. CHAPTER V
DISCUSSION

CCA are the highest incident liver cancer especially in Northeast Thailand (Srivatanakul, 2001). Not only surgical resection of all detectable tumors improves five-year survival of the patients but chemotherapy or the other palliative therapies could also improve the survival of these patients. To date, evidence from clinical studies demonstrate that the response rate of CCA to chemotherapy is relatively poor with partial response of only 10-20 % of cases for single chemotherapy (Lee, 2004; Martin, 2003; Patt, 2001). Therefore, better understanding in mechanism of drug sensitivity to chemotherapy in CCA is very essential. 5-FU is the commonly used chemotherapy in CCA, we therefore investigated the correlation of enzymes involved in the 5-FU function and the sensitivity of CCA tissues to 5-FU as well as explore the determinants that can be use as a predictor of 5-FU responsiveness *in vitro.*

The response of CCA to 5-FU in the present study was determined using a technique called "Histoculture Drug Response Assay" which cultures a three-dimensional tumor tissue in culture medium instead of a culture of free cells. This assay maintains inter-cellular contact and interaction with stromal cells in a condition of native architecture, and thus is believed to assess the chemosensitivity of individual tumors in a state that close to the *in vivo* condition (Hasegawa, 2007). It has been shown in several cancers such as head and neck, stomach, colon, urinary tract, ovary, lung and breast that HDRA chemosensitivity could predict clinical outcomes with high sensitivity in cancers (Ariyoshi, 2003; Hirano, 2001; Isshi, 2002; Ohie, 2000; Singh *et al.*, 2002; Tanino, 2001). Consistent with the previous studies, the present study demonstrated that the success rate for culturing CCA tissues was 90.3 % (28 of 31). However, the HDRA requires large amount of specimens so only tissues from hepatectomy could be used but not from the needle biopsy. In addition, there was no contamination in all of the CCA tissues that we cultured.

In several reports, cell viability in cancer tissue after culture using HDRA is determined by MTT colorimetry. MTT assay is based on the principle of the

reduction of MTT by mitochondrial succinate dehydrogenase which presented only in living cell, resulting in the production of the colored compound formazan which could be measured by spectrophotometry. Although this MTT assay seems to be convenient, rapid, and economical (Yasuda, 1998), in the present study we found that the MTT signal was not directly correlated with viability of CCA cells. These may due to the fact that other stromal cells particularly fibroblast cells can also give rise to MTT signal. In addition, these fibroblasts were less sensitive to 5-FU than CCA cells therefore MTT colorimetric signal obtained from cancer cells and normal stromal cells could not be differentiated. Therefore, we decided to evaluate the viability of cancer cells by estimation the percentage of viable cancer cells in the CCA tissue based on their morphology under microscope. In the present study, the CCA tissues were fixed with formalin and stained with H&E before and after 4 day-culture in the culture medium with or without 5-FU. By using this technique, the cancer cells could easily be distinguished from other cells. However, this method was time consuming and the scientist who estimated the percentage of the cells need to be trained.

By using HDRA technique for CCA tissues, we could demonstrated the dose response curve of 5-FU. At 5-FU concentration ranging from 100-1,000 μg/ml of culture medium, the relationship between % inhibition index (I.I.) and 5-FU concentrations were observed (Figure 11). However, the responses of CCA tissues to 5-FU at 400 and 1,000 μg/ml of culture medium were not significant different. The liner relationship between the response of CCA tissues to 5-FU was observe at the 5-FU concentration range from 100 to 400 μg/ml of culture medium. According to the dose response curve, the concentration of 200 μg/ml and 50% I.I. were selected as a cut-off drug concentration for evaluating 5-FU sensitivity in CCA tissues. Prior studies from gastric cancer has identified the HDRA to have a significant predictive value by using 5-FU concentration of 150 μg/ml (Kodera, 2007). In breast cancer, 5-FU concentration of 300 μg/ml with 60% cut-off I.I. was used (Kakimoto, 2005). According to the evaluation of optimal drug concentration in head and neck cancer (HNC), the dose response curve between % I.I and 5-FU at the concentration ranging from 60-1500 μg/ml was constructed. The liner relationship between the response of HNC tissues to 5-FU was observe at the 5-FU concentration range from

60 to 300 μg/ml of culture medium, according to the dose response curve, 5-FU concentration of 120 μg/ml seems to be optimal for HNC (Hasegawa, 2007).

Expressions of several target genes involved in metabolism and action of 5-FU (i.e. TS, TP, DPD and OPRT) have been proposed as determinants for the response of several cancers to 5-FU. However, there is no data about the expression of these genes are available for CCA. In the present study, level of expressions of TS, TP, DPD, OPRT as well as house keeping gene, GAPDH were determined in 35 CCA tissues. The level of expressions of DPD and TP mRNA among individual samples were markedly different (169-fold; range 0.04-6.76 and 100-fold; range 0.03-3.01, respectively). The markedly difference in DPD expression has also been found in colorectal cancer (204-fold; range 0.02-4.08) (Inoue *et al.*, 2005) and esophageal carcinoma 1,353-fold; range 0.01-13.53) (Ando 2007). Similarly, the level of TP mRNA expression was also markedly difference in esophageal carcinoma (1,780-fold; 0.01-17.8) (Ando 2007).

Compare to DPD and TP, the expression level of TS and OPRT mRNA among CCA tissues were only moderately difference (31-fold; range 0.04-1.22 and 34-fold; range 0.03-1.02, respectively). The moderate difference of OPRT expression levels were similar as those observed in 75 gastric carcinoma tissues (12.9-fold) (Sakurai, 2006). In addition, a moderately different in TS expression has been observed in colorectal (24.3-fold) (Inoue, 2005).

These high variations in DPD and TP mRNA expression and moderately variations in TS and OPRT mRNA expression may resulting from the genetic polymorphism of DPD, TP, TS and OPRT genes in CCA patients and this polymorphism may affect the expression of these genes in CCA patients (Kawakami *et al.*, 1999). In addition to hypoxia and cytokines, genetic changes may elevate TP. Elevated TP will favour tumor progression by increasing angiogenesis and thymidine salvage (Brown, 1998). The evidence of TP gene mutations in tumor cell is also reported. Loss of chromosome 22, on which the TP gene is located, has been observed in Kaposi sarcoma, a disease characterized by up-regulation of TP (Kindblom *et al.*, 1991). In the mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) patients, up to 21 distinct TP mutations have been identified from 27 probands (Gamez, 2002; Kocaefe *et al.*, 2003; Nishino *et al.*,

2000). Mutations in the TP gene within the chromosome segment 22q 13.32-qter were identified as the cause of MNGIE phenotype and compound heterozygous and homozygous mutations diminish leukocyte TP activity to less than 5% of normal control (Nishino *et al.*, 1999). These results imply that TP mutation may affect TP gene expression and TP activity.

A molecular basis for DPD deficiency has been identified in several patients with severe 5-FU toxicity (Ridge *et al.*, 1998a). A G to A mutation in the exon 14 splice donor site has been identified which leads to skipping of the 165 bp exon (Ridge *et al.*, 1998b; Wei *et al.*, 1996). About 13 polymorphisms of DPD gene have been indentified in a Japanese population, of which 9 were previously unknown. A novel polymorphism 2303 C→A in exon19 results in change of an amino acid at 768 from Thr to Lys (Okamoto, 2005). However, the influence of the different polymorphisms on DPD protein expression, activity, and substrate specificity is still unknown.

The human TS gene is polymorphic with either double-or triple-tandem repeats of a 28-bp sequence downstream of the cap site in the 5′-terminal regulatory region (Horie *et al.*, 1995). In *in vitro* studies, the activity of a reporter gene linked to the 5′-terminal fragment of the TS gene with triple-tandem repeats was 2.6 times higher than that with double-tandem repeats (Horie *et al.*, 1993). Thus, this polymorphic region appears to be functional and may modulate TS gene expression. TS protein expression in gastrointestinal cancer was found to be associated with this TS polymorphism in the 5′- untranslated region (Horie, 1995). It is also suggested that this TS polymorphism may be a significant predictor of TS gene and protein expression (Leichman, 1997; Pullarkat *et al.*, 2001). Pullarkat *et al.* reported that higher intratumoral TS mRNA expression was observed with an increasing number of the 28-bp tandem repeats (Pullarkat, 2001). Recently, the molecular mechanism by which the tandem-repeat polymorphism enhances transcription has been studied. Mandola *et al* identified new proteins, USF-1 and-2, that bind within the tandem-repeat polymorphism of TS 5′-regulatory region. They also found a novel single-nucleotide polymorphism (G→C) within the tandem repeats that determines the binding and transactivating ability of USF complex (Mandola *et al.*, 2003). In a population-based experiment, this novel single-nucleotide polymorphism was

commonly found in non-Hispanic whites, Hispanic whites, African Americans, and Singapore Chinese. Recent studies have shown TS polymorphism to vary among world populations (Marsh and McLeod, 2001). Reportedly, triple-tandem repeat (L/L) is significantly higher in Asian populations compared with other ethnic groups. TS polymorphism was found to be a significant prognostic factor for patients with stage II and III colon cancer. Patients with homozygous for the triple repeat had a worse prognosis than those with heterozygous or homozygous for the double-repeat variant (Suh *et al.*, 2005). These results imply that triple-tandem repeat of a 28-bp sequence may produce a higher level of TS protein in the tumor tissue. The other polymorphism has been described in the TS gene was a 6-bp deletion at the 3′UTR region (Horie, 1995; Kawakami *et al.*, 2001; Ulrich *et al.*, 2000). Regarding the TS-3′UTR polymorphism, the genotype del-6 bp/del-6 bp was only present in patients showing a poor response to the treatment (Salgado *et al.*, 2007). It would be possible that the deletion of 6 bp in the 3′UTR region affects mRNA stability or secondary RNA structure, and could thus ultimately affect protein levels and/or regulation (Ulrich, 2000).

The evidence for OPRT gene mutations in tumor cells is limited. A single nucleotide polymorphism (SNP) in the human OPRT gene has been identified in exon 3. The presence of G213A increase *in vitro* OPRT activity compared to wild type (Ichikawa *et al.*, 2002). A direct sequence analysis of DNA from patients with colorectal cancer, the SNP was classified into 3 types including homozygous for G213A (A/A), heterozygous (A/G), and homozygous for wild type (G/G) (Ichikawa, 2002). Among these types, OPRT activity in A/A tumors was twice higher than G/G tumors. In addition, OPRT mRNA expression measured by real-time PCR had a linear correlation with OPRT activity. Clinical study in patients with metastatic colorectal cancer receiving UFT/leucovorin regimen demonstrated that tumors with good response to chemotherapy had higher expression of OPRT mRNA compared with tumors with no response. The median survival dropped from 12 months for high OPRT tumors to 8 months for low OPRT. Tumors of low 2-years survivors were both A/A tumors. These results suggest that polymorphisms of OPRT gene may affect the expression of OPRT gene and OPRT activity which might be a predictive marker for 5-FU sensitivity.

According to the previous reports and our results, the variations in DPD, TP, TS and OPRT gene expression may cause by the genetic polymorphism which lead to up-or down regulation of gene and protein expression. The polymorphisms of these genes may influence mRNA and protein expressions as well as activity, and substrate specificity. In addition, the genetic polymorphism of these genes may also affect the pharmacokinetic profile of 5-FU among individual patients.

In this study, the correlation in the mRNA levels of enzymes involved in 5-FU function, DPD, TP, TS and OPRT, were examined. There was a significant correlation between the mRNA expressions of OPRT and TS, with a coefficient of correlation of 0.602 (Figure13). OPRT and TS are the key enzymes for de novo synthesis of DNA (Danenberg, 1977; Navalgund, 1980). Ikenaka and colleagues reported that the level of OPRT activity increased rapidly in growing cells such as tumor cells (Ikenaka, 1981). Previous study in bladder carcinoma showed that the activity of OPRT in tumor tissues was significantly higher compared with normal tissues. Those findings suggest that OPRT may be necessary for carcinogenesis as well as proliferation of bladder carcinoma. Our study has shown that OPRT mRNA level was positively correlated with the TS mRNA level, which is another key enzyme for de novo DNA synthesis. This result is consistent with previous studies in gastric cancer and bladder carcinoma (Kodera, 2007; Mizutani, 2004). These findings suggest that de novo synthesis of DNA in CCA is coregulated by both OPRT and TS.

In addition, OPRT and DPD mRNA expressions were also significantly correlated with a coefficient of correlation of 0.621 (Figure14). These results was contrast to those observed in gastric cancer (Sakurai, 2006).

DPD and TP mRNA expressions were also significantly correlated with a coefficient of correlation of 0.596 (Figure 15). DPD is involved in the pyrimidine catabolism while TP is a salvage phosphorylating enzyme. This result is consistent with the previous report in colorectal cancer in which a very closed correlation ($r = 0.915$ and $P<0.0001$) between DPD and TP expression levels were observed (Mori *et al.*, 2000). These results suggest that these salvage pathways may work together to cover the requirement for de novo DNA synthesis.

In this study, we examined the correlation of TP, OPRT, DPD and TS mRNA expressions with sensitivity of CCA tissues to 5-FU measured by HDRA. We found that mean level of TP expression was apparently higher in the poorly response group compare to the well response group. In colorectal cancer, TP gene expressions varied over a range of more than 200-fold, and that those tumors with the highest basal levels of TP gene expression were nonresponsive to a 5-FU based protocol, whereas the response rate among tumors with lower TP expressions was greater than the overall response rate. The apparent discrepancy between these results and the concept that cells with higher levels of TP should be more sensitive to 5-FU (Eda, 1993; Elias and Sandoval, 1989; Kato *et al.*, 1997; Schwartz, 1995b; Schwartz *et al.*, 1992) could be explained in several ways: (a) the relationship between the amount of TP mRNA and TP protein levels in the tumors remains to be established. If some of the regulation of TP protein expression takes place at the translational level, high TP gene expression may not be precisely reflected in the TP protein levels. Alternately, even if TP protein levels were proportional to gene expression, the rate of conversion of 5-FU to FdUrd by TP could be limited by the availability of deoxyribose-1-phosphate in tumors. The addition of deoxyribose donor cosubstrates of TP has been shown to greaty increase the incorporation of thymine into DNA as well as the growth-inhibitor potency of 5-FU (Schwartz *et al.*, 1995a). However, although both of these possibilities might explain a lack of increased 5-FU sensitivity in tumors with high TP expression, they would not readily account for the observed inverse relationship. Although, the contribution of the different pathways to 5-FU activation is still unclear. Phosphorylation is necessary to activate 5-FU into its nucleotides by one or more of the following three pathways in the three distinct pathways by which 5-FU is activated into a nucleotide have been identified : (1) directly converted to FUMP by OPRT (2) indirectly converted to FUMP in a sequence of reactions with conversion of 5-FU to 5-fluorouridine (FUR) catalyzed by a pyrimidine nucleoside phosphorylase (UP) (3) indirectly to FdUMP by 2′-deoxy-5-fluorouridine (FUdR) catalyzed by TP in the presence of deoxy-Rib-1-P (dRib-1-P). *In vitro* and *in vivo* studies have shown that the main origin of TS-directed FdUMP metabolite comes from the reduction of FUDP by ribonucleotide reductase after prior conversion of 5-FU to FUDP via OPRT, but not from FUdR phosphorylated by TP (Inaba, 1996;

Koyama *et al.*, 2000; Peters *et al.*, 1986; Peters, 1991). Consistent with our results, it has also been demonstrated in head and neck squamous cell carcinoma and colorectal cancer that the mean level of TP mRNA of cancer tissues with low sensitivity to 5-FU was higher compared with the well response group (Aubry *et al.*, 2008; Yoshinare, 2003). Our results suggest that pathway (3), phosphorylation of 5-FU to FdUMP through FUdR, is not important for anabolism of 5-FU because of the lack of the TP cofactor dRib-1-P at physiological concentrations (Barankiewicz and Henderson, 1977). Conversely, high TP concentrations in conjunction with the normally low dRib-1-P drive the reaction in the opposite direction (Ackland and Peters, 1999). These data support our findings that TP expression is a negative predictor of the result of 5-FU sensitivity test in CCA. Among these pathways, the direct pathway converting 5-FU to FUMP by using OPRT has been shown to be predominant in tumor tissues (Peters, 1991), indicating that OPRT rather than TP activity is mainly the rate limiting step in the phosphorylation process of 5-FU. OPRT directly converted 5-FU to FUMP (in the presence of 5-phosphoribosyl-1-pyrophosphate (PRPP) as a cosubstrate), followed by FUDP conversion to FUTP and subsequent uptake by RNA. The preferential use of the OPRT pathway (1) was revealed to correlate with a high sensitivity to 5-FU in cell lines and human xenograft models (Peters, 1986; Peters, 1991). It has been reported in urinary bladder cancer (Mizutani, 2004) and colorectal cancer (Isshi, 2002) that high OPRT enzymatic activity is associated with high sensitivity to 5-FU. Recent study in gastric found that the level of OPRT mRNA was correlated with chemosensitivity of 5-FU determined by HDRA in which OPRT level appeared to be higher among potential 5-FU responders (Kodera, 2007). Moreover, in colorectal cancer patients treated with oral-5-FU base adjuvant chemotherapy shown that the disease-free and overall survival of OPRT mRNA high expression group were significantly longer than that of the OPRT mRNA low expression group (Yamada *et al.*, 2008). Our results in CCA tissues showed that the mean OPRT mRNA level were higher in well response group compared to those of the poorly response group. These results support the previous reports, indicating that OPRT mRNA expression may contributes to 5-FU sensitivity *in vitro*. Moreover, we also found that the ratio of OPRT/TP mRNA expression were statistically significant higher in the well response

group compare to the poorly response group. According to previous study, the TS, DPD and OPRT expression were quantitatively estimated in gastric cancer biopsy specimens and the expression ratios of OPRT/TS, OPRT/DPD and OPRT/(DPD+TS) were higher in responder than non-responder. In particular, estimations using OPRT/(DPD+TS), in which all three factors were combined, conferred the strongest correlation with responsiveness to S-1 (Kai *et al.*, 2007). Our results suggest that the ratio of OPRT/TP mRNA expression in CCA tissues may be a promising indicator for the sensitivity to 5-FU *in vitro* using modified HDRA technique. Whether OPRT/TP gene expression ratio could be used as an indicator to predict the response of CCA patients to 5-FU need to be investigated in the future.

DPD is associated with 5-FU catabolic action. This enzyme is present in quantity in the human liver, and some several reports suggest a negative correlation between 5-FU sensitivity and DPD activity within tumor cells in many cancers (Beck, 1994; Inaba, 1996). It has been reported in several cancers that the antitumor effects of 5-FU or 5-FU based chemotherapy are reduced in the patient with high level of DPD enzyme (Etienne, 1995; Salonga, 2000). Salonga and colleagues have conducted a study in 33 patients with advanced colorectal cancer treated with 5-FU/leucovorin (Salonga, 2000). They found that patients who response to 5-FU showed low levels and a narrow distribution range for DPD mRNA expression before chemotherapy, whereas non-responders showed a broader distribution range of DPD mRNA levels and many of these non-responders showed low DPD mRNA. In our present study, we found no relationship between the DPD mRNA expression and the sensitivity to 5-FU. The mean values of DPD mRNA expression levels in well response and poorly response groups were very closed. This finding may suggest that DPD may not play a role in the sensitivity of CCA tissues to 5-FU *in vitro*.

TS is a key enzyme that catalyzes the methylation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), which is an important step in DNA synthesis (Johnston *et al.*, 1992; Van der Wilt *et al.*, 1992). TS had been investigated as a predictor of the response to chemotherapies based on 5-FU and as a prognostic marker, particularly in gastric (Kuniyasu *et al.*, 1998), colorectal (Edler, 2002), pancreatic (Hu *et al.*, 2003) and bladder cancers (Mizutani

et al., 2001b). Patients with high TS expression were reportedly associated with non-response to 5-FU and poor prognosis (Allegra *et al.*, 2002; Aschele *et al.*, 1999; Edler, 2002). An association between TS overexpression and decreased survival was also reported in esophageal cancer, including adenocarcinoma and squamous cell carcinoma tumors (Harpole *et al.*, 2001). Etienne *et al* measured both TS and DPD activity in tumor biopsy specimens from 62 head and neck cancer patients before administration of 5-FU based chemotherapy and reported that DPD activity was a factor significantly related to 5-FU responsiveness, but no relationship was demonstrated between TS activity and response to 5-FU therapy (Etienne, 1995). In contrast, Danenberg and colleagues reported that tumoral expression of TS mRNA is association with response to protracted infusion of 5-FU based chemotherapy and survival in gastric and disseminated colorectal cancer patients (Leichman, 1997; Lenz *et al.*, 1996). It was also reported that the clinical efficacy of 5-FU was good in pancreatic cancer patients who showed low expression of TS (Hu, 2003). Although it has been suggested that TS is an important prognostic factor of 5-FU response in colon cancer (Edler, 2002; Leichman, 1998; Popat *et al.*, 2004), our present study found no relationship between TS expression and 5-FU sensitivity of CCA *in vitro*.