tepsiri N**, Tassaneeyakul W, Sripa B, Kongyingyoes B, Kukongviriyapan V, Wongkham S, Bhudisawasdi V. Investigation of chemotherapeutic drug resistant gene expression in cholangiocarcinoma cell lines. Abstract in: *Second Regional APOCP Conference – South East Asia “Custom, Environment and Cancer”,* Kosa Hotel, Khon Kaen, Thailand, Feb 9-11 2004.

3.4 Chaturat L, **Tepsiri N**, Sripa B, Bhudhisawasdi V, Tassaneeyakul W. Prediction of response to chemotherapeutic agents in cholangiocarcinoma cell lines using gene expression profiles. Abstract in: *The 5th Princess Chulabhorn International Science Congress “ Evolving Genetics and its Global Impact”*, Shangri-La Hotel, Thailand, August 16-19, 2004.

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CHAPTER I

INTRODUCTION

1.1 BASIC AND REASONS

Cholangiocarcinoma (CHCA) is a relatively rare cancer which accounts for an estimated 15% of liver cancers worldwide (Parkin *et al.*, 1993). This cancer however is the most common disease particularly in Northeastern part of Thailand (Vatanasapt *et al.*, 1995). The etiology is rather obscure, but identified risk factors including hepatolithiasis, inflammatory bowel disease (Parkin *et al.*, 1993). A strong association between the liver flukes *Clonorchis sinensis* and *Opisthorchis viverrini* infection and the incidence of CHCA has been observed in certain areas of Southeast and Eastern Asia. The mechanisms of carcinogenesis in *O. viverrini* infection are the subject of considerable research. It appears that the presence of such parasite inducing DNA damage and mutations as a consequence of the formation of carcinogenesis or free radicals and of cellular proliferation of the intrahepatic bile duct epithelium (Parkin *et al.*, 1993).

CHCA is a malignant tumor arising from bile duct epithelium, at any portion of the biliary tree from the level of bile duct at the ampulla of Vater. CHCA can be mainly divided into 2 groups; intrahepatic and extrahepatic CHCA. Intrahepatic CHCA refers as carcinoma developing from the intrahepatic biliary tree including the right and left hepatic ducts (Nakanuma *et al.*, 1997). Intrahepatic CHCA are classified as peripheral or hilar type according to their main location along the biliary tree. Extrahepatic CHCA includes the cancer that occur in common hepatic duct, cystic duct and common bile duct whereas the gall bladder and the ampulla of Vater are excluded in the definition (Mairiang, 2000). Curative treatment of patients with CHCA is only possible with complete resection (Casavilla *et al.*, 1997). For patients with anatomically resectable intrahepatic CHCA and without advanced cirrhosis, partial hepatectomy is the protocol of choice (Casavilla *et al.*, 1997; Pitt *et al.*, 1995). Approximately 40% of patients with resectable intrahepatic CHCA have either multiple tumors or tumors involving both hepatic lobes (Casavilla *et al.*, 1997). Hepatic resection is planned to remove all tumor completely with an adequate margin. Generally, this surgery involves a formal lobectomy or trisegmentectomy (Mairiang, 2000). Care must also be taken to achieve a negative bile duct margin if the tumor approaches the hilum. In patients with perihilar and distal CHCA, palliative therapy

is directed at relieving obstructive jaundice and pruritus to prevent recurrent cholangitis, and avoiding hepatic failure caused by unrelieved biliary obstruction (Mairiang, 2000). In patients with distal CHCA, palliative therapy is also aimed at preventing or relieving gastric outlet obstruction. Palliation can be achieved either non-surgically with percutaneous or endoscopic techniques or surgically (Mairiang, 2000).

Chemotherapy has been chosen for adjuvant trials in biliary tract cancers including CHCA from all sites as well as gallbladder cancers (Pitt *et al.*, 1995). Common regimens have used 5-fluorouracil (5-FU) alone or in combination with other potent chemotherapeutic agents (Mairiang, 2000). The use of 5-FU alone gives partial responses in 8% of patients and median survival time of 26 weeks in patients with CHCA (Falkson *et al.*, 1984). Combination chemotherapy using 5-FU together with cisplatin, carboplastin, or interferon-alpha2b has produced modest improvement in survival and response rate (Gil-Benso *et al.*, 2001; Sanz-Altamira *et al.*, 1998). Palliative treatment by Chemotherapy in CHCA patients at Srinakarind Hospital has not been shown to be promising. Clinical trials with new anticancer drugs may need to provide the most effectiveness in cancer chemotherapy.

In 1985, Yamaguchi and co-worker established a human cholangiocarcinoma cell line, designated as HChol-Y1. Six years later, (Sirisinha *et al.*, 1991) isolated and established another human cholangiocarcinoma cell line (HuCCA-1) from Thai pateint with intrahepatic bile duct cancer. Such cell lines then provided for an experimental basis for the planning of clinical studies. Data of *in vitro* sensitivity of human cholangiocarcinoma cell lines have been accumulated to provide preclinical pharmacology. (Casavilla *et al.*, 1997) and co-workers (1997) demonstrated the *in vitro* study in chemosensitivity in human CHCA cell line (SG231) with 6 anticancer drugs such as mitoxantrone, taxol, mitomycin C, doxorubicin, cisplatin and 5-fluorouracil by the sulforhodamine B assay. Results showed SG231 was sensitive to cisplatin, to doxorubicin and to mitomycin C but resistant to 5-fluorouracil. It has been found that a CHCA cell line (SK-ChA-1) contained a resistant phenotype to 5-FU by increasing thymidylate synthase (TS) activity, the target enzyme of 5-FU, but not that of dihydropyrimidine dehydrogenase (DPD), the initial and rate-limiting enzyme of pyrimidine catabolism (Habara *et al.*, 2001). Glutathione-S-transferase π (GST- π), a detoxifying enzyme, has been investigated in SG231 CHCA cell line but its association to drug sensitivity was not reported (Villa *et al.*, 1997). Recently, GST- π has been reported to be involved in cisplatin resistance of breast

cancer cell line MCF-7 and head and neck squamous cell carcinoma cell lines (Cullen *et al.*, 2003; Rudin *et al.*, 2003).

Multidrug resistance in cancer confers resistance to multiple drugs that are frequently encountered during chemotherapy of many types of cancer. One of the factors involved in this phenomenon is a transport of anticancer drugs out from cells leading to the decrease in intracellular levels of drugs. To date, 2 major types of multidrug resistance factors have been classified: multidrug resistance protein (MDR) and multidrug resistance related protein (MRP) (Borst *et al.*, 1999). Both MDR and MRP have been investigated from normal and cancer cells. MDR1 was found to be expressed in various tumor cells that possess intrinsic or acquired cross-resistance to diverse chemotherapeutic agents (Tan *et al.*, 2000). MRP1 is present in non-expressing MDR1 tumor cells (Tan *et al.*, 2000) but in low amount in liver (Borst *et al.*, 1999). MRP2 expression was found in human liver and gallbladder epithelia (Rost *et al.*, 2001). In human bile duct, MRP3 mRNA level is rarely found in liver but is present mainly in the proliferative cholangiocytes and its levels are up-regulated in intrahepatic cholestasis (Scheffer *et al.*, 2002). In rat liver, MRP2 protein localized to the canaliculr membrane, is down-regulated after bile duct ligation or cholestasis, but there is an increase in MRP3 protein in the basolateral membrane of hepatocytes surrounding the central veins and of cholangiocytes (Donner & Keppler, 2001). The physiological role of MRP3 may be to compensate for the biliary obstruction and impaire canalicular MRP2 function by clearing cholephilic anionic substrates into the blood (Donner & Keppler, 2001).

The association between expression of multidrug resistance genes and genes involved in drug metabolism have been investigation. TS and DPD have been reported to correlate with resistance to 5-FU in many cancer cells both in vitro and in tumor tissues (Habara *et al.*, 2001; Kogure *et al.*, 2004; Nishiyama *et al.*, 1999). High TS activity was suggested to influence the high resistance to 5-FU of biliary tract carcinoma cell lines (Habara *et al.*, 2001). DPD expression in hepatocellular carcinoma has been suggested as a predictable factors in 5-FU chemotherapy (Kogure *et al.*, 2004). The associations between expression of MDR1, MRP1, -2, -3, -4 and –5 have been recently examined (Kool *et al.*, 1997). MDR1 was found to associate with doxorubicin resistance in human sarcomas (Hoffmann *et al.*, 1999) and MRP2 was found to correlate with cisplatin resistance in many human cell lines (Kool *et al.*, 1997).

Given that in CHCA the overall response rate to chemotherapy is still low (Chen *et al.*, 2001), there has not as yet been experimental study of the relationship between enzymes involved in drug metabolism (TS, DPD and GST- π), multidrug resistance gene expression and resistance to various anticancer drugs in human intrahepatic CHCA cell lines. Using a preclinical approach to identify relevant specific drug resistance mechanisms in CHCA cell lines, we examined the cytotoxic effect of chemotherapeutic agents on five intrahepatic CHCA cell lines employing the MTS assay. Direct correlation was evaluated between chemosensitivity profiles of those cell lines and their expression levels of drug resistance markers in order to determine *in vitro* drug resistance factors.

1.2 THE OBJECTIVES OF THIS STUDY

1.2.1 To examine the cytotoxic effect of chemotherapeutic drugs in the sensitivity of five established CHCA cell lines

1.2.2 To investigate the molecular mechanisms of chemotherapeutic drugs resistance of these CHCA cell lines by investigating the expression of several genes involved in cell proliferation process (TS), drug metabolism (DPD and GST- π) and multidrug resistance (MDR1, MRP1, -2 and –3)

1.2.3 To examine the reverse drug resistance using an inhibitor of multidrug resistance proteins.

1.3 SCOPE OF RESEARCH

1.3.1 Five commercial chemotherapeutic drugs were chosen to test against 5 CHCA cell lines for the cytotoxic effect.

1.3.2 Two of chosen drugs were examined the synergistic effect *in vitro*.

1.3.3 Apoptotic detection was performed to prove the effect of anticancer drugs on CHCA cell lines.

1.3.4 Pattern of gene expression involving drug resistance was characterized from 5 CHCA cell lines.

1.3.5 Direct correlation was evaluated between drug sensitivity profiles of those cells and their expression levels of drug resistance genes.

1.3.6 Reverse drug resistance was performed to demonstrate the synergistic effect.

1.4 ANTICIPATED OUTCOME

1.4.1 The result of *in vitro* drug sensitivity test in CHCA cell lines, which were isolated from CHCA Thai patients, can provide the basic data for clinical trial.

1.4.2 The effective drug concentration gained from *in vitro* experiment could represent the potency of chemotherapeutic drugs whether it kills tumor cells of CHCA patients or not.

1.4.3 New chemotherapeutic agents may use as a palliative treatment with surgery and they may improve patient's survival rate better than the old regimen.

1.4.4 Drug combination provides the synergistic effect *in vitro*, which can help clinicians to set up the new combination regimen.

1.4.5 The pattern of drug sensitivity and gene expression profiles can provide the data for the molecular mechanism of drug resistance in CHCA cells.

1.4.6 Drug sensitivity method that is set up in our laboratory may offer the service in anticancer drug screening for CHCA in the future.

CHAPTER II

LITERATURE REVIEWS

2.1 CHOLANGIOCARCINOMA (CHCA)

2.1.1 Incidence, epidemiology and classification

In developing country as Thailand, liver cancer is the most frequent malignancy, but there are large regional differences in the incidence and in histological type, with very high rate of cholangiocarcinoma in the northeastern part (Vatanasapt *et al.*, 1995).

The etiology of CHCA is rather obscure, but several risk factors such as hepatolithiasis, inflammatory bowel disease have been identified (Falchuk *et al.*, 1976). In certain areas of southeast and eastern Asia, incidence rates are very high. There is a strong association with infection with the liver flukes *Clonorchis sinensis* and *Opisthorchis viverini* (Parkin *et al.*, 1993; Thamavit *et al.*, 1978). It has been proposed that the presence of parasites induces DNA damage and mutations as a consequence of the formation of carcinogenesis/free radicals and of cellular proliferation of the intrahepatic bile duct epithelium (Parkin *et al.*, 1993).

CHCA is a malignant tumor developing from bile duct epithelium, at any portion of the biliary tree from the level of bile duct at the ampula of Vater. CHCA can be devided into the intrahepatic and extrahepatic CHCA. The extrahepatic CHCA includes the cancer that occur in common hepatic duct, cystic duct and common bile duct whereas the gall bladder and the ampula of Vater are excluded by the definition (Mairiang, 2000). The intrahepatic CHCA refers as carcinoma arising from the intrahepatic biliary tree including the right and left hepatic ducts (Nakanuma *et al.*, 1997). The intrahepatic CHCA are classified as peripheral or hilar type according to their main location along the biliary tree. Peripheral intrahepatic CHCA is defined as carcinoma of the intrahepatic bile duct periphery to the bifurcation of the right and left hepatic ducts (Nakanuma *et al.*, 1997; Uttaravichien *et al.*, 1999). In addition, (Uttaravichien *et al.*, 1999) had included the CHCA that originated from the intrahepatic portion of the right or left hepatic duct referred as the central type of the intrahepatic CHCA. The tumor mass of the intrahepatic CHCA is usually located within the liver. In the hilar type, the tumor is located mainly at the hepatic hilus usually derived from the right or left hepatic duct or its direct branches (Nakanuma *et al.*, 1997) or in the vicinity of hepatic duct confluence (Klatskin, 1965). Tumor usually

grows and spreads slowly and often invades the liver parenchyma in segment III, V or VII, and manifests as hilar mass (Uttaravichien *et al.*, 1999).

At the early stage of tumor, the location of the intrahepatic CHCA with respect to the biliary anatomy is recognizable. However, the differentiation between the peripheral and hilar CHCA is often difficult at a relatively advanced stage, particularly that arising from the segmental area of ducts or that showing periductal infiltration (Okuda *et al.*, 1977). Obstructive jaundice is the common clinical feature of hilar CHCA, which more closely resemble the extrahepatic CHCA, and basically is classified including as proximal (upper) extrahepatic bile duct cancer and the perihilar cancer. Perihilar cancer is a primary tumor of the extrahepatic bile duct arising in the region of hepatic ducts, common hepatic duct, cystic duct and common bile duct at the level above pancreas. Cancer of the lower third extrahepatic bile duct is called distal extrahepatic bile duct cancer.

After being diagnosed, only 20% of patients can be treated by surgery, which offers the only chance for cure (Harder & Blum, 2002). Due to high recurrence rates, liver transplantation is not indicated. Patients with advanced unresectable carcinoma have a dismal prognosis with an overall survival rate of only 6-8 months (Harder & Blum, 2002). For patients who are not eligible for curative surgery, the prevention or treatment of cholestasis is the main choice (Harder & Blum, 2002). This can be achieved by endoscopically, percutaneously or surgically biliodigestive anastomosis. Palliative chemotherapy results in response rates up to 20% (Harder & Blum, 2002). The most frequently used agents are 5-fluorouracil and gemcitabine that can be combined with external or internal radiation (Harder & Blum, 2002). By using the combination of different treatments, modalities shows significant survival which can be achieved in some patients (Harder & Blum, 2002).

2.1.2 Staging of CHCA

Staging of CHCA is commonly conformed to the TNM classification of tumor in liver and intrahepatic bile ducts (Nakanuma *et al.*, 1997). The classification of CHCA regarding the tumor (T), regional lymph nodes (N) and distant metastasis (M) when determined clinically (cTNM) and histopathologically (pTNM) as following descriptions. Stage I contains solitary tumors with a diameter less than 2 cm but without vascular invasion, lymph node and remote metastasis. Stage II regards the solitary tumor with a diameter less than 2 cm but with vascular invasion. In addition, it can be multiple tumors which has been limited to one lobe, and a dimeter less than 2 cm in greatest dimension but without vascular invasion or solitary tumor with diameter more than 2 cm but without

vascular invasion. Stage III A, there are solitary tumor with a diameter more than 2 cm in greatest dimension with vascular invasion. Stage IIIA can be multiple tumors limited to one lobe, not more than 2 cm of diameter with vascular invasion. Stage IIIB is defined as multiple tumors limited to one lobe with any diameter more than 2 cm with or without vascular invasion but emphasized in regional lympnode metastasis. For stage IV, it can be divided into IVA and IVB. Stage IVA, there is no distance metastasis but there are multiple tumors in more than one lobe. It can be tumors involve a major branch of the portal or hepatic veins, or tumors with direct invasion of other adjacent organs except gallbladder. It can also be tumors with perforation of visceral peritoneum whereas stage IVB focus only the present of distant metastasis.

Ultrasonography, a computed tomography and magnetic resonance imaging are useful in the detection of primary tumor and metastases. Endosonography (EUS) gives 83% accuracy in preoperative staging (Cory *et al.*, 1991). The EUS is more accurate in detecting distal bile duct tumor than proximal bile duct tumor (Rosch *et al.*, 1992).

2.1.3 Overview of treatments

Treatment of stage I and II CHCA is defined a curative resection (Thuluvath *et al.*, 1997), whereas the more advanced stage (III and IV) which the treatment of choice are surgical bypass or non-surgical management (Terblanche, 1994). There are many methods for non-surgical management including percutaneous transhepatic drainage (PTBD), endoscopic retrograde biliary drainage (ERBD), external radiation, brachytherapy, chemotherapy and phytodynamic therapy.

2.1.4 Chemotherapeutic treatment

Single chemotherapy used for unresectable CHCA give a poor response rate. Drug response of 10% was obtained from patients treated with mitomycin C (Taal *et al.*, 1993) or with 5-FU (Falkson *et al.*, 1984). Eight percent of drug response was observed from patients who were treated with cisplatin (Okada *et al.*, 1994). Although combined chemotherapy gave a better response rate, most regimens gave partial response with median survival of 2-26 months (Mairiang, 2000). Clinical trials in CHCA patients can be summarized in Table 2.1.

Table 2.1 The summary of drug regimens and response rate in the treatment of CHCA patients.

Regimen	No. of patients	Response rate (%)	References
5-FU alone	1	100	(Hasuike *et al.*, 1999)
5-FU + cisplatin	25	24	(Ducreux *et al.*, 1998)
5-FU + mitomycin C + doxorubicin	19	26	(Asamoto *et al.*, 2001)
5-FU + doxorubicin + ciplatin + interferon alpha-2b	18	39	(Gil-Benso *et al.*, 2001)
5-FU + leucovorin + carboplatin	14	2	(Sanz-Altamira *et al.*, 1998)
Gemcitabine	30	27	(Tsavaris *et al.*, 2004)

2.2 CHOLANGIOCARCINOMA CELL LINES

CHCA cell lines have been interesting to be established in order to use as an experimental model to study CHCA. CHCA cell lines can be served as a model which can used for investigating a specific tumor marker, *in vitro* drug sensitivity, signalling pathway, etc. In 1985, the first CHCA cell line named Hchol-Y1 was established by (Yamaguchi *et al.*, 1985). Hchol-Y1 was subcultured in a protein-free, chemically defined medium after a very short period of primary culture in 0.1% fetal bovine serum (FBS)-containing medium. The cell line had been propagated in this medium for 2 years. The cells grew as a monolayer and the doubling time was about 52 hours. Addition of FBS did not stimulate cell growth (population-doubling time = 50 hr) or increase saturation density. The cells grown in a protein-free medium secreted small amounts of carcinoembryonic antigen (CEA) and large amounts of carbohydrate antigen (CA) 19/9 (CEA: 12.5 +/- 2.1 ng/10(6) cells/48 hr; CA 19/9: 760 +/- 52 IU/10(6) cells/48 hr. These tumor markers were immunohistochemically demonstrated in HChol-Y1 cells. Two years later, a human CHCA cell line named KMCH-1 was established and demonstrated that it was an adenocarcinoma which was the feature of CHCA (Murakami *et al.*, 1987).

In 1991, (Sirisinha *et al.*, 1991) and coworkers established a CHCA line isolated from CHCA tissue slices which were surgically removed from a Thai patient with intrahepatic bile duct cancer. The cells were subcultured in Ham's F12 supplemented with 10% FBS. A CHCA cell line was then named HuCCA-1 which was a mucin secreting tumor of epithelial cell origin. A CHCA cell line was also then identified as a cytokeratin-positive cell. As being facilitated by cell culture technology, there are many lines of research regarding the establishment of CHCA lines have been accumulated to characterize them and study CHCA in terms of tumor biology and molecular genetics as summarized in Table 2.2 and 2.3.

Table 2.2 Summary of the establishment of human CHCA cell lines listed in chronologically order

CHCA cell line	Doubling time (hr)	Morphological type	The study	Reference
Hchol-Y1	50	N/R	Characterisation of the cell	(Yamaguchi *et al.*, 1985)
KMCH-1	55	Moderately differentiated	Chromosomal breakpoints IL-6 involved MAPK P38 mitogen & MAPK pathway	(Murakami *et al.*, 1987) (McAleer *et al.*, 1999) (Tadlock & Patel, 2001)
HuCCA-1	39	N/R	Characterisation of the cell Identified tumor markers Ultrastructure	(Sirisinha *et al.*, 1991) (Sirisinha *et al.*, 1994) (Sriurairatana *et al.*, 1996)
CC-SW-I	72	Moderately differentiated	Cytogenetics & response to growth factors	(Shimizu *et al.*, 1992)
CC-LP-I	180	Moderately differentiated	Cytogenetics & response to growth factors Phospholipase A2 & COX-2	(Shimizu *et al.*, 1992) (Wu *et al.*, 2002)
HuCC-T1	N/R	N/R	IL-6 as autocrine growth factor Growth inhibition by quercetin **Carboplatin sensitivity** Phospholipase A2 & COX-2 **Reverse drug resistance**	(Okada *et al.*, 1994) (Kudo *et al.*, 1999) **(Mezawa *et al.*, 2000)** (Wu *et al.*, 2002) **(Nakajima *et al.*, 2003)**

Table 2.2 (continue)

CHCA cell line	Doubling time (hr)	Morphological type	The study	Reference
SK-ChA-1	N/R	Well-differentiated	Acid/base transport **Molecular chemotherapy** **Growth inhibition by Tamoxifen**	(Strazzabosco *et al.*, 1994); (Knutter *et al.*, 2002) **(Pederson *et al.*, 1997)** **(Sampson *et al.*, 1997)**
KMC-1	N/R	N/R	Herbal effect on cell cycle	(Yano *et al.*, 1994)
Mz-ChA-1	N/R	N/R	ATP-activated chloride permeability Regulation of cell swelling Ca2+-activated C1 channel Alpha2-adrenagic receptor	(McGill *et al.*, 1995) (Roman *et al.*, 1996) (Schlenker & Fitz, 1996) (Kanno *et al.*, 2003)
SG231	**36**	**N/R**	***In vitro* drug sensitivity** IL-6, hepatocyte growth factor Phospholipase A2 & COX-2	**(Casavilla *et al.*, 1997)** (Yokomuro *et al.*, 2000) (Wu *et al.*, 2002)
TK	N/R	N/R	Identified tumor markers	(Abe *et al.*, 2000)
SCK	N/R	N/R	Chromosome abberration	(Kim *et al.*, 2001)
JCK	N/R	N/R	Chromosome abberration	(Kim *et al.*, 2001)
Cho-CK	N/R	N/R	Chromosome abberration	(Kim *et al.*, 2001)
Choi-CK	N/R	N/R	Chromosome abberration	(Kim *et al.*, 2001)
QBC939	N/R	N/R	Fas expression Celecoxib inhibits growth	(Ajiki *et al.*, 2001) (Wu *et al.*, 2003)
CCKS1	N/R	Intrahepatic	Fibrous stroma formation Galectin-1,3 & metastasis	(Sabit *et al.*, 2001) (Sabit *et al.*, 2001)
KMBC	N/R	N/R	COX-2 induction	(Yoon *et al.*, 2002)

N/R = No report

Table 2.3 Summary of animal CHCA cell lines

CHCA cell line	Doubling time (hr)	Morphological type	The study	Reference
C611B (rat)	N/R	N/R	Characterization Hepatocyte growth factor	(Courtois *et al.*, 1999) (Abe *et al.*, 2000)
CC-62 (rat)	23	Combined hepatocellular CA and CHCA	Characterization	(Gil-Benso *et al.*, 2001)

N/R = No report

2.3 *IN VITRO* DRUG SENSITIVITY ON CHCA CELL LINES

Few reports demonstrate *in vitro* drug sensitivity against CHCA cell lines. A bile duct cancer cell line (BDC-SN) was transplanted into nude mice (Tsubono *et al.*, 1992). The xenograft cell line was then exposed to a series of anticancer drugs such as cisplatin, 5-FU, VDS, mitomycin C, adriamycin, epirubicin, CQ and etoposide. Results showed that when used singly, cisplatin, 5-FU and VDS were effective against BDC-SN transplanted nude mice. Among the various two-agent combinations, cisplatin and 5-FU was the most effective against BDC-SN. However, three-agent combinations cosisting of cisplatin, 5-FU and another agent were less effective than the cisplatin and 5-FU double regimen and cause significant loss of weight as well as high mortality. *In vitro* drug sensitivity in CHCA cell lines has been demonstrated by using tamoxifen which was an anti-estrogen receptor (Sampson *et al.*, 1997). Tamoxifen in 5-10 μM could inhibit SK-ChA1 growth. The sensitivity of a human CHCA cell line (SG231) to various chemotherapeutic drugs has then been investigated (Casavilla *et al.*, 1997). Results showed that SG231 was sensitive to cisplatin, to doxorubicin and to mitomycin C but resistant to 5-fluorouracil.

2.4 CHEMOTHERAPEUTIC AGENTS

To date, chemotherapeutic agents have been continuously developed to treat several types of cancer. Six anticancer drugs, which have been used to treat solid tumors, are summarized in this chapter as described below.

2.4.1 5-Fluorouracil (5-FU)

5-FU has been used as a standard regimen to treat in human colorectal cancer (Longley *et al.*, 2003). It can particularly effect on adenocarcinoma, cancers of the aerodigestive tract (Araki *et al.*, 2001). The mechanism of action of 5-FU has been

proposed as it is the inhibitor of thymidylate synthase (TS) resulting in the inhibition of DNA synthesis and causes cells die by apoptosis (Aota K, 2000). In addition, 5-FU leads cells to apoptosis *via* NF-kappaB inhibition and the increase of caspases activity. Tumor cells with P53 mutation revealed less sensitivity to 5-FU (Yukimoto *et al.*, 2001). It is so far reported that 5-FU treatment in CHCA patients gave contradictory outcome (Gil-Benso *et al.*, 2001; Hasuike *et al.*, 1999).

2.4.2 Etoposide (VP-16)

Etoposide is used in treatment of both small cell and non-small cell lung carcinoma (Tamura, 2001). The drug is semisynthetic derivative of podophyllotoxin, which is extracted from the root of the mayapple (*Podophyllum peltatum*). Etoposide block cells in the late S-G2 phase of the cell cycle. The mode of action involves inhibition of topoisomerase II, which results in DNA damage through strand breakage induced by the formation of a ternary complex of drug, DNA and enzyme. In addition, etoposide induces protein kinase Cdelta resulting in cellular apoptosis (Blass *et al.*, 2002).

2.4.3 Cisplatin

Cisplatin (cis-diamminedichloroplatinum [III] is a neutral inorganic platinum complexes which was firstly reported that it inhibited cell division and induced filamentous growth of *E.coli.* The certain mechanism of action of cisplatin is still underinvestigated, it is purposed to act analogously to alkylating agents. It kills cells in all stages of the cell cycles, inhibits DNA biosynthesis, and binds DNA through the formation of interstrand cross-links. Cisplatin has major antitumor activity in genitourinary cancers, especially testicular, ovarian, and bladder carcinoma.

2.4.4 Oxaliplatin

Oxaliplatin is a third generation platinum compound which has shown a wide antitumor effect both *in vitro* and *in vivo*. Oxaliplatin has been registered in treatment of patients with metastatic colon cancer (Raymond *et al.*, 1998). Oxaliplatin induces apoptosis by inhibiting DNA synthesis *via* the stimulation of poly (ADP-ribose polymerase (PARP) (Guichard *et al.*, 2001). Oxaliplatin combined with 5-FU and/or irinotecan showed a good response in CCA patients (Stemmler *et al.*, 2002; Ychou *et al.*, 2003).

2.4.5 Irinotecan

Irinotecan is the camptothecin derivatives. It can inhibit DNA synthesis *via* pyrimidine salvage pathway by the inhibition of topoisomerase I and thymidine kinase activities (Voeller *et al.*, 2000). Irinotecan revealed that it reduced the regression of metastatic stage of colon cancer (Shinohara *et al.*, 2000). Irinotecan induced tumor cell apoptosis by elevating $p16^{INK4}$ which was a tumor suppressor (Fukuoka *et al.*, 2000). One report suggested that using oxaliplatin combined with irinotecan gave a good response in two CHCA patients (Ychou *et al.*, 2003).

2.4.6 Paclitaxel

Paclitaxel is an alkaloid ester derived from the Western yew (*Taxus brevifolia*) and the European yew (*Taxus baccata*). The drug functions as a mitotic spindle poison through the stimulation of tubulin polymerisation. This promotion of microtubule assembly by paclitaxel occurs in the absence of microtubule-associated proteins (MAPs) and guanosine triphosphate. Paclitaxel has substantial activity in ovarian and advanced breast cancer.

2.4.7 Doxorubicin

Doxorubicin is a anthracycline antibiotics. It is isolated from *Streptomyces peucetius* vas caesius. It is among the most useful cytotoxic anticancer drugs due to it has a broad spectrum of potent activity against many different types of cancers. There are three major actions documented for the tumor cytotoxicity including (1) high-affinity binding to DNA through intercalation, with consequent blockade of the synthesis of DNA and RNA, and DNA strand scission through effects on topoisomerase II; (2) binding to membranes to alter fluidity and ion transport; and (3) generation of the semiquinone free radical and oxygen radicals through an enzyme-mediated reductive process. Doxorubicin is one of the most important anticancer drugs, with major clinical application in carcinomas of the breast, endometrium, ovary, testicle, thyroid, and lung and in treatment of many sarcomas. It is generally used in combination with other agents such as cyclophosphamide, cisplatin and nitrosoureas.

2.5 MECHANISMS OF ANTICANCER AGENT RESISTANCE

Although chemotherapy is the treatment of choice in many malignant diseases, drug resistance is a major impediment in successful treatment of malignancies. Failure of chemotherapy can be caused by intrinsic resistance of the tumor and/or acquired resistance

developed during drug treatment (Stavrovskaya, 2000). Recent developments in molecular pharmacology and cell biology have suggested that resistance may be due to failure of drug uptake or activation, alteration in target enzymes including thymidylate synthase (TS), activation of enzymatic systems involved in the enhanced expression of detoxyfying enzymes including dihydropyrimidine dehydrogenase (DPD) and glutathione-S-transferase, or increased anticancer drug efflux (Stavrovskaya, 2000). The acquisition of the multidrug resistance phenotype against anticancer drugs in patients after chemotherapy has also been reported in acute myeloid leukemia, acute lymphoblastic leukemia, non-Hodgkin's lymphoma and breast, gastrointestinal, gynecological, and genitourinary tumors (Goldstein, 1996; Marie *et al.*, 1996). Clinically, multidrug resistance must be multifactorial, but is often discussed in reference to the expression of the anticancer drug efflux protein MDR1 (P-glycoprotein) encoded by the *MDR1* gene, and its related proteins including multidrug resistance-associated protein 1 (MRP1), multidrug resistance-associated protein 2 (MRP2) and multidrug resistance-associated protein 3 (MRP3). These efflux proteins activity expel structurally and functionally unrelated anticancer drugs from cells, decreaseing their intracellular accumulation to noncytotoxic levels (Aszalos & Ross, 1998; Cole & Deeley, 1998; Huet & Laval, 1985; Loe *et al.*, 1996; Stein, 1997). During the past decade, a correlation between MDR1 expression in patient tumor specimens have led to poor chemotherapy treatment prognosis (Goldstein, 1996; Marie *et al.*, 1996).

2.5.1 Decrease of drug accumulation in cell

A priori decrease of a drug accummulation by cells may result both from decrease of drug efflux and increase of drug efflux from cells (Stavrovskaya, 2000). Since most chemotherapeutic drugs enter cells by passive diffusion through the plasma membrane, cell changes in drug influx can be connected with changes in the cell membrane structure (Stavrovskaya, 2000). Indeed, both electron microscopy and analysis of the lipids of the membranes of multidrug resistance cells revealed differences between some drug-sensitive and drug-resistant cells (Simon & Schindler, 1994). The modifications found could cause either changes in drug traffic through the cell membrane or an influence on signaling pathways controlling apoptosis (Stavrovskaya, 2000). However, data concerning alterations of drug uptake by cells are scarce, so another mechanism (drug efflux from the resistant cell) is considered to be the main mechanism of the decreased drug accumulation in cells (Stavrovskaya, 2000).

A major form of resistance against a variety of the antineoplastic agents currently used involves the function of a group of membrane proteins called multi-drug transporters that extrude cytotoxic molecules, thus keeping intracellular drug concentration below a cell-killing threshold. These transporters belong to the superfamily of ATP Binding Cassette (ABC) proteins, present in almost all organisms from bacteria to humans (Holland & Blight, 1999). The medical significance of ABC proteins exceeds their role in cancer therapy resistance. The transport function of several members of these proteins was found to hinder the effective therapy of many other widespread diseases (e.g. malaria, AIDS), and inherited diseases were also linked to mutations in these genes (Stavrovskaya, 2000). The transport activity of ABC proteins has an important effect in general pharmacology, that is, in modulating the absorption, distribution and excretion of numerous pharmacological compounds (Stavrovskaya, 2000). ABC transporter proteins are located in the plasma membrane of the cells or in the membrane of different cellular organelles, and mediate the translocation of various molecules across these barriers. These substrate molecules exhibit a wide variety of chemical structures. Some ABC proteins facilitate the transport of inorganic ions, whereas others pump various organic compounds, including lipids, bile acids, glutathione and glucuronide conjugates, or even short peptides (Stavrovskaya, 2000). Most ABC proteins utilize the energy of ATP hydrolysis for this transport activity (active transporters), but some ABC transporters form specific membrane channels (Stavrovskaya, 2000).

The typical structure of an ABC protein consists of membrane-embedded transmembrane domains (TMD) and ATP binding domains (ABC) (Klein *et al.*, 1999). Typically, the transmembrane regions anchor the protein to the membrane and form a pore through which the transport of a surprisingly large variety of substrates occurs. The cytoplasmic nucleotide binding domains provide the molecular compartment where the energy of ATP is released. It is not known how the energy is conveyed from the ABC domains to the site of the transport and the precise mechanism of transport also remains unclear.

Numerous clinical data revealed that the multi-drug resistance phenotype in tumours is associated with the overexpression of certain ABC transporters, termed MDR proteins (Cole *et al.*, 1992; Grant *et al.*, 1994; List *et al.*, 1996). The P-glycoprotein (P-gp, MDR1, ABCB1)-mediated multidrug resistance was the first discovered and probably still is the most widely observed mechanism in clinical multidrug resistance (Stavrovskaya, 2000). Soon after the cloning and characterization of MDR1, it became evident that other efflux-pumps may also play a significant role in transport-associated drug resistance. There

are two other ABC transporters, which have been definitively demonstrated to participate in the multi-drug resistance of tumours: the multi-drug resistance protein 1 (MRP1, ABCC1), and the mitoxantrone or half-transporter resistance protein (MXR/BCRP, ABCG2) (Litman *et al.*, 2000).

(A) P-Glycoprotein (P-gp)

Human P-gp is coded by MDR1 gene that is a member of the multidrug resistance family. The gene is located on chromosome 7 (7q21.1) (Stavrovskaya, 2000). The MDR family includes two genes in man (MDR1 and MDR2) and three in rodents (mdr1, 2, 3). By means of gene transfer it was shown that only one human gene (MDR1) and two rodent genes cause multidrug resistance (Stavrovskaya, 2000). Mutations in some sites of the MDR1 gene can result in the changes in the cross-resistance pattern, i.e., in the binding of certain substrates by the protein (Stavrovskaya, 2000). The introduction of MDR2 gene into cells did not result in drug resistance. The product of the MDR2 gene (mouse *mdr2*) is present at high amount on the surface of cells of bile canaliculi, functions as a flipase, and transports phosphatidylcholine into the bile (Smit *et al.*, 1993). Multidrug resistance can occur both due to alteration of MDR1 gene expression and to increase in the dose of a gene amplification of a fragment of the genome containing the MDR1 gene (Borst *et al.*, 1999). Amplification is found usually in cultured cell lines with high levels of P-gp-MDR, but not in a samples from patients. Multidrug resistance can also result from stabilization of MDR1 mRNA, regulation at the level of synthesis, and alterations of protein processing (Bosch & Croop, 1996).

MDR1 substrates

One of the important features of P-gp is its broad substrate recognition pattern. Over the past decade the substrate list expanded from the original description of P-gp as conferring resistance to the vinca alkaloids and anthracyclines to the currently large list of compounds which includes structurally unrelated anticancer agents, antihuman immunodeficiency virus (HIV) agents and fluorophores as shown in Table 2.4 (Litman *et al.*, 2000).

Table 2. 4 Lists of P-gp substrates

Chemical group	Example
Anthracyclines	Daunorubicin, doxorubicin and epirubicin
Anthracenes	Bisantrene and mitoxantrone
Vinca alkaloids	Vinblastine, vincristine, vinorelbine and vindesine
Camptothecin derivatives	CPT-11 and topotecan
Epipodophyllotoxins	VP-16 (etoposide) and VM-26 (teniposide)
Tubulin polymerizing drugs	Colchicine, paclitaxel and docetaxel
Chromopeptide antibiotics	Actinomycin D
HIV-1 protease inhibitors	Ritonavir, Saquinavir and Indinavir
Fluorophores	Calcein-AM, fluo-3 AM, fura-2 AM, rhodamine 123 and Hoechst 33342/33258

Inhibitor of MDR1 (P-gp)

The clinical significance of P-gp called the attention of many researchers and pharmaceutical companies to the examination of ways to inhibit P-gp activity. These include calcium channel blockers (e.g. verapamil, nifeldipine), hypotensive drugs (reserpine), antibiotics (cephalosporins, gramicidin, puromycin), immunosuppressors (cyclosporin A and its derivatives) and many other lipophilic compounds (Sandor *et al.*, 1997). All of these diverse compounds are hydrophobic and have a low-molecular-weight aromatic ring in the molecule. Some of these compounds such as verapamil bind to the P-gp molecule and inhibits it in a competitive manners the binding or transport of drugs that are P-gp substrates (Beck *et al.*, 1986). Evidence from several experiments suggest that the P-gp reversal is rather complex; for example, in cells with 200-fold resistance to vinblastine, verapamil induces two-fold decrease of vinblastine resistance, but cross resistance to doxorubicin remaines unchanged (Beck *et al.*, 1986). Clinical testing showed that it is difficult to obtain the necessary concentration in the patient blood for most of the P-gp-MDR modifiers because of their toxicity and side effects (Sandor *et al.*, 1997). Less toxic and more effective P-gp inhibitors are now being developed, among them are cyclosporin derivatives, PSC-833 and verapamil R, a variant of verapamil (Stavrovskaya, 2000). Recently, anti-P-gp monoclonal antibodies were suggested as modifiers of P-gp-MDR; however, they are still under clinical testing (Stavrovskaya, 2000).

(B) The MRP family

General characteristics

In 1992, the second major ABC transporter involved in MDR; the MDR-associated protein (MRP) has been discovered (Almquist *et al.*, 1995). It is a 190-kDa membrane protein containing 1531 amino acids, with only 15% homology to P-gp. MRP is more closely related to the cystic fibrosis transmembrane regulator gene product CFTR. Until now, nine homologs of MRP have been reported. These are termed MRP1 to MRP9 (Borst *et al.*, 2000; Litman *et al.*, 2001). MRP1 is the original MRP, whereas MRP2 was originally identified as the canalicular multispecific organic anion transporter, cMOAT. A new nomenclature scheme was implemented for the human ABC and mouse Abc genes in October 1999, according to which MRP1–6 is designated *ABCC1–6*, whereas MRP7 listed as *ABCC10*. MRP1 shares only between 34% and 58% sequence identity with MRP2–6, but the overall membrane topology is thought to be similar in all members of the MRP family (Litman *et al.*, 2001). In addition to the 12 transmembrane segments characterizing P-gp, MRP has an additional 5TM (TMD_0) attached to the N-terminal. This additional domain has an apparent role in the organic anion affinity of the MRP family (Litman *et al.*, 2001). Although at least nine homologs of MRP have been reported, none have yet been linked as clearly with drug resistance as MRP1 (Litman *et al.*, 2001). Interestingly, the homolog MRP1, 2 and 3, in which resistance has been observed with transfectants also contain the 5 transmembrane segments (Litman *et al.*, 2001). However, the membrane topology of MRP is still under debate. Some studies suggest that the third membrane-spanning domain (equivalent to the second half of P-gp) has only four TM helices, whereas other studies favor the more 'conventional' six TM configuration of MSD3 (Litman *et al.*, 2001). MRP1 contains 12 potential glycosylation sites, but these appear not to be of importance for its function (Almquist *et al.*, 1995). On the other hand, phosphorylation of MRP seems to play a role for its transport function (Feller *et al.*, 1995).

MRP2 expression was found in human liver and gallbladder epithelia (Rost *et al.*, 2001). In human bile duct, MRP3 mRNA level is rarely found in liver but is present mainly in the proliferative cholangiocytes and its levels are up-regulated in intrahepatic cholestasis (Scheffer *et al.*, 2002). In rat liver, MRP2 protein localized to the canalicular membrane, is down-regulated after bile duct ligation or cholestasis, but there is an increase in MRP3 protein in the basolateral membrane of hepatocytes surrounding the central veins and of cholangiocytes (Donner & Keppler, 2001). The physiological role of MRP3 may be to compensate for the biliary obstruction and impaire canalicular MRP2 function by clearing cholephilic anionic substrates into the blood (Donner & Keppler, 2001).

MRP substrates

While P-gp has its greatest affinity for large, hydrophobic cations, MRP appears most effective in transporting organic anions. It has been demonstrated that MRP was able to transport glutathione, glucuronide and sulfate conjugated compounds (Jedlitschky *et al.*, 1996; Jedlitschky *et al.*, 1994). Since historically it had been reported that pretreatment of cells with buthionine sulfoximine (which depletes the intracellular GSH by inhibiting the γ-glutamylcysteine synthetase) was able to sensitize cells to anthracyclines, but difficult to demonstrate formation of glutathione conjugates, It was then hypothesized that a model for MRP included cotransport of GSH without actual glutathione conjugation (Litman *et al.*, 2001). Such a finding would indicate that the cell did not have to conjugate compounds with glutathione in order to have the compound transported out of the cell. Although the data for this model are convincing, it is not known to what extent MRP substrates are metabolized, or cotransported (Rappa *et al.*, 1997). It is well accepted that substrates for MRP include doxorubicin, vincristine and etoposide (VP-16) (Litman *et al.*, 2001). MRP overexpression has emerged in cell lines exposed to these compounds, and developing non-P-gp-mediated drug resistance. However, mice in which MRP has been genetically deleted display only an increased sensitivity to etoposide (Wijnholds *et al.*, 1997). This finding is perhaps easily explained by the overlap in substrate specificity between MRP and P-gp, and VP-16 being a better substrate for MRP than for P-gp. In a recent study by Bakos and coworkers (Bakos *et al.*, 2000), both the ATPase and transport properties of MRP1 and MRP2 were compared. Leukotriene C4 and *N*-ethylmaleimide glutathione were transported by both proteins and stimulated their ATPase activity, MRP1 having the highest affinity for these substrates. On the other hand, MRP2 was a more efficient transporter of methotrexate. Interestingly, probenecid, sulfinpyrazone, indomethacin, furosemide and penicillin G activated the ATPase activity of MRP2, whereas they acted more as inhibitors of the MRP1-associated ATPase (Litman *et al.*, 2001). From this study it was concluded that MRP1 is a more efficient glutathione conjugate transporter than MRP2, whereas the latter transports organic anions. Transfection studies demonstrated that the overexpression of MRP3 was able to confer drug resistance, whereas the overexpression of MRP 5 was not (Konig *et al.*, 1999; Kool *et al.*, 1999; McAleer *et al.*, 1999). Like MRP1 and MRP2, MRP3 has been shown to transport adriamycin, vincristine, and methotrexate. MRP2 is expressed in the liver, on the cannalicular (apical) surface of

the hepatocyte (Keppler & Konig, 2000), whereas both MRP1 and MRP3 are found on the lateral membrane (Konig *et al.*, 1999). List of MRP substrates is shown in Table 2.5.

Table 2.5 Substrates of MRP

MRP	Substrates/ Drugs
MRP1	Organic anions, GSH conjugates (LTC4), adriamycin, vincristine, methotrexate
MRP2	Organic anions, cisplatin, adriamycin, vincritine, methotrexate
MRP3	Glucuronides, bile salts, adriamycin, vincritine, methotrexate
MRP4	Organic anions, nucleotide drugs (PMEA)
MRP5	Organic anions, nucleotide analogs, GSH conjugates
MRP6	Anionic peptides (BQ-123)
MRP7	?
MRP8	?
MRP9	?

The role of MRP in drug resistance

The studies of P-gp served as a model for evaluating the role of MRP in clinical drug resistance. Similar to P-gp, the extent to which MRP plays a role in resistance has been difficult to define. It has been found that MRP mRNA is expressed in most cell and tumour types (Nooter *et al.*, 1995). Levels of expression in tumours are frequently low and correlation studies between expression and survival have frequently been unrewarding. This may be due to redundancy with the other MRPs, which have not been analyzed in the same tumour types. Whereas a functional MRP activity is detectable in acute myeloid leukemia, convincing evidence that it has a major impact on treatment outcome has not been forthcoming (Filipits *et al.*, 1999). In non-small-cell lung cancer, high levels of MRP protein expression are observed in approximately one-third of the cases, and in lung cancer cell lines, expression of MRP appears to correlate with resistance to MRP substrates including doxorubicin, vincristine and VP-16 (Young *et al.*, 2001). In primary breast cancer, significant MRP protein expression has been reported in 25–30% of samples by immunohistochemistry, and in a series of 259 patients, increased in MRP protein expression appear to confer an increased risk of treatment failure (Filipits *et al.*, 1999). Recent development of the RT-PCR-ELISA, (reverse transcription-polymerase chain reaction multiplex assay with enzyme-linked immunosorbent assay) detection system failed to confirm such correlation in 85 patient samples (Ferrero *et al.*, 2000). Whereas

expression of MRP has been detected in ovarian cancer, studies have both confirmed and rejected a correlation between outcome and expression (Yokoyama *et al.*, 1999). Thus, the role of MRP in clinical drug resistance remains poorly defined. Interestingly, and in support of a role for MRP at least in intrinsic resistance, are the studies of Allen and others in mouse cell lines in which the ortholog for MDR1 (*mdr1a* and *mdr1b*) as well as MRP had been genetically deleted. In these studies, absence of the ortholog rendered cells several fold times more sensitive than the expressing counterparts (Allen *et al.*, 2000). Knockout of MRP in these cells then resulted in an increase in sensitivity by as much as 20- fold to several drugs (Litman *et al.*, 2001). Since the control cells express only low levels of MDR and MRP, it can be concluded that the low level found in unselected cells confers a significant amount of drug resistance (Litman *et al.*, 2001).

Greater complexity has been added to the definition of the role of MRP in drug resistance by the identification of the nine additional MRP family members. The most well understood to date is MRP2 or cMOAT, which has been shown to be the bilirubin glucuronide transporter at the cannalicular membrane of the hepatocyte. Mutations in the cMOAT gene are responsible for the Dubin-Johnson syndrome of hyperbilirubinemia. Despite clear identification of this important role in normal physiology, convincing evidence for a role in drug resistance has not yet been forthcoming. There is a suggestion that cMOAT can confer resistance to cisplatin and to SN38, the active metabolite of CPT-11 (Chu *et al.*, 1997). Recently, transfection experiments with human and rat MRP2 demonstrated that expression of MRP2 confers resistance to etoposide, vincristine, cisplatin, doxorubicin and epirubicin, and that this resistance could be partially reversed by buthionine sulfoximine, suggesting a role of the intracellular glutathione level (Cui *et al.*, 1999). Antisense nucleotides to cMOAT sensitized cells 5-fold to cisplatin and over 10-fold to SN-38 (Koike *et al.*, 1997). Since cMOAT is normally involved in the pathway for heme metabolism, it is possible that it may be similarly involved in a mechanism of drug metabolism. In addition to being of major relevance in clinical resistance to anticancer drugs, MRP1 has an important and overlapping role in protecting normal mammalian tissue from a wide range of xenobiotic-induced damage (Leslie *et al.*, 2001). MRP1 was originally cloned from a multidrug resistant human small cell lung cancer cell line (Cole *et al.*, 1992). It works as an active transporter of GSH, glucuronate and sulfate conjugated organic anions such as the inflammatory mediator leukotriene C4 (LTC4), anionic drugs as methotrexate but also neutral organic drugs not known to be conjugated to acidic ligands by transporting these drugs together with free GSH (Borst *et al.*, 2000). Obvious overexpression of MRP2–5 was not found in a screen of cisplatin-resistant cell lines (Kool

et al., 1997). MRP4 and MRP5 resemble each other more closely than they resemble MRPs 1-3 and confer resistance to purine and nucleotide analogs, which are either inherently anionic, as in the case of the anti-AIDS drug. Given their capacity for transporting cyclic nucleotides, MRP4 and MRP5 have also been implicated in a broad range of cellular signaling processes (Kruh *et al.*, 2001). Taken together, the findings suggest that the MRP subfamily of ABC transporters has a role in drug disposition from the liver, and probably from other normal tissues as well, with a lack of specificity that may allow subversion for drug resistance after the onset of the malignant process.

MRP inhibitors

Inhibitor for MRP have not been as readily identified as for P-gp. Most of the second-generation P-gp antagonists have little or no effect on MRP-mediated drug efflux (Litman *et al.*, 2001). In contrast the second-generation inhibitor agent VX710 does show antagonistic ability for MRP (Germann *et al.*, 1997). Like VX-710, the compound PAK-104P, a pyridine carboxylate, is able to inhibit both P-gp and MRP in *in vitro* models (Chen *et al.*, 1999). Thus, clinical trials could be designed to treat tumours in which both MRP and P-gp may be expressed, and drugs such as doxorubicin and VP-16 may be chosen as substrates for both transporters. MK571, a leukotriene LTD4 receptor antagonist which modulates MRP-mediated multidrug resistance, has been used as a research tool for the inhibition of MRP *in vitro* (Gekeler *et al.*, 1995).

Other MRP inhibitors include difloxacin (Gollapudi *et al.*, 1997), an inhibitor of organic anion transport (probenecid) (Gollapudi *et al.*, 1997), indomethacin (Draper *et al.*, 1997), the leukotriene LTD4 receptor antagonist ONO-1078 (Nakano *et al.*, 1998), pluronic block copolymer (Miller *et al.*, 1999), the nucleoside transport inhibitor (dipyridamol)(Curtin & Turner, 1999), the progesterone antagonist RU486 (Payen *et al.*, 1999), rifampicin (Courtois *et al.*, 1999), antimicrobial agents (erythromycin and ofloxacin) (Terashi *et al.*, 2000).

2.5.2 Increase the detoxification/inactivation of drug

(A) Glutathione system

The cellular glutathione (GSH) system is a critical component of detoxification of cytostatics in the cell (Stavrovskaya, 2000). Glutathione, a non-protein thiol, can interact

via its thiol with the reactive site of a drug, resulting in conjugation of the drug with glutathione. The conjugate is less active and more water-soluble, and it is excluded from the cell with the participation of transporter proteins named GS-X (including MRP) (Stavrovskaya, 2000). Increased levels of glutathione were found in cell lines resistant to alkylating agents (e.g., nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, BCNU) (Tew, 1994). The enzymes glutathione-S-transferases (GST) catalyze the interactions between glutathione and alkylating drugs, increasing the rate of a drug detoxification. The activation of these enzymes thus can cause cellular drug resistance (Tew, 1994). In addition, enzymes which catalyze glutathione synthesis could also mediate drug resistance; however, their role in this phenomenon is not yet clarify (Stavrovskaya, 2000). Resistance of tumour cells to drugs of P-gp-MDR group (anthracyclines, vincristine) can also be connected with alterations of the GSH system (Sinha *et al.*, 1989; Tew, 1994). In cells with P-gp-MDR, increased levels of a GST isoenzyme, GST-π, is frequently found (Tew, 1994). It is probable that genes of several defense systems are coordinately regulated in these cells. Introduction of the genes of various GSTs into mammalian cells has produced some cell lines with modest levels of resistance to alkylating agents (Tew, 1994). Transfection of human GSTs genes into the cells of the yeast *Saccharomyces cerevisiae* conferred significant levels of resistance to adriamycin and chlorambucil (Black *et al.*, 1990). Inhibitors of glutathione S-transferases are considered as potent modifiers of this type of drug resistance (Stavrovskaya, 2000). Thus ethacrynic acid (usually used as diuretic), a potent inhibitor of GST, is now under clinical trial as a modulator of GSH-mediated drug resistance (Shen *et al.*, 1997). Buthionine sulfoximine decreases intracellular levels of glutathione and thereby overcomes resistance to alkylating agents (Tew, 1994). GSH is implicated in the results of the treatment of lymphomas. Increased GST activity has been observed in patients with chlorambucil-resistant chronic lymphocytic leukemia (Schisselbauer *et al.*, 1990).

(B) Dihydropyrimidine dehydrogenase

Dihydropyrimidine dehydrogenase (DPD; EC 1.3.1.2) is the initial and rate-limiting enzyme in the 3-step pathway of uracil and thymidine catabolism and in the pathway leading to the formation of beta-alanine (Diasio & Harris, 1989). Berger *et al* (Berger *et al.*, 1984) presented findings in 3 unrelated pedriatic patients with a nonspecific clinical picture of cerebral dysfunction and persistent urinary excretion of excessive amounts of uracil, thymine, and 5-hydroxymethyluracil. The excretory pattern suggested deficiency of

DPD. Autosomal recessive inheritance was supported by the finding that the parents of one patient were first cousins (Ferrero *et al.*, 2000). DPD is also the principal enzyme involved in the degradation of the chemotherapeutic drug 5-FU which acts by inhibiting thymidylate synthase (Santi *et al.*, 1974). In addition to its role in 5-FU toxicity, DPD activity may be a potential factor for controlling 5-FU responsiveness at the tumoral level (Milano & McLeod, 2000). A high level of tumour DPD would metabolise 5-FU to inactive products before cytotoxic nucleotides can be formed. The domain of fluoropyrimidines is currently expanding quickly with the clinical development of oral 5-fluorouracil (5-FU) prodrugs such as UFT, S1, capecitabine or oral 5-FU combined with 5-ethynyluracil (Meropol, 1998). DPD inhibition has become a major goal of the strategy for the development of oral fluoropyrimidines like UFT, S1 or 5-FU-ethynyluracil.

(C) Thymidylate synthase

Thymidylate synthase (TS; EC 2.1.1.45) catalyzes the reductive methylation of dUMP by CH_2H_4 folate to produce dTMP and H_2 folate (Carreras & Santi, 1995). This enzyme uses the 5,10-methylenetetrahydrofolate (methylene-THF) as a cofactor to maintain the dTMP (thymidine-5-prime monophosphate) pool critical for DNA replication and repair (Milano & McLeod, 2000). Knowledge of the catalytic mechanism and structure of TS has increased substantially over recent years. Major advances were derived from crystal structures of TS bound to various ligands, the ability to overexpress TS in heterologous hosts, and the numerous mutants that have been prepared and analyzed (Carreras & Santi, 1995). These advances, coupled with previous knowledge, have culminated in an in-depth understanding of many important molecular details of the reaction. The enzyme has been of interest as a target for cancer chemotherapeutic agents. It is considered to be the primary site of action for 5-fluorouracil, 5-fluoro-2-prime-deoxyuridine, and some folate analogs (Milano & McLeod, 2000).

Since TS is the target for 5-FU, it was hypothesized that the large variation in TS levels would be related to 5-FU sensitivity (Tanaka-Nozaki *et al.*, 2003). Correlation between TS level and 5-FU sensitivity were controversy. Grem *et al.* observed a lack of correlation between 5-FU sensitivity and TS levels in the National Cancer Institute 60-cell line panel, either evaluated as TS protein or TS-mRNA expression (Grem *et al.*, 2001). Also with Beck *et al* found a weak correlation between 5-FU sensitivity and TS levels, but in the subpanel of colon cancer cells this relation was not present (Beck *et al.*, 1994). However, there are many reports found this correlation; such as: TS catalytic activity was significantly correlated to both FdUMP binding and TS protein measured by Western

blotting as described previously (Johnston *et al.*, 1995) and TS levels were also related to TS-mRNA expression. Recently, higher TS activity in primary cultured renal cell carcinoma predicted higher sensitivity to 5-FU (Mizutani *et al.*, 2003). The lack of correlation might have several reasons, such as the relatively short drug exposure time (48 h), after which growth inhibition was determined. This continuous presence of 5-FU possibly leads to a complete inhibition of TS independently of the endogenous TS levels as was observed in cells with induced TS (Peters *et al.*, 1999). Thus, growth inhibition might be dependent on additional parameters, such as the extent of induction of DNA damage (van Triest *et al.*, 1999). Alternatively, sensitivity to 5-FU might also be dependent on other parameters in addition to TS levels, such as toxicity mediated by incorporation of 5-FU into RNA. Scherf *et al.* (Scherf *et al.*, 2000) investigated a large panel of mechanistically different drugs using cluster analysis after running microarrays. 5-FU clustered with drugs with an RNA-directed effect, rather than with other TS inhibitors. It seems that very high amplified TS levels as found in cells with acquired resistance to 5-FU are only related to 5-FU sensitivity.

CHAPTER III

RESEARCH METHODOLOGY

3.1 HUMAN CHCA CELL LINES AND CELL SUBCULTURE

Five established CHCA cell lines were kindly supplied by Assoc.Prof.Dr.Banchob Sripa; Department of Pathology, Faculty of Medicine, Khon Kaen University. These cell lines include KKU-100 and KKU-055 (poorly differentiated adenocarcinoma), KKU-M156, KKU-M214 (moderately differentiated adenocarcinoma) and KKU-OCA17 (well differentiated adenocarcinoma). All cell types grew in an adherent manner in Ham's F12 medium (Invitrogen) and supplemented with 10% heat inactivated fetal calf serum (Seromed), 100 U/ml of penicillin G (Invitrogen) and 100 µg/ml streptomycin (Invitrogen). Cells were incubated in a 5% CO_2 incubator at 37°C and subcultured twice a week. At 70% confluence, cells were detached from culture flask using trypsin-EDTA and collected for further experiments.

3.2 CHEMOTHERAPEUTIC DRUGS

Seven chemotherapeutic drugs were given by Assoc.Prof.Vajarabhongsa Bhudisawasdi; Department of Surgery, Faculty of Medicine, Khon Kaen University, as shown in Table 3.1.

Table 3.1 Chemotherapeutic drugs that were used in this project.

Generic name	Molecular weight
5-Flurouracil (I.V)	130
Etoposide (I.V)	589
Cisplatin (I.V)	300
Oxaliplatin (I.V)	395
Irinotecan (I.V)	623
Paclitaxel (I.V)	854
Doxorubicin (I.V)	558

3.3 DRUG SENSITIVITY ASSAY

The assay on CHCA cell lines to chemotherapeutic drugs was determined by cytotoxic test using the CellTiter 96 Aqueous Assay reagents (MTS assay, Promega). Assays were performed in 96-well plates at a cell density of 5,000/well. Drugs, diluted in 2-fold dilution, were added to each well containing cells in triplicate manner, 72 hours exposure. In this homogenous, colorimetric assay 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) was reduced to a soluble formazan in the presence of an electron-coupling reagent (phenazine ethosulfate; PES) as a result of (succinate) dehydrogenase activity found in metabolically active cells. Absorbance by formazan at 492 nm measured in an ELISA reader (TECAN Austria Ges.m.b.H) is directly proportional to the number of viable cells. The percent cell viability was calculated using this formula: [(Ti)/(C)]x100, Ti = OD of test growth in the present of drug at the end point, C = OD of control growth without drug. At the drug concentration which causes 50% cell death was defined as the IC_{50} (IC = growth inhibition concentration) (Mizutani *et al.*, 2002). The plot of cell viability versus drug concentration was shown as Figure 3.1.

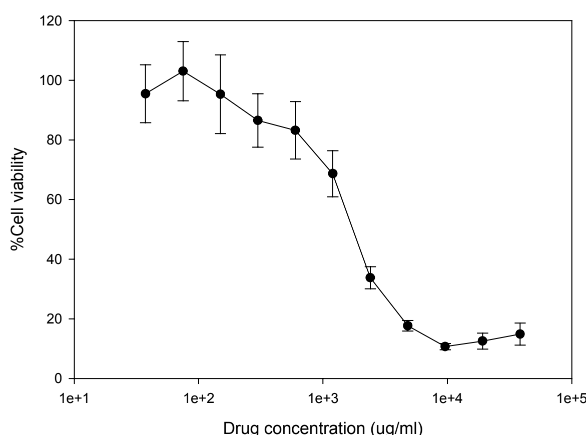

Figure 3.1 Plot between % cell viability and drug concentration in µg/ml. Inhibition concentration was determined as IC_{50} defined from the drug concentration which allowed 50% cell survival.

3.4 DRUG COMBINATION ASSAY

Twenty five µl of 1st with series-fixed concentrations and 25 µl of 2nd drug with varied concentration and diluted in 2-fold dilution, were added into 50 µl of 5,000 cells per well, in triplicate manner, 72 hours exposure. The OD measurement and % cell viability were calculated as the same manner as described in 3.3. The new IC_{50} of paclitaxel and/or

cisplatin has been examined with or without adding 5-FU in corresponding panel. The graph between concentrations of a fixed drug (x-axis) and IC_{50}s of a varied drug (y-axis) was plotted.

Table 3.2 The layout of drug combination adding on 96-well plate

Fixed 1st drug conc. -------------→

		5-FU 0 μM			5-FU 381 μM			5-FU 763 μM			5-FU 1,527 μM		
Varied 2nd drug (Paclitaxel)		1	2	3	4	5	6	7	8	9	10	11	12
0 μM	A												
3.8 μM	B												
7.3 μM	C												
14.6 μM	D												
29.3 μM	E												
58.5 μM	F												
117 μM	G												
	H												

3.5 APOPTOSIS ASSAY

Apoptotic cells were obtained by culturing cells in 96-well plate with or without drugs. After 72 hours exposure, cells were visualized under inverted microscope and photographed. Cell death detection ELISAplus kit was then used for measuring the level of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) after induced cell death by apoptosis, following the manufacturer's instruction (Roche Applied Science). Brief protocol was as follows. A hundred microliters of CHCA cell lines (5,000 cells/well, duplicated) were incubated with 100 μl anticancer drugs for 72 hr corresponding to the time of drug exposure in cytotoxicity assay. A microtiter plate (MP) containing incubated cells was then centrifuged with 200xg at 15°C. Cell pellet containing DNA fragments was lysed by resuspending in 200 μl lysis buffer and incubated for 30 min at 15-25°C. Lysate was subsequently centrifuged at 200xg for 10 min. Twenty μl of cytoplasmic fraction was carefully transferred to the streptavidin coated MP for analysis. The ELISA was developed and evaluated with the use of 20 μl sample and 80 μl immunoreagent per MP-well. Twently μl of samples including cytoplasmic fraction (lysate) of treated cells, culture supernatants after centrifugation of treated cells, positive control, negative control

(culture supernatant and lysate after centrifugation of untreated cells), and background control (incubation buffer) were added into the MP. Eighty µl of the immunoreagent was then added to the MP. The MP which was covered with an adhesive cover foil was incubated on a MP shaker under gently shaking (300 rpm) for 2 hr at 15-25°C. After the time of incubation, the solution was removed thoroughly by tapping or suction. Each well was 3 time-rinsed with 250 µl incubation buffer by gently removing solution. A hundred µl of ABTS solution was pipette into each well and the MP was incubated on a plate shaker at 250 rpm 15-25°C until the color development was sufficient for a photometric analysis (approximately after 10-20 min). The O.D. was measured at 405 nm against ABTS solution as a blank (reference wavelength was approximately 490 nm). Data analysis was done as the following calculation in Table 3.3.

Table 3.3 The data analysis of ELISA

Step	Action
1	Average the values from the double absorbency measurements of the samples.
2	Subtract the background value (incubation buffer + ABTS solution) of the immunoassay from each of these averages.
3	Calculate the specific enrichment of mono- and oligonucleosomes released into the cytoplasm from these values using the following formula: Enrichment factor = $\frac{\text{mU of the sample (dying/dead cells)}}{\text{mU of the corresponding negative control (cells without drug treatment)}}$

The results of the experiment after analysis of the samples by the ELISA were plotted between absorbency [$A_{405nm} - A_{490nm}$] and drug concentration (µM).

3.6 RNA EXTRACTION AND SEMI-QUANTITATIVE RT-PCR REACTION

Total RNA was extracted from five human CHCA cell lines ($2x10^6$ cells) using QIAamp® RNA blood mini kit, following the manufacturer's instructions (QIAgen), and quantified by spectrophotometry. Total RNA (5 µg) from each cell line was reverse-transcribed in 50 µl containing 250 pmole of oligo$(dT)_{15}$ primer (Promega), 40 units of rRNasin ribonuclease inhibitor (Promega) and 250 units of M-MLV reverse transcriptase (Promega) in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 0.5

mM dNTPs. Initially, total RNA and oligo(dT) were mixed together and heated at 70°C for 10 min and immediately chilled on ice. Other reagents was then added and incubated for 15 min at 30°C. First-strand cDNA were synthesized at 42°C for 60 min. PCR amplification was performed using specific primers for genes of interest. The PCR primers was designed based on human cDNA sequences including GST-π, MDR1, MRP1 and MRP2 were selected with Vector NTI Suite 8 software (InforMax, Inc.). MRP3, TS, MRP1, DPD and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were selected from respective references (Ishikawa *et al.*, 1999; Nies *et al.*, 2001; Takechi *et al.*, 1998). The PCR primer sequences were shown in Table 3.4.

Table 3.4 Primers used in RT-PCR

Gene	Sense (5') primer	Antisense (3') primer	PCR product (bp)
TS	5'-GAATCACATCGAGCCACTGAAA-3'	5'-GTGTTACTCAGCTCCCTCAGA-3'	579
DPD	5'-TCCTCCAGGTATGCAGTGCCA-3'	5'-GTTATGGTGGGCAGGTGGGTT-3'	514
GST-π	5'-TACGGGCAGCTCCCCAAGTT-3'	5'-TGCCCGCCTCATAGTTGGTG-3'	199
MDR1	5' GTCTTTGGTGCCATGGCCGT-3'	5'-ATGTCCGGTCGGGTGGGATA-3'	206
MRP1	5'-CTGACAAGCTAGACCATGAATGT 3'	5'-CCTTTGTCCAAGACGATCACC-3'	262
MRP2	5'-GCCAGATTGGCCCAGCAAA-3'	5'-AATCTGACCACCGGCAGCCT-3'	202
MRP3	5'-GGGACCCTGCGCATGAACCTG-3'	5'-TAGGCAAGTCCAGCATCTCTGG-3'	453
GAPDH	5'-CAACAGCCTCAAGATCATCAGC-3'	5'-TTCTTAGACGGCAGGTCAGGTC-3'	328

Optimization of number of cycles and of first stranded cDNA concentration was determined for linear amplification condition. The expression levels of mRNA were measured with human GAPDH mRNA as the internal standard by being co-amplified to each gene in order to minimize tube-to-tube variation in the efficiency of PCR. PCR reaction was carried out in a final volume of 25 µl containing first-strand cDNA (1:20 dilution), 10 pmole of each primer, 2 pmole of each GAPDH primer, 1.25 units of Superscript II *Taq* polymerase (Invitrogen) in 2.5 µl of 10x *Taq* buffer, 2 mM $MgCl_2$ and 0.2 mM dNTPs, using a RoboCycler gradient 40 (Stratagene). The amplification was initiated by one cycle at 94°C for 3 min followed by 28 cycles of denaturation at 94°C for 1 min, a 1-min annealing at 62°C (GST-π, MRP1, MRP2 and MDR1) or 56°C (TS, DPD and MRP3) and polymerization at 72°C for 1 min and a final 10-min extension at 72°C.

PCR products were separated on Tris-borate-EDTA 2% agarose gel (Seakem LE) containing ethidium bromide 100 ng/ml. Gels were visualized on a UV transilluminator and photographed on ULTRALUM Dual Light Transilluminator. The bands were scanned with an image scanner (CANON) and analyzed with Image Master 1D (Pharmacia Biotech). The relative amount of mRNA was expressed as the ratio of a gene of interest to GAPDH.

3.8 COMBINATION OF DOXORUBICIN WITH MRP/MDR INHIBITOR

Cells were incubated with different concentrations of MRP/MDR inhibitor and doxorubicin. The tested inhibitor was verapamil (0.1, 1 and 10 μM). The IC_{50} changed was compared between doxorubicin alone and doxorubicin + verapamil. The procedure was followed similarly as Drug combination assay in 3.4.

3.9 STATISTICAL ANALYSIS

Statistical analysis was performed using Microsoft Excel 97 version 8.0 for mean± S.D. Student *t*-test used for observing statistical differences of IC_{50} changed between single and combined drugs (inhibitors) and linear regression determined for the relationship between IC_{50} and ratio of gene expression were performed by Sigma Plot version 8.0. The level of statistical significance was set at 0.05.

CHAPTER IV

RESULT

4.1 CYTOTOXIC EFFECT STUDIES

The cytotoxic effects of 6 chemotherapeutic agents on 5 human intrahepatic CHCA cell lines were evaluated using the MTS assay. Different degrees of drug sensitivity were obtained from different CHCA cell lines (Table 4.1). Different drug sensitivity was however obtained in the same histological type, such as KKU-100 and KKU-M055, or KKU-M156 and KKU-M214. The most sensitive cell line to most drugs was KKU-M055, which was classified as poorly differentiated CHCA whereas the most resistant cell was KKU-100, which was classified as the same type as KKU-M055. KKU-100 was the most sensitive cells to doxorubicin (2.3 μM), but it was the most resistant cell to etoposide (416 μM) and 5-FU (1,020 μM). KKU-M156, which was moderately resistant to most compounds, showed high sensitivity to doxorubicin (2.69 μM) and oxaliplatin (4.7 μM) but it was highly resistant to etoposide (273 μM), cisplatin (222 μM) and 5-FU (121 μM). KKU-M214 was highly sensitive to doxorubicin (0.25 μM) and paclitaxel (2.62 μM). It was shown that KKU-M214 was mildly resistant to irinotecan, oxaliplatin, etoposide and 5-FU. KKU-M214 showed the highest resistance to cisplatin (130 μM) compared to IC_{50} of other drugs. KKU-OCA17 was sensitive to most drugs in average range of 0.84-12.5 μ M but it was markedly resistant to etoposide (125 μM). It was the most sensitive cells to 5-FU (3.46 μM) compared to other cells. The last cell line, KKU-M055, was the most sensitive cells to most drugs. It showed markedly sensitive to paclitaxel (4 nM), doxorubicin (40 nM), cisplatin (50 nM) and irinotecan (3.73 μM) but it was mildly resistant to 5-FU (46 μM). Regarding to the potency of each compound to the cytotoxic effect on CHCA cells, we defined that drugs with high potency should be a concentration below 5 μM. Results revealed that doxorubicin had the highest potency but 5-FU showed the least potency due to its cytotoxicity was much higher than 5 μM up to 1,020 μM. Only one cell line that was sensitive to 5-FU was KKU-OCA17. Oxaliplatin was given less potency in KKU-100 and KKU-M214 but it showed high potency in KKU-M055. Etoposide had less potency obtained from KKU-100, KKU-M156, KKU-M214 and KKU-

OCA17. Histopathological types of CHCA cell lines did not show any significant relationship with drug sensitivities.

Table 4.1 IC_{50} values for chemotherapeutic agents in five intrahepatic CHCA cell lines

Drugs		CHCA Cell lines				
		Poorly differentiated		Moderated differentiated		Well differentiated
		KKU-100 IC_{50}	KKU-M055 IC_{50}	KKU-M156 IC_{50}	KKU-M214 IC_{50}	KKU-OCA17 IC_{50}
5-Fluorouracil	μM	1020±290	46±9	121±52	29±16	3.5±0.83
Etoposide	μM	416±119	0.04±0.01	273±20	54±21	125±78
Cisplatin	μM	37±3	0.05±0.01	222±31	130±10	6.2±0.16
Oxaliplatin	μM	71.7±31.5	0.11±0.00	4.7±2.0	38±5	6.8±1.1
Irinotecan	μM	23.3±5.0	3.7±0.42	13.1±1.8	43±7	3.6±0.95
Paclitaxel	μM	39±5.5	0.0004±0.0003	10.9±4.7	2.6±0.61	12.5±1.7
Doxorubicin	μM	2.3±0.71	0.04±0.02	2.7±0.94	0.25±0.05	0.84±0.76

The IC_{50} represents the mean±S.D. of triplicate experiments.

4.2 DRUG COMBINATION STUDIES

4.2.1 Drug combination between 5-FU and cisplatin

Drug combination was analysed by constructing an inhibitory response graph. Fixed concentration of cisplatin at 10.8 μM was mixed with a series of 5-FU concentration (0-3,077 μM). KKU-100 cells were incubated with such 2 drugs for 72 hr and measured O.D. at 492 nm for MTS reaction. Percent cell viability of each culture with drugs was referred to a culture without any drug. Cytotoxic effect was shown in Figure 4.1. The synergistic effect was significantly observed when increasing 5-FU concentration. At 5-FU concentration was 769 μM, that was mixed with 10.8 μM cisplatin, gave the new IC_{50} that was 2-fold less than those using 5-FU alone. Result suggested that cisplatin could reduce the amount of 5-FU when used in CHCA treatment.

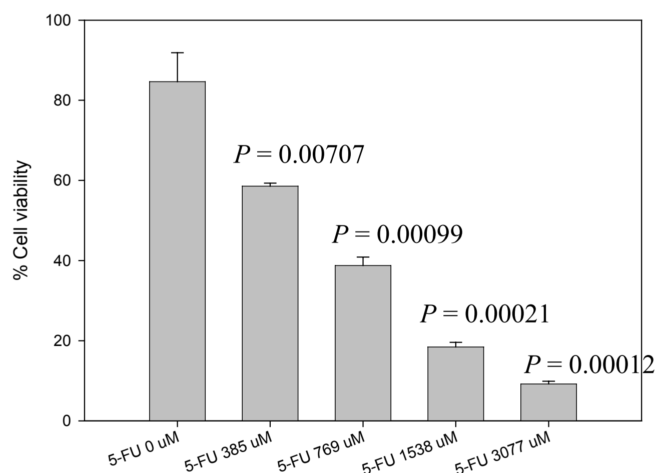

Figure 4.1 Cytotoxic effect on KKU-100 when incubated with 5-FU (varied doses) and cisplatin (fixed single dose = 10.8 μM).

4.2.2 Drug combination between 5-FU and paclitaxel

Fixed concentration of paclitaxel at 3.81 μM was mixed with a series of 5-FU concentration (0-3,053 μM). KKU-100 cells were incubated with such 2 drugs for 72 hr and measured O.D. at 492 nm for MTS reaction. Percent cell viability of each culture with drugs was referred to a culture without any drug. Cytotoxic effect was shown in Figure 4.2. The synergistic effect was significantly observed when increasing 5-FU concentration from 763 μM to 3053 μM. At 5-FU concentration was approximate 800 μM, that was mixed with 3.81 μM paclitaxel, gave the new IC_{50} that was 2-fold less than those using 5-FU alone. Result suggested that paclitaxel could reduce the amount of 5-FU when used in CHCA treatment.

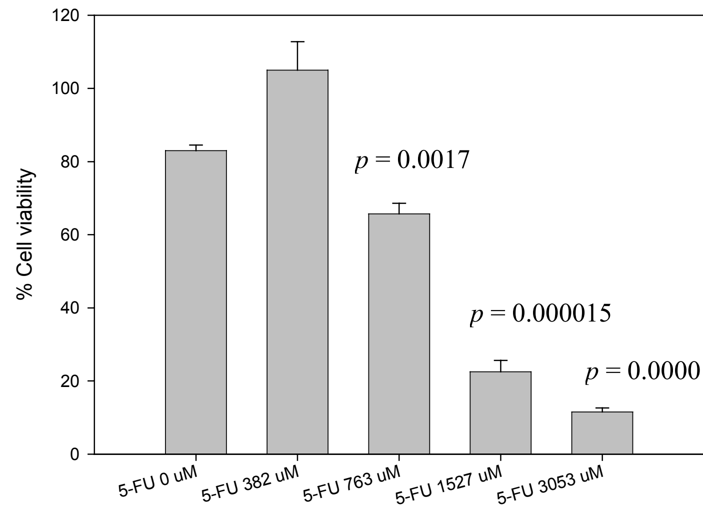

Figure 4.2 Cytotoxic effect on KKU-100 when incubated with 5-FU (varied doses) and paclitaxel (fixed single dose = 3.81 μM).

4.3 APOPTOSIS ASSAY

4.3.1 Morphology of apoptotic cells

Apoptotic cells were obtained by culturing cells in 96-well plate with or without drugs. After 72 hours exposure, cell were visualized under inverted microscope and photographed. Result showed that the apoptotic bodies were obtatined when KKU-100 cells treated with 5-FU (1,020 μM) as shown in Figure 4.3.

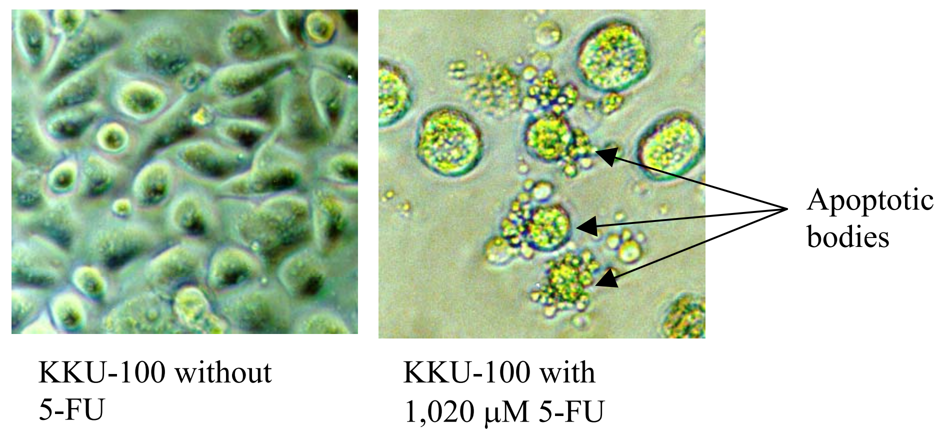

Figure 4.3 Apoptosis detection when exposed KKU-100 with 5-FU (1020 μM) for 72 hr. Apoptotic bodies were obtained as indicated with arrows.

4.3.2 In vitro determination of cytoplasmic histone-associated-DNA-fragments

To determine the level of DNA fragments generated after induced cell death by apoptosis, the detection of cytoplasmic histone-associated-DNA-fragments was performed and the result was shown in Figure 4.4

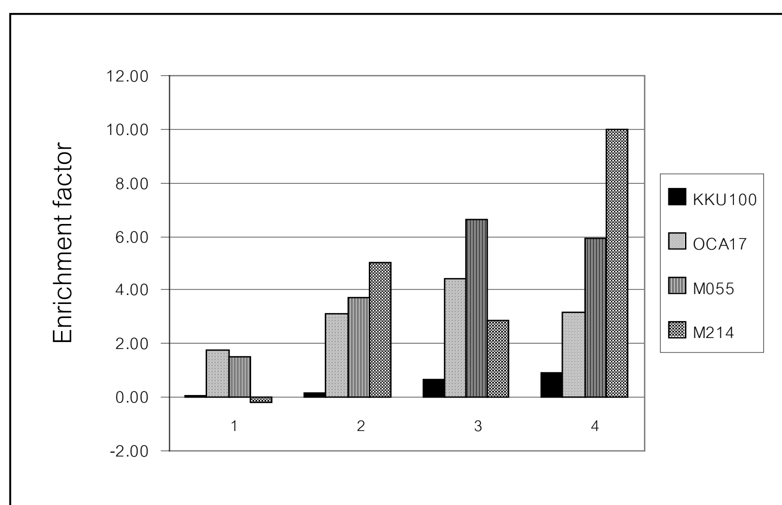

Figure 4.4 Enrichment of nucleosomes in the cytoplasm of CHCA cells treated with 5-FU. 1 = Culture supernatant treated with 25 µM 5-FU, 2 = culture supernatant treated with 100 µM 5-FU, 3= cell lysate treated with 25 µM 5-FU and 4 = cell lysate treated with 100 µM 5-FU.

Four CHCA cell lines including KKU-100, KKU-OCA17, KKU-M055 and KKU-M214 were treated with 25 and 100 µM of 5-FU for 72 hr. Cell death by apoptosis was assayed by the detection of nucleosome composed of histone-associated DNA fragments. Results showed that 5-FU caused cell death by apoptosis in both 25 and 100 µM concentrations. Cells treated with 100 µM gave higher amount DNA fragments than those treated with 25 µM, indicating dose-dependent apoptosis. DNA fragments were also detected from culture supernatant in both treated with 25 and 100 µM due to it was released from cells to culture medium.

Linear regression analysis was performed to make the association between 5-FU sensitivity (IC_{50} values) and the level of apoptosis. Results demonstrated that four CHCA cell lines containing different IC_{50} values had a moderately significant association with the enrichment of nucleosomes (r = 0.776) as shown in Figure 4.5.

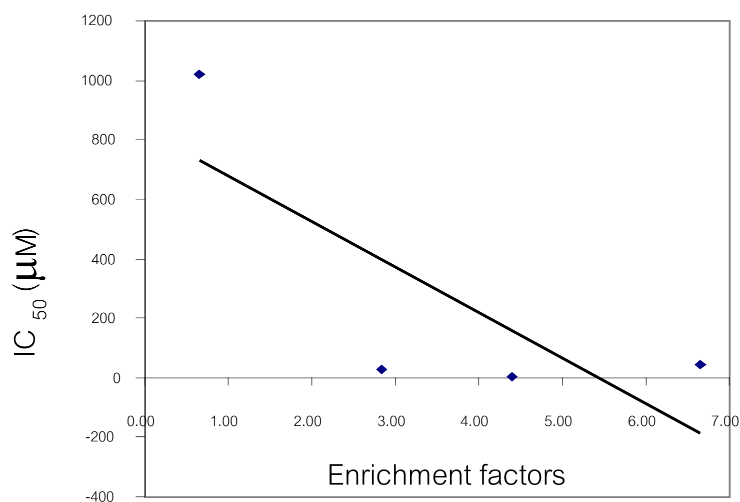

Figure 4.5 Association between IC_{50} values of 5-FU treated CHCA cell lines and enrichment factors which represented for the level of apoptosis. The moderate significance was obtained from linear regression analysis (r = 0.776).

4.4.1 Expression of mRNAs for TS, DPD, GST-π, MDR1, MRP1, -2 and –3

Gene expression of TS, DPD, GST-π, MDR1, MRP1, -2 and –3 in CHCA cell lines was investigated using semi-quantitative RT-PCR. Expression of mRNA in CHCA cells was determined using primer sets specific to these genes as described in Materials and methods. As shown in Figure 4.6A, mRNA for TS, DPD, GST-π, MDR1, MRP1, -2 and –3 were detected in all cells. High level of MDR1 was observed in KKU-OCA17 but not in other cell lines. All cells expressed low level of MRP2 mRNA. MRP3 mRNA was moderately expressed in KKU-100, KKU-M156, KKU-OCA17 and KKU-M214, but was rarely observed in KKU-M055.

4.4.2 Association between relative mRNA expression levels and the IC_{50} values

As shown in Figure 4.6 B-C, strong significant associations were found between MRP3 mRNA expression and the IC_{50} value of doxorubicin (r = 0.94, P < 0.05) and etoposide (r = 0.95, P < 0.05). No associations were observed for GST-π, MRP1and MRP2 and IC_{50} values of the anticancer drugs tested (data not shown). No significant correlation was found between TS (r = 0.13) and DPD (r = 0.11) with 5-FU IC_{50} value.

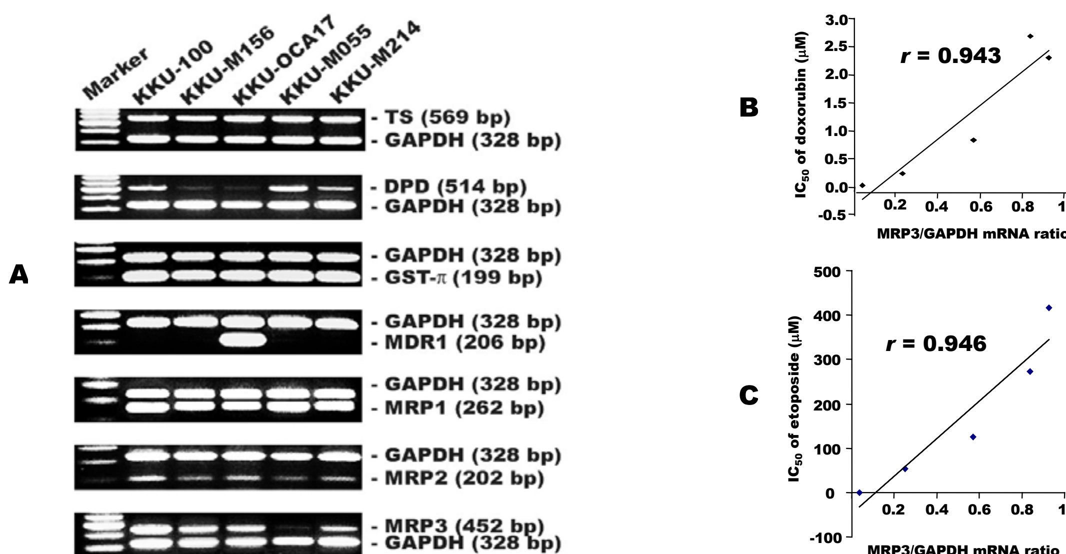

Figure 4.6 A, Gene expression of TS, DPD, GST-π, MDR1, MRP1, -2 and –3 in CHCA cell lines. GAPDH mRNA was an internal control. **B-C,** Linear regression analysis of relative mRNA expression of MRP3 and (A) doxorubicin and (B) etoposide in five human CHCA cell lines.

4.5 THE REVERSE DRUG RESISTANCE

As shown in Figure 4.7, verapamil decreased IC_{50} of doxorubicin gradually. Verapamil at 0.1, 1 and 10 μM reduced IC_{50} of doxorubicin to 2.3, 1.9 and 0.6 μM, respectively compared to doxorubicin alone (IC_{50} = 2.9 μM).

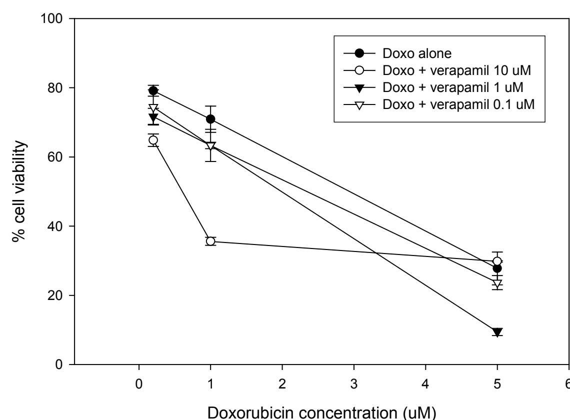

Figure 4. 7 Effect of verapamil on IC_{50} of doxorubicin

Verapamil at 0.1, 1 and 10 mM was added with varying concentration of doxorubicin to KKU-100 CHCA cell lines. Cells and drugs with and without verapamil were incubated for 72 hr. MTS assay was performed to analyse cell viability. Each point represents the mean±S.D of data from three wells.

CHAPTER V

DISCUSSION AND CONCLUSION

We established the *in vitro* drug sensitivity to test against human CHCA cell lines, which are the intrahepatic type of CHCA, preferentially occurred in Northeastern Thailand. We investigated the sensitivity of human CHCA cells to different anticancer agents including drugs frequently used in chemotherapy regimens for CHCA (5-FU) (Asamoto *et al.*, 2001; Gil-Benso *et al.*, 2001), as well as new drugs in order to provide a preclinical rationale for a new local treatment of this cancer. Our results demonstrated that CHCA cells had varying degrees of drug susceptibility. All cell lines were markedly sensitive to new drugs including doxorubicin, paclitaxel and campthothecin derivative (irinotecan). On the other hand, the inherent resistance of CHCA cells to 5-FU was observed, corresponding to poor response rates generally obtained in CHCA patients treated with this chemotherapy (Hasuike *et al.*, 1999). Resistance to 5-FU in CHCA cells was to a much higher degree than that observed in human colon carcinoma cells (HCC-48, IC_{50} = 8.6 μM) and human colon adenocarcinoma cells (COLO201, IC_{50} = 16 μM) (Nishiyama *et al.*, 1999). Low sensitivity was observed for most cell lines to other groups of drug, which have hardly been used in CHCA treatment (oxaliplatin and etoposide).

Drug combination between 5-FU and other anticancer agents was also demonstrated in this study. As it has been frequently indicated that 5-FU is a standard regiment to treat CHCA patients, it has been proved that the resistance to 5-FU occurred in most cases. To overcome the resistance of 5-FU, drug combination must be considered to perform. Results showed that 5-FU combined with either cisplatin or paclitaxel can reduce IC_{50} of 5-FU dramatically. Likewise, adding 5-FU to cisplatin or paclitaxel can reduce IC_{50} of each drug. These data suggest that drug combination between 5-FU and cisplatin or paclitaxel may be managed in clinical treatment of CHCA.

We have also investigated the expression of genes involved in chemotherapeutic drug resistance. Genes of TS, DPD, GST-π, MRP-1. -2, and –3 were expressed in all CHCA cells albeit at different levels. MDR1 expression was found only in one cell line. To determine whether those genes could be candidates for determining drug resistance phenotype in CHCA cells, linear regression analysis was performed and results

demonstrated that the expression of MRP3 is significantly correlated with resistance to doxorubicin and etoposide suggesting that this may pose a suitable target in drug reversal studies. Results confirmed that verapamil, the inhibitor of MDRs and MRPs was markedly reduced IC_{50} of doxorubicin. Due to MRP3 associates in the drug sensitivity of doxorubicin, verapamil blocks MRP3 and subsequently improves its sensitivity. No correlation was observed between the intrinsic expression of TS and DPD in five CHCA cell lines with the cytotoxic effect of 5-FU, controversy to data reported in SK-ChA-1 that, a metastatic CHCA cell line (Ajiki *et al.*, 2001). Further investigations of TS and DPD gene expression under 5-FU drug exposure need to be performed.

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