CHAPTER II
LITERATURE REVIEWS

2.1 Cholangiocarcinoma

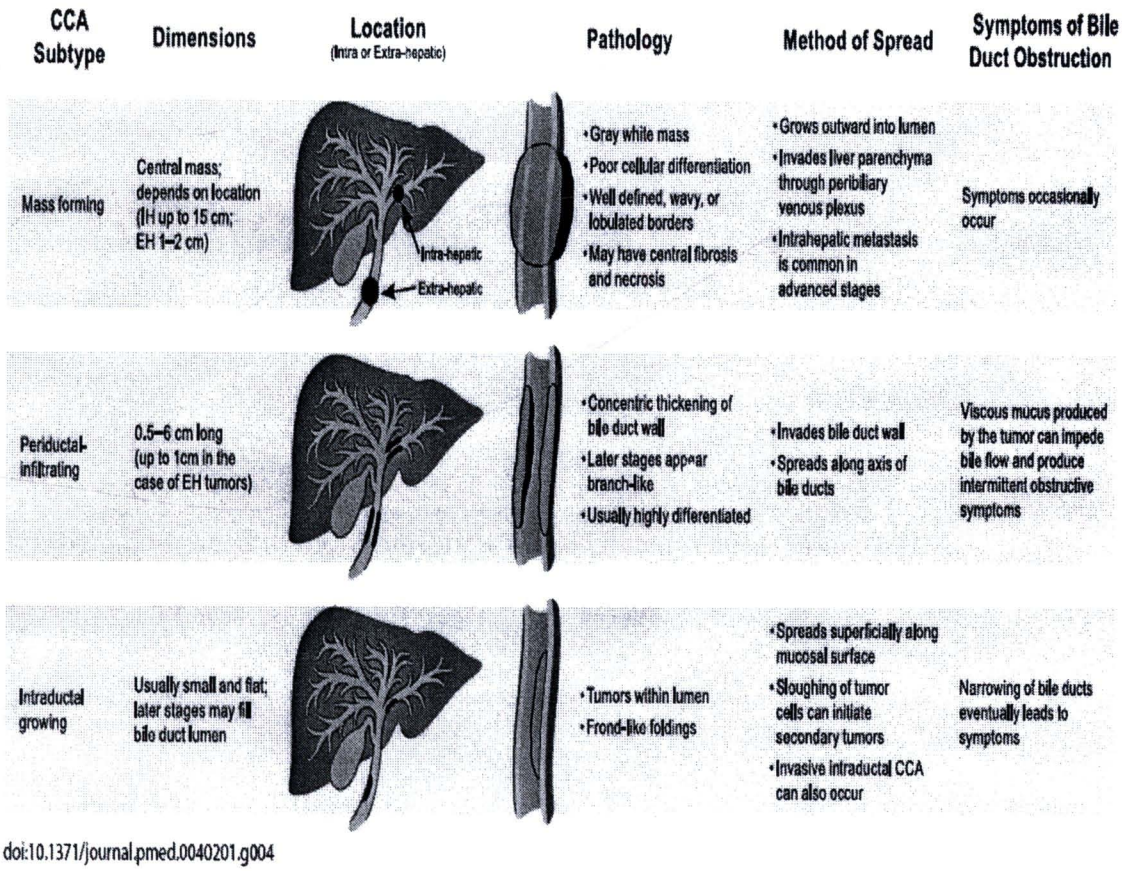
2.1.1 Definition

Cholangiocarcinoma (CCA) is a bile duct tumor arising from cholangiocytes or bile duct epithelial lining cells anywhere in the biliary tree, excluding the gallbladder and the ampular of vater (Vatanasapt, 1990). CCA can be classified into three major types including intrahepatic, perihilar and distal extrahepatic CCA (Malhi and Gores, 2006). Intrahepatic or peripheral CCA arises from any portion of the intrahepatic bile duct epithelium, i.e. from intrahepatic large bile ducts (the segmental and area ducts and their finer branches) or intrahepatic small bile ducts. CCA arising from the right and the left hepatic ducts at or near their junction is called hilar CCA and is considered as extrahepatic lesion (Nakanuma *et al.*, 2000). The morphology of this cancer can be classified into 3 subtypes: mass forming, periductal infiltrating and intraductal types as shown in Figure 1 (Sripa *et al.*, 2007). This classification is independent of the location of the affected bile duct, which may be intrahepatic or extrahepatic.

2.1.2 Epidemiology

The high percentage of liver cancers from Northeaster region of Thailand was observed. In addition, the prevalence of *Opisthorchis viverrini* infection in this regions higher than another region in Thailand (Srivatanakul, 2001). It has been showed that the incidence of CCA in the five major regions of Thailand varied by at least 12-fold and had a strong positive correlation with the prevalence of *O. viverrini* infection (Sriamporn *et al.*, 2004). The prevalence of liver fluke infection in each regions of Thailand is not uniform, in the northern region occurred 19.3% and northeastern region occurred 15.7% whereas central and southern regions found only 3.8% and 0% respectively (Jongsuksuntigul and Imsomboon, 2003). However, it has been reported that the incidence of this type of cancer in Khon Kaen province which located in northeast of Thailand was the highest in the world (Parkin and Muir, 1992;

Vatanasapt, 1990). CCA is responsible for about 71% of liver cancers in Khon Kaen, Thailand compared to those only 19% in the United states (Parkin, 1992).



doi:10.1371/journal.pmed.0040201.g004

Figure 1 characteristic of CCA. It is classified into 3 subtype: mass forming, periductal infiltrating and intraductal (Lim, 2003; Lim and Park, 2004)

2.1.3 Etiology

There are several factors which involve with the developing of CCA. These factors include parasitic infestation, inflammatory bowel disease with/without primary sclerosing cholangitis (PSC), hepatolithiasis, hepatitis C infection, cirrhosis, deposition of thorotrast, toxin and drugs (Vatanasapt, 1990). The liver fluke infection is the greatly influence factor which has been demonstrated to be closely related to the high incidence of CCA (Flavell, 1981; Srivatanakul *et al.*, 1991b). The liver flukes *Clonorchis sinensis,* which is endemic in southern China and Korea and *O. viverrini*, endemic in northeast Thailand and Laos, have been demonstrated to be closely related to the high incidence of CCA. The infection by these liver flukes are leading to chronic inflammation (Holzinger *et al.*, 1999; Kawanishi and Hiraku, 2006; Sirica,

2005). After liver flukes infection, its metabolic products can cause pathologic change leading to inflammation, periductal fibrosis, and proliferative responses, including epithelial hyperplasia, goblet cell metaplasia, and adenomatous hyperplasia, may represent predisposing lesions that enhance susceptibility of DNA to carcinogens (Kim, 1984; Thamavit *et al.*, 1978). Moreover, *N*-nitroso compounds (e.g. nitrosamine), which occur at low levels in fermented food such as preserved mud fish paste, *pla-ra*, a condiment of the cuisine of northeast Thailand and Laos (Sripa, 2007) have been hypothesized to be a primary carcinogen leading to CCA (Migasena *et al.*, 1980). Several *N*-nitroso compounds and their precursors were reported to be occurred during liver fluke infection, *Opisthorchis* infection in hamsters can induce NO synthase expression by immune effector cells in inflamed areas surrounding the bile ducts and increased endogenous nitrosation of thiazolidine-4-carboxylic acid (thioproline) (Oshima *et al.*, 1994). Moreover, several human studies suggest that infected people have a higher endogenous nitrosation potential than uninfected people (Srianujata *et al.*, 1987; Srivatanakul *et al.*, 1991a).

Patients with primary sclerosing cholangitis and ulcerative colitis have an increased association with CCA and extrahepatic bile duct carcinoma (Broome *et al.*, 1995). Anabolic steroids and thorotrast have been reported to be associated with this tumour. And also chronic hepatitis C virus (HCV) infection was reported as a risk factor for intrahepatic CCA. In the United States, HCV core protein, detected in patient tissues, was also identified as a risk factor of CCA (Chen *et al.*, 2005).

2.1.4 Inflammation and carcinogenesis

Molecular perturbations that lead to emergence of a cancerous phenotype involve the following pathways, growth autonomy, escape from senescence, unlimited replication, blockade of growth inhibitory signals, altered microenvironment and evasion of cell death. In chronically inflamed biliary epithelium several changes culminate in the upregulation of growth and prevention of cell death. In a chronic inflammatory environment, epithelial cells are constantly stimulated to participate in the inflammation by generating chemokines and cytokines (Moss and Blaser, 2005).

The cancer arising in this background retain secretion of these inflammatory mediators presumably because they provide growth and survival advantages.

In this regard, interleukin 6 (IL-6) appears to be a pivotal cytokine for cholangiocarcinogenesis. Indeed cholangiocarcinoma cells constitutively secrete IL-6 (Isomoto *et al.*, 2005). In a presumably autocrine fashion, IL-6 activates pro-survival p38 mitogen activated protein kinase (Park *et al.*, 1999). Upregulation of Mcl-1, an anti-apoptotic protein of the Bcl-2 family, and Akt activation are downstream consequences of this cytokine signaling cascade (Isomoto, 2005; Kobayashi *et al.*, 2005). Indeed, transforming growth factor β (TGFβ), a tumor suppressor, can inhibit carcinogenesis by blocking IL-6 signaling (Becker *et al.*, 2004). Loss of TGFβ signaling, therefore may allow full prooncogenic activity of IL-6 (Figure 2). Furthermore, IL-6 also plays a role in senescence abeyance by promoting expression of telomerase (Yamagiwa *et al.*, 2006). Periductal-infiltrating and papillary cancers grow in an environment enriched in bile, suggesting not only resistance to the toxicity of bile but perhaps a tropism for bile. Indeed, bile acids transactivate the epidermal-derived growth factor receptor (EGFR) and also enhance expression of Mcl-1 (Werneburg *et al.*, 2003). Both IL-6 and EGFR have been shown to influence the expression of cyclooxygenase-2 (COX-2) (Yoon *et al.*, 2002). COX-2 and inducible nitric oxide synthase (iNOS) activation are the end result of this pro-inflammatory biliary epithelial milieu. Increased iNOS expression occurs in cholangiocytes in primary sclerosing cholangitis (PSC) and CCA and elevated circulating nitrate levels occur in patient with liver fluke infestation (Jaiswal *et al.*, 2001a). Nitric oxide (NO) has known pleiotropic effects. It can directly or via formation of reactive peroxynitrite species lead to deamination of guanine and DNA adduct formation promoting DNA mutations (Jaiswal *et al.*, 2000; Jaiswal *et al.*, 2001b). NO can also nitrosylate and inactivate DNA repair proteins permitting the accumulation of DNA mutations necessary for cancer development. NO can also nitrosylate caspase-9 leading to inhibition of apoptosis (Torok *et al.*, 2002).

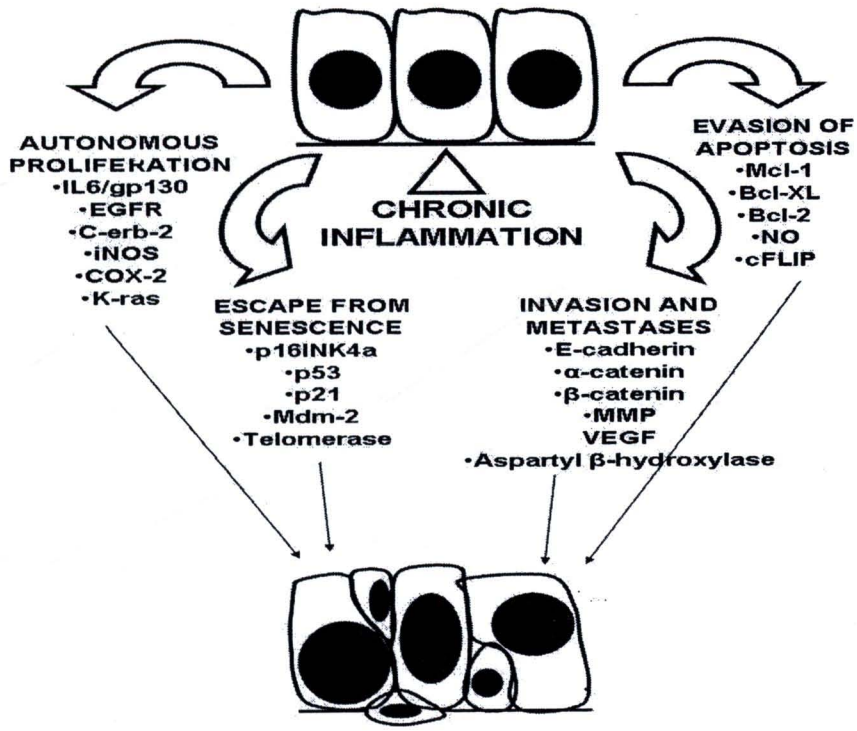


Figure 2 Molecular features of cholangiocarcinogenesis. Epithelial changes driven by chronic inflammation that promote tumor formation are depicted here. Autonomous proliferation is promoted by the following: interleukin 6 (IL-6), its receptor glycoprotein (gp130), epidermal growth factor receptor (EGFR), Her-2 (neu) gene product (c-erb-2), inducible nitric oxide synthetase (iNOS), cyclooxygenase-2 (COX-2), and the oncoprotein K-ras. Evasion of apoptosis is promoted by: myeloid cell leukemia 1 (Mcl-1), Bcl-XL, Bcl-2, nitric oxide (NO) and cellular FLICE inhibitory protein (cFLIP). Escape from senescence is promoted by: p16INK4a, p53, p21, Mdm-2 and telomerase. Lastly, invasion and metastases are promoted by alterations of: E-cadherin, β-catenin, α-catenin, matrix metalloproteinase (MMP), vascular endothelial growth factor (VEGF) and aspartyl β-hydroxylase (Malhi, 2006)

2.1.5 Histopathology and histopathologic classification

According to histological types, there are two major histological types, common type CCA (adenocarcinoma) and special (unusual) types. The majority of CCAs are adenocarcinoma with variable different desmoplasia (common type CCA). Including well-differentiated, moderately-differentiated and poorly-differentiated adenocarcinoma. Some cases present with uncommon histologic features (special or

unusual CCA). These include subtypes adenosquamous and squamous cell carcinomas, musinous carcinoma, sarcomatous type, signet ring cell carcinoma, cholangiocellular carcinoma and biliary papillomas. The latter histologic types have been reported to be related to poor prognosis of CCA patients. Particularly, the squamous cell or sarcomatous elements and mucinous variants confer a poor prognosis (Nakajima and Kondo, 1990; Nakajima *et al.*, 1993). Patients with well-differentiated adenocarcinoma seem to survive longer than those with moderately- or poorly-differentiated ones. A few cases of well-differentiated peripheral CCA with bland features resembling bile duct adenoma show a good prognosis (Foucar *et al.*, 1979). This report correlated with the report from Kosuge and colleagues (1999). The latter reported that patients with papillary and well-differentiated tubular adenocarcinoma had significantly better survival than those with moderately- and poorly-differentiated adenocarcinoma.

2.1.6 Clinical features and diagnostic approaches

The clinical features of CCA appear to differ according to the location of the tumor along the intrahepatic biliary tree, whether the CCA is the hilar or the intrahepatic types (Okuda *et al.*, 1977). The hilar type tends to present with a slow, relentless, obstructive jaundice or cholangitis, while the intrahepatic type presents with abdominal pain and weight loss. It usually presents symptomatic after the disease is advanced. Common symptoms include abdominal pain, weight loss, fever (Nagorney *et al.*, 1993; Nakeeb *et al.*, 1996) and biliary obstruction: jaundice, pale stool, dark urine and pruritus. (de Groen *et al.*, 1999; Gores, 2000).

At present, there are no tumor markers specific for CCA. CA 19-9, carcinoembryonic antigen (CEA), and CA-125 are currently the most widely used serum tumor markers. CA19-9 is evaluated in up to 85% of patients with CCA. CA19-9 value greater than 100 U/ml has a sensitivity of 75% and specificity of 80% in patients with PSC has been reported. Although CA19-9 is a promising test for detecting patients predisposed to the development of CCA but it does not discriminate between CCA, pancreatic, gastric malignancy or in hepatic injury from any cause. CEA and CA-125 are also elevated in 30% and 40-50% of CCA patients, respectively. These tumor markers may be useful for the diagnosis of CCA but further

studies are needed (de Groen, 1999; Hultcrantz *et al.*, 1999; Patel *et al.*, 2000; Ramage *et al.*, 1995). Moreover, several imaging techniques are available for the detection of CCA. It is usually visible by ultrasound sonography (US), magnetic resonance imaging (MRI), and computed tomography (CT). The US typically reveals dilation of the intrahepatic ducts in proximal lesions, or both intrahepatic and extrahepatic ducts in more distal lesions (Saini, 1997). One limitation of US is that the bile ducts may not be visibly dilated in patients with CCA who have PSC or underlying cirrhosis, while CT scans are sensitive for detecting intrahepatic bile duct tumors (Valls *et al.*, 2000), however it does not usually define the extent of CCA. Endoscopic retrograde cholangiography (ERCP), arteriography, and portography are also available to detect and characterize CCA, particularly that arising in the large intrahepatic bile ducts including the hepatic hilus. Magnetic resonance cholangiopancreatography and ERCP give details of level and degree of the bile duct obstruction (Guibaud *et al.*, 1995; Slater *et al.*, 1995) and also help in differentiation between CCA and other biliary diseases (Harell *et al.*, 1981). At present, the combination of cytologic study of the biliary tree with cholangiography may be another useful approach in the diagnosis of CCA. The sensitivity of this method varies from 18-70%. The specificity of the diagnosis of CCA is reported to be very high for these cytologic approaches (Martins *et al.*, 1994).

2.1.7 Treatment of CCA

There are various methods for treatment of CCA patients includes both surgical or non-surgical management such as liver transplantation, symptom palliation, radiotherapy and chemotherapy (Anderson *et al.*, 2004; Malhi, 2006). Surgical resection is indicated in the absence of underlying liver or biliary tract disease. The resection remains the mainstay of treatment in patients with CCA, although prognosis after resection is poor compared with that of hepatocellular carcinoma.

Early detection of CCA is difficult and survival depends largely on feasibility of complete surgical resection. The best predictors of survival are the absence of lymph node involvement, negative tumor margins up to 1 cm, solitary lesions, and lack of microscopic vascular invasion. Five-year patient survival ranges from approximately 20 to 43%, the higher survival stems from careful patient

selection (Berdah *et al.*, 1996; Isaji *et al.*, 1999; Nakagohri *et al.*, 2003; Yeh *et al.*, 2004). However, survival rate under surgery is reported to vary with site and macroscopic type of CCA (Khan *et al.*, 2005). Those with hilar involvement show a median survival of 12-24 months, compared with 18-30 months for those without hilar involvement, although 5-year survivals of up to 40% have been reported for intrahepatic CCA in Japan (Khan, 2005), especially since the advent of an increasingly radical approach over the past 10 years. Regional lymph-node metastases are common with hilar CCA but there is no evidence that extended nodal dissection improves survival. Liver transplantation for CCA is controversial and because of the high recurrence rate published by most authors (Meyer *et al.*, 2000). However, some reports of success have been published (Kubo *et al.*, 1995). The most recent review of 207 patients who underwent liver transplantation for CCA reported 1, 2 and 5 year survival rates of 72%, 48% and 23%, respectively. But more than 50% of patients had recurrence within 2 years (Meyer, 2000). Since the natural history of this disease is short and most patients present with advanced disease or recurrence, they are candidates mostly for palliative therapy. The goals then are symptom resolution and quality of life. Biliary obstruction is the major cause of morbidity in this population. This can be treated by a surgical, endoscopic or percutaneous approach. Surgical biliary bypass is associated with a high perioperative morbidity and mortality. Endoscopic biliary stenting is associated with negligible morbidity and mortality. Biliary drainage can also be easily achieved percutaneously, however it has the disadvantage of external drains, bile leakage and patient discomfort. It may be the only option in patients with complete biliary obstruction (De Palma *et al.*, 2001). For given the high rate of post-resection disease recurrence, adjuvant radiation therapy and chemotherapy have been explored as a means of improving disease free survival. There are no randomized controlled trials of either modality for post-operative therapy in patients with curative resection. In case series, radiation beam does not improve survival (Pitt *et al.*, 1995), and may even lead to hepatic decompensation (Cherqui *et al.*, 1995). Conflicting data exist regarding the role of chemotherapy. Some studies have shown no benefit, whereas others shown improved survival (Kelley *et al.*, 2004; Yoshida *et al.*, 2002). However, during the past 25 years, patients

with CCA have been received chemotherapy after surgery resulting in an attempt to increase their prognosis and a decrease in recurrence of cancer (Todoroki, 2000).

2.1.7.1 Chemotherapeutic treatment in CCA

Various adjuvant chemotherapy regimens have been proposed to improve the postoperative survival. The majority of reports use 5-FU alone or in combination with methotrexate, leucovorin, cisplatin, mitomycin C, or interferon alpha (IFN-α) (Anderson, 2004).

Clinical trials in CCA patients are summarized in Table 1. CCA are relatively chemosensitive, wih most studies being 5-FU based, and 10-20% partial response rates to single agents whereas partial response rates to newer single agents such as gemcitabine, very from 20% to 40%. Gemcitabine in combination with cisplatin show 30-50% partial response rates (Burris *et al.*, 1997). Currently, an European study of infusional 5-FU with cisplatin compared with infusional 5-FU is recruiting. Oral analogues of 5-FU are also now available (UFT-tegafur or capecitabine) (Ducreux, 1998).

Table 1 The summary administration of drug resistance and response rate in the treatment of CCA patients.

Regimen	Number of patients	Response rate(%)	Reference
Gemcitabine *Gemcitabine 1000 mg/m^2 days 1,8,15 every 4 weeks*	24	12.5	(Lin *et al.*, 2003)
Gemcitabine + Capecitabine *Gemcitabine 1000 mg/m^2 days 1,8 + capecitabine 1300 mg/m^2 /day days 1-14 every 3 weeks*	45	31	(Knox *et al.*, 2005)
Gemcitabine + 5-FU *Gemcitabine 900 mg/m^2 days 1,8,15 + 5-FU 200 mg/m^2/day 21 day continuous infusion every 4 weeks*	27	33	(Knox *et al.*, 2004)
5-FU + INF-α *5-FU 750 mg/m^2/d on days 1–5 and a subcutaneous injection of 5 MU/m^2 of rIFN-alpha-2b on days 1, 3 and 5. Treatment cycles were repeated* every 14 days for 8 weeks	25	38	(Nakeeb and Pitt, 2005)
5-FU + Epirubicin + Cisplatin *Epirubicin 50 mg/m^2 and cisplatin 60 mg/m^2 were administered i.v., repeated every 21 days. 5-FU (200 mg/m^2/day was given continuous i.v. via an ambulatory infusion pump throughout the treatment course*	24	10	(Lee, 2004)
5-FU + Leucovorin + cisplatin *Leucovorin 200 mg/m^2, a 400 mg/m^2 bolus of 5-FU followed by a 22-h continuous infusion of 5-FU 600 mg/m^2 on two consecutive days and cisplatin 50 mg/m^2 on day 2*	10	34	(Taieb *et al.*, 2002)

2.1.8 5-Fluorouracil

5-Fluorouracil (5-FU) has been used more than 40 years for treatment of various cancers. It has been so far still the standard first-line treatment of colorectal cancer, breast cancer and CCA (Salonga *et al.*, 2000). This drug is also commonly used for treatment of CCA in Thailand.

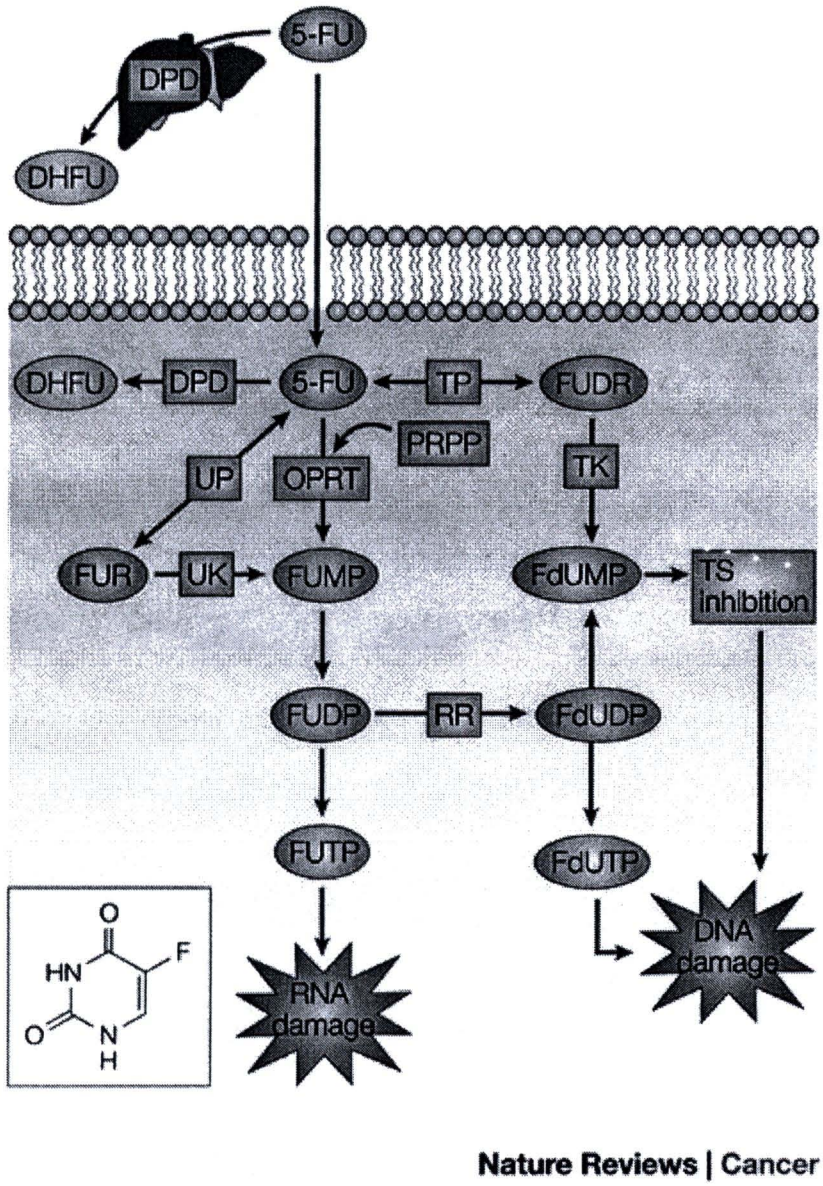


Figure 3 Mechanism of 5-fluorouracil (5-FU) action including catabolism and Anabolism (Longley *et al.*, 2003)

5-FU is an analogue of uracil with a fluorine atom at the C-5 position in place of hydrogen. It rapidly enters the cell using the same facilitating transport mechanism as uracil. The inhibition of RNA function occurs when 5-FU is phosphorylated by orotate phosphoribosyl transferase (OPRT) to form 5-fluorouridine monophosphate (FUMP), followed by 5-fluorouridine diphosphate (FUDP) conversion to 5-fluorouridine triphosphate (FUTP), which can incorporated into RNA strand in placed of uridine triphosphate (UTP). The other way, 5-FU is

phosphorylated by 5-fluorouridine phosphorylase (UP) to 5-fluorouridine (FUR) and then phosphorylated by 5-fluorouridine kinase (UK) to form FUMP, followed by FUDP conversion to FUTP and subsequent uptake by RNA. The inhibition of DNA synthesis occurs when 5-FU is converted by thymidine phosphorylase (TP) through 5-fluorodeoxyuridine (FudR) to 5-fluorodeoxyuridine monophosphate (FdUMP) and then is converted to be 5-fluorodeoxyuridine diphosphate (FdUDP), which can produce anticancer effect by inhibited DNA synthesis whereas in form of FdUMP is a multistep process as a result thymidine synthetase (TS) inhibition (Figure 3). FdUMP forms a covalent ternary complex with the DNA de novo synthesizing enzyme thymidine synthetase (TS) blocking the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) and thus inhibiting DNA synthesis. In addition, the degradation pathway for 5-FU involves conversion by dihidropyrimidine dehydrogenase (DPD) into inactive form, α-fluoro-β-alanine (FBAL) and excretion through the urine (Isshi *et al.*, 2002).

2.1.9 Enzymes involved in the 5-FU function

Metabolic pathway and the enzymes involved in the metabolic process of 5-FU have been studied extensively (Sakurai *et al.*, 2006). Some of the enzymes involved in the metabolic process of 5-FU, including TS, DPD, TP and OPRT have been reported to correlate with the efficacy of 5-FU in various cancers. The details are discussed below.

2.1.9.1 Dihydropyrimidine dehydrogenase (DPD)

DPD is the initial and rate-limiting enzyme in 5-FU degradation, with most administered 5-FU being degraded by DPD in the liver (Yokota *et al.*, 1994). However, 5-FU is metabolized not only in the liver, but also in tumors and normal tissue. DPD is widely expressed in most normal tissues, and its content is highest in the liver and peripheral blood mononuclear cells (PBMC) (Ho *et al.*, 1986).

Several authors have reported that 5-FU clearance is mainly correlated with DPD activity in human PBMC or hepatic tissue (Davis *et al.*, 1993; Diasio and Lu, 1994; Fleming *et al.*, 1993; Lu *et al.*, 1993). There are several reports that in blood cells lymphocyte has the greatest activity (Fleming, 1993; Milano and Etienne, 1994). In contrast, it has been demonstrated that DPD is more highly

expressed in monocytes than in lymphocytes (Van Kuilenburg *et al.*, 1997a). With regard to the location of DPD in cell, it has been reported that almost all the activity of DPD was located in the cytosolic fraction (Van Kuilenburg *et al.*, 1997b). Takenoue and colleagues examined DPD expression by using immunohistochemistry in 47 resected colon cancer specimens, 4 colon cancer cell lines, 2 xenografts by colon cancer cell lines, and human mononuclear cells. They found that DPD was strongly expressed in the cytoplasm of cancer cells, and in the cytoplasm of macrophage and plasma cells (Takenoue *et al.*, 2000).

The expression of DPD in tumor cells has been reported to vary considerably compared with its expression in normal tissues (Naguib *et al.*, 1985). DPD activity in renal cell carcinoma (RCC) tissues was widely distributed from 1.9 to 380.4 pmol/mg protein/min (mean value 68.8 pmol/mg protein/min). The DPD activities reported for cancers from other organs are as follows: colorectal cancers ranged from 27.9 to 206.9 pmol/mg protein/min (McLeod *et al.*, 1998), metastatic liver cancers 87.9-219.6 (McLeod, 1998), and head and neck cancers 13-193 (Etienne *et al.*, 1995).

When the tumor DPD/normal DPD ratio of 29 paired specimens, from which both RCC specimens and normal kidney tissue specimens were calculated, the mean ratio was found to be 1.33 (range 0.41-4.75). In addition, 5 of the 29 (17%) specimens had a ratio greater than 2 (Hirano *et al.*, 2003). In head and neck carcinomas, the mean ratio was 1.04 (range 0.26-6.6) and 7 of 42 (17%) had a ratio greater than 2 (Etienne, 1995). For colorectal carcinomas, a mean ratio of 0.76 (range 0.19-3.32), and 3 of 63 (5%) tumors had a ratio greater than 2 was observed (McLeod, 1998) while Guimbaud and colleagues reported that colorectal carcinomas had a mean ratio of 0.97 (range 0.19-3.32) and that 9 of 70 (13%) had a ratio greater than 2 (Guimbaud *et al.*, 2000).

There are several papers reporting that DPD activity or expression of DPD mRNA were correlated with 5-FU chemosensitivity both *in vitro* or *in vivo* in xenografts (Ishikawa *et al.*, 1999a; Kirihara *et al.*, 1999). Scherf and colleagues at the National Cancer Institute have used a cDNA microarray to screen anticancer drugs on 60 human tumor cell lines, and have investigated the correlation between expression of 9073 genes and the *in vitro* activity of 1400 compound species

(Scherf *et al.*, 2000). Of these genes and compounds tested in the context of sensitivity to anticancer agents, the strongest correlation was observed between DPD and 5-FU.

Moreover, several investigators have reported that a high intra-tumoral DPD level was associated with the low anti-tumor activity of 5-FU due to increased 5-FU inactivation (Beck *et al.*, 1994; Fischel *et al.*, 1997; Ishikawa, 1999a). Ando and colleagues examined the relationship between gene expression of 5-FU metabolic enzymes and 5-FU sensitivity in 25 esophageal carcinoma (ESCC) cell lines (Ando 2007). They found that high levels of DPD mRNA expression were significantly correlated with resistance to 5-FU in ESCC cell lines. Yoshinare and colleagues predicted the sensitivity of colorectal cancer to 5-FU by comparing the gene expression of 88 surgically obtained colorectal cancer specimens with chemosensitivity to 5-FU. The correlations between the variables were analyzed, and the predictive value of these mRNA was assessed statistically using a receiver operating characteristic (ROC) curve. They found that high DPD expression was associated with low sensitivity to 5-FU ($P<0.022$). ROC curves indicated that DPD mRNA was possible predictor of sensitivity to 5-FU, with cutoff value of 0.6 (Yoshinare *et al.*, 2003). This result suggests that the sensitivity of colorectal cancer to 5-FU may be regulated by DPD, the rate-limiting enzyme of catabolism. Moreover, Ishikawa and colleagues reported that high DPD mRNA level resulted in low sensitivity to 5-FU *in vivo* using human tumor xenografts in nude mice (Ishikawa, 1999a).

A significant inverse correlation between DPD activity and 5-FU sensitivity in carcinoma tissues has also been reported in bladder cancer (Mizutani *et al.*, 2001a). Consistent with the results obtained from other cancer tissues, the significant correlation between DPD levels and 5-FU chemosensitivity was also reported in gastrointestinal cancer cells (Ma, 2004). For gastric cancer, the correlation between 5-FU sensitivity and DPD was controversy (Kodera *et al.*, 2007). No correlation between DPD and 5-FU sensitivity was found in renal cell carcinoma (Hirano, 2003). Several clinical studies on gastrointestinal and other cancers which have been widely treated by 5-FU-based chemotherapy have demonstrated that intratumoral DPD expression is associated with 5-FU resistance and poor outcomes

(Fujiwaki *et al.*, 2000; Horiguchi *et al.*, 2002; Huang *et al.*, 2000; Inada *et al.*, 2000; Ishikawa, 2000; Mizutani, 2001a; Salonga, 2000). Etienne and colleagues measured 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 significantly related to 5-FU responsiveness (Etienne, 1995).

The relationship between intratumoral DPD expression and response to 5-FU liver perfusion chemotherapy LPC in pancreatic cancer patients was also reported. Of the 68 tumors studied, 27 carcinomas (39.7%) were DPD positive, and 41 (60.3%) were DPD negative. In the DPD positive group, there was no significant difference between the response group to liver perfusion chemotherapy (LPC +) and non-response group to liver perfusion chemotherapy (LPC -), whereas in the DPD (-) group the LPC (+) subgroup showed a significantly higher survival rate than the LPC (-) subgroup. Moreover, in the LPC (+) group, overall survival in the DPD (-) subgroup significantly better than in the DPD (+) subgroup (Nakayama *et al.*, 2004). These results suggested that an intratumoral DPD expression might be useful in predicting responsiveness to 5-FU-based chemotherapy in pancreatic cancer patients. In addition, it has been reported in advanced colorectal cancer patients treated with 5-FU/leucovirin that patients who responsed to the treatment showed low and a narrow distribution range for DPD mRNA expression before chemotherapy, whereas nonresponders showed a broader distribution range. However, they also noted that many nonresponders also showed low DPD mRNA, making it difficult to predict drug sensitivity based exclusively on DPD expression. However, when both TS and DPD were evaluated, responders were most commonly found among patients showing low values for both TS and DPD. This made it feasible to predict favorable response based on these two parameters (Salonga, 2000).

Terashima and colleagues determined the sensitivity of gastric cancer to 5′-deoxy-5-fluorouridine (5′-DFUR) by comparing TP and DPD expression levels of 93 surgically resected gastric cancer specimens with *in vitro* sensitivity to 5′-DFUR detected by an adenosine triphosphate (ATP) assay (Terashima *et al.*, 2002). They found a weak inverse correlation between the DPD level and the sensitivity to 5′-DFUR (r_s = -0.361). Furthermore, a significant correlation between the TP/DPD ratio and 5′-DFUR sensitivity (r_s = 0.634) was observed. In a subgroup of patients

with postoperative 5′-DFUR administration, the survival rate was significantly longer in patients with a high TP/DPD ratio (n = 8) than in those with low TP/DPD ratio (n = 14) (p = 0.0140). These results suggest that sensitivity to 5′-DFUR may be predictable by measurement of both TP and DPD levels.

In conclusion, In some clinical settings such colorectal cancer (Ichikawa, 2003a; Leichman *et al.*, 1997; Salonga, 2000) and gastric cancer (Terashima *et al.*, 2003), patients with low levels of DPD mRNA expression were found to respond to 5-FU-based chemotherapy.

2.1.9.2 Thymidine phosphorylase (TP)

Thymidine phosphorylase (TP) is one of the key enzyme in the metabolism of 5-FU, since it is required for the initial conversion of 5-FU to 5-fluoro-2′-deoxyuridine (FdUrd) (Hartmann and Heidelberger, 1961). TP is also known to be identical to platelet-derived endothelial cell growth factor (PDECGF), which has potent angiogenic activity (Ishikawa *et al.*, 1989). TP activity is indispensable for its angiogenic action *in vivo* (Brown and Bicknell, 1998) and stimulates chemotaxis of endothelial cells *in vitro* (Haraguchi *et al.*, 1994; Ishikawa, 1989). Although neovascularization is a necessity for tumor growth and metastasis (Folkman, 1990), studies on the prognostic role of microvessel density (MVD) counts in colorectal cancer are inconclusive. MVD of 178 colon tumor samples (Bossi *et al.*, 1995) was higher in adenoma *versus* carcinoma but was not prognostic. However, MVD was prognostic for hematogenous spread in 133 patients with colorectal cancer (Tanigawa *et al.*, 1997). In colon cancer, MVD correlated with TP immunostaining, but TP was inversely correlated with vascular endothelial growth factor (VEGF) (Takahashi *et al.*, 1996). High TP immunostaining also correlated with more extensive angiogenesis and poor clinical outcome of patients with colorectal cancer (Takebayashi *et al.*, 1996). These data indicate that TP levels and TP-associated neovascularization may play a role in the prognosis of cancer. High levels of this enzymatic activity have been observed in various malignant tumors (Hirano *et al.*, 2000; Imazano *et al.*, 1997; Marchetti *et al.*, 2001).

A high TP level seems to be an important indicative parameter for a shorter disease-free survival (DFS). In addition, patients with renal cell carcinoma with high TP levels, evaluated by enzymatic method or by immunoblotting and

immunostaining (Imazano, 1997), had a 4-fold higher risk for death than patients with low or no TP expression. Patients with node-negative non-small cell lung cancer and positive TP immunostaining had a poor prognosis (Koukourakis *et al.*, 1997). TP expression was elevated in node-positive primary breast carcinoma as measured with immunohistochemistry (n = 240) and by RNase protection assay (Fox *et al.*, 1996). In addition, data obtained from 163 colorectal cancer patients, high TP levels were associated with poor clinical outcome (Takebayashi, 1996). It seems that TP levels are related with overall survival or DFS, although the correlation is different among the various tumor types. Different regulatory pathways of TP activation may play a role, possibly leading to development of liver metastases, explaining the shorter DFS.

In the previous study, a significantly higher TP expression was observed in tumor than normal tissues (Terashima, 2002). It has also been reported in breast cancer (Toi *et al.*, 1995), gastric cancer (Kakeji *et al.*, 1999; Maeda *et al.*, 1996; Tanigawa *et al.*, 1996), renal cell carcinoma (Hirano, 2003) and colorectal cancers (Takebayashi, 1996) that the expression level of TP in these tumor tissues were higher than those observed in the surrounding normal tissues.

A point of major interest in the role of infiltrating cells in TP expression. Van Triest and colleagues reported that, 44% of the colorectal cancer cells were TP positive as measured by immunohistochemical staining (IHS) (van Triest *et al.*, 2000). Furthermore, 51% breast cancer tissues were positive for TP with nuclear and/or cytoplasmic staining, which was occasionally focal but often up-regulated at the infiltrating tumor edge (Fox *et al.*, 1997). In non-small cell lung cancer patients, 25% of the tumors were positive with invariably stained alveolar macrophages and weak immunoreactivity of the stromal fibroblasts (Koukourakis, 1997). TP expression was also high in infiltrating cell (83%, macrophages and lymphocytes) of 96 colorectal cancer patients (Takahashi, 1996), whereas only 5% of the tumor epithelium was positive for TP. Similarly in patients with gastric cancer, only 10% of the tumors were positive for TP, but 54% of the infiltrating cells (predominantly macrophages) were TP positive. TP expression was high in infiltrating cells of intestinal-type gastric cancer (66%) but not in diffuse-type gastric cancer (40%) (Takahashi *et al.*, 1998). In colorectal cancer, 41% of the samples showed TP-positive infiltrating cells (mostly fibroblast and plasma cells), but no

positive stromal cells were found in separate normal tissue (van Triest, 2000). In particular, because infiltrating cells in the tumor were highly positive, this suggests a specific role of the infiltrating cells in the tumor area in producing angiogenic factors such as TP, although TP in tumor cells might also affect expression of angiogenic factors in tumor cells.

Although the regulating mechanism for TP expression in tumors remains unclear, TP expression is reportedly accelerated by hypoxia (Griffiths *et al.*, 1997), cytokines such as tumor necrosis factor-alpha (TNF-alpha), Interferon (INF), interleukin-1 (IL-1) (Eda *et al.*, 1993) or anticancer drugs such as cyclophosphamide, paclitaxel, and docetaxel (Endo *et al.*, 1999).

A correlation between the activity of this enzyme and malignant potential of tumors has also been observed (Hirano, 2000; Imazano, 1997; Marchetti, 2001). Furthermore, studies on gastric cancer report that TP expression closely correlates with tumor invasion, haematogenous metastasis, lymph node metastasis, venous invasion, lymphatic invasion, and microvascular density (Kakeji, 1999; Maeda, 1996; Ogawa *et al.*, 1999; Tanigawa, 1996). A significantly higher TP expression in tumors with a deeper tumor invasion, lymphatic and venous invasion was also reported in gastric cancer suggesting an angiogenesis-inducing role for TP (Terashima, 2002). These results suggest that TP activity is an important predictive parameter for malignant potential and poor prognosis.

Hirano and colleagues demonstrated that a significant positive correlation was found between TP activity in RCC tissue and 5-FU sensitivity, though no correlation was found between DPD activity in RCC and 5-FU sensitivity. In addition, there was a stronger correlation between TP/DPD ratio and 5-FU sensitivity than between TP activity in RCC tissue and 5-FU sensitivity. These results suggest that TP rather than DPD may regulate 5-FU sensitivity in RCC (Hirano, 2003).

It is known that TP is also a metabolic enzyme for the fluoropyrimidines including capecitabine, an analogue of 5-FU, and 5′-DFUR (Cook *et al.*, 1979). It is also reported that TP shows a significant affinity to 5′-DFUR, but that cells transfected with the TP gene show no change in sensitivity to 5-FU (Kanyama *et al.*, 1999; Patterson *et al.*, 1995). Correlation between TP expression and 5′-DFUR efficacy has also been proven clinically. For example, among gastric cancer

patients treated by drug regimens including 5′-DFUR, those with a high TP expression in their tumor tissues showed a significantly higher response rate to the regimens (Koizumi *et al.*, 1999). The higher TP expression in tumor tissues than in normal tissues in the basis behind the rationale of tumor-selective activity of these anticancer drugs. Moreover, the relationship of TP and DPD activities in tumor and normal tissues from 19 colorectal cancer patients given capecitabine with 5-FU concentrations was found that the TP activity was 3.7 times higher in the tumor tissues than in the normal tissues and the 5-FU concentration in the tumor tissues was 3.2 times higher than in the normal tissues (Schuller *et al.*, 2000).

An enhancing effect of combined chemoimmunotherapy with interferon α and 5-FU was reported (Gebrosky *et al.*, 1997; Schwartz *et al.*, 1995b). The mechanism of the enhancing effect is thought that interferon α functions as an up-regulator of TP activity (Schwartz, 1995b) and/or down-regulator of DPD activity in carcinoma cells (Milano *et al.*, 1994). Recently, the combined treatment of RCC cells with tumor necrosis factor-related apoptosis-including ligand (TRAIL) and 5-FU could overcome 5-FU resistance of RCCs. The mechanism of enhanced cytotoxicity of 5-FU reported that TRAIL down-regulated the TS and DPD activities and up-regulated the activity of OPRT in RCCs (Mizutani *et al.*, 2002).

In addition, S-1, a new oral FU drug which includes 5-chloro-2,4-dihydroxypyridine (CDHP) as a strong DPD down-regulator, has recently been developed. The correlation between *in vitro* tumor sensitivity to either 5′-DFUR and TP/DPD expression was observed (Terashima, 2002), that tumors with a high DPD expression were resistant to 5′-DFUR and the TP/DPD ratio demonstrated a strong correlation with the tumor sensitivity to 5′-DFUR. In colorectal cancer, the TP/DPD ratio was significantly higher in tumour tissues than in adjacent normal tissues and suggested that there is a preferential activation of fluoropyrimidines in tumor cells (Collie-Duguid *et al.*, 2001). Moreover, TP was also reported as a prognostic factor in gastric cancer patients (Maeda, 1996). However, Tanigawa and colleagues reported that the TP expression showed a relationship to imcrovascular density and heamatogenous metastasis, but no difference was seen in prognosis according to the TP expression level (Tanigawa, 1996). The findings of these studies may result from

the different analytical methods employed and differences in patient selection. Thus, the prognostic significance of TP expression in gastric cancer patients is still unclear.

2.1.9.3 Thymidylate synthase

Thymidylate synthase (TS) is an important target enzyme for 5-FU, which catalyzes a key step in DNA synthesis. When 5-FU is administered, it is converted to fluorodeoxyuridine-5′-monophosphate (FdUMP), which prevents methylation of dUMP by TS in the ternary complex form of 5,10-methylene-tetrahydrofolate (m-THF), and consequently inhibits DNA synthesis (Beck, 1994; Peters *et al.*, 1995; Spears *et al.*, 1982). This is a very crucial reaction because TS provides the sole intracellular *de novo* source of thymidylate, and is irreversibly blocked by 5-FU (Santi *et al.*, 1974). Moreover, TS itself may function as cell proliferation from the G0 to S phase in association with the grade of tumor malignancy (Mirjolet *et al.*, 1998). It was noted in various types of tumor tissues that advanced-stage cases showed higher TS levels than early-staged cases, suggesting that TS levels in tumor tissues are strongly associated with tumor progression and prognosis (Ishikawa *et al.*, 1999b; Ishikawa, 2000; Otake *et al.*, 1999).

There still be some controversial about the prognostic or predictive value of TS. TS has been evaluated in patients who did not receive subsequent adjuvant chemotherapy. In some studies, TS has been reported as a prognostic marker and low TS values have been related to a longer survival (Johnston *et al.*, 1994). However, when patients were adjuvantly treated with 5-FU-containing chemotherapy, high TS levels appeared to be a predictive marker for a longer survival (Edler *et al.*, 2002; Johnston, 1994; Kornmann *et al.*, 2003). In contrast, others found no survival difference between low and high TS expressions (Nanni *et al.*, 2002; Yamachika *et al.*, 1998). The predictive value of TS in treatment of patients with advanced disease is more straightforward, since in most studies a low TS level was associated with a longer survival and/or a better response rate to 5-FU containing therapy (Aschele *et al.*, 2000; Johnston *et al.*, 2003; Paradiso *et al.*, 2000). However, this relationship appeared to be limited to TS levels determined in metastases, rather than in primary tumors (Aschele, 2000; Johnston, 2003).

In patients with metastatic colorectal cancer, TS mRNA expression as measured by reverse transcription-PCR was also prognostic for the outcome of

5-FU therapy (Leichman, 1997) and in patients treated with hepatic artery infusion therapy (Kornmann *et al.*, 1997). With immunohistochemically staining using the monoclonal antibody TS-106, a significant relationship was found between TS levels and prognosis in rectal cancer (Johnston, 1994). A high TS level seems to be one of the important prognostic parameters; poor response to 5-FU-based chemotherapy in patients with colorectal cancer was associated with a high TS level (Peters *et al.*, 1994). High TS levels, as determined by immunostaining in 294 patients with rectal cancer (Johnston, 1994) or biochemically and by immunostaining (Yamachika, 1998) in 58 patients with colorectal cancer, were correlated with poor survival. However, van Triest *et al* found a relationship between low FdUMP-binding levels and both longer survival and the natural behavior of the disease (van Triest, 2000). In conclusion, TS levels may not only be predictive for 5-FU response but also may be of prognostic value for survival in non-treated patients, possibly because low TS levels correspond to less aggressive tumor types or to a low growth potential.

2.1.9.4 Orotate phosphorybosyl transferase

OPRT is the key enzyme in the *de novo* DNA and RNA synthetic process. OPRT may reflect the clinicopathological background of malignant diseases. In fact, it was reported that OPRT activity in primary lesion was a powerful predictor of nodal involvement in resectable gastric cancer (Ochiai *et al.*, 2005). Furthermore, the OPRT mRNA levels were significantly higher in liver metastases than in primary colorectal cancer (Inokuchi *et al.*, 2004). In urologic malignancies, OPRT activity levels were correlated positively with the stage and grade of bladder and renal carcinomas (Mizutani *et al.*, 2004). In prostatic cancer, the OPRT expression level was significantly higher in carcinoma samples than in benign prostatic hypertrophy (Miyoshi *et al.*, 2005). The activity of OPRT in bladder carcinoma was significantly higher compared with OPRT activity in normal bladder.

High OPRT activity in bladder carcinoma may be a reflection of the rates of tumor cell proliferation (Mizutani, 2004). Ikenaka and colleagues reported that the level of OPRT activity increased in rapidly growing cells, including tumor cells and normal cells, such as testis (Ikenaka *et al.*, 1981). These findings suggest that OPRT may be necessary for carcinogenesis as well as cell proliferation in bladder carcinoma. However, the precise reasons responsible for the correlation between

OPRT levels and clinical outcome remain unclear. Because OPRT is the principal *de novo* DNA and RNA synthetic enzyme associated with cell division and proliferation, it is reasonable to assume that clones of cells that overexpress OPRT can grow more easily and rapidly after implantation compared with clones that do not overexpress OPRT.

It has been shown that OPRT activity in bladder carcinoma is correlated positively with the activity levels of TS and Thymidine kinase (TK), which are the key enzymes for DNA synthesis in the *de novo* and salvage pathways, respectively (Mizutani, 2004). These findings suggest that DNA synthesis in both the *de novo* and salvage pathways is up-regulated in high-stage/high-grade of tumor. Accordingly, simultaneous inhibition of the activity of OPRT, TS and TK may provide therapeutic means of preventing growth and recurrence in patients with bladder carcinoma. These results demonstrated that OPRT activity in bladder carcinoma was correlated positively with histologic stage and grade and that low OPRT activity was a good prognostic sign.

Furthermore, elevated levels of OPRT activity in bladder carcinoma were associated with high response to 5-FU. These findings suggest that the assessment of OPRT activity may be useful in the management of bladder carcinoma and it has been proposed that the level of OPRT activity may be used both as prognostic parameter in patients and as a predictive indicator for 5-FU efficacy (Mizutani, 2004).

In vivo, OPRT is the enzyme that catalyzes the final two steps in the process of *de novo* pyrimidine synthesis (Suttle *et al.*, 1988). Three distinct pathways by which 5-FU is activated into a nucleotide have been identified (Peters *et al.*, 1991) : (1) directly converted to FUMP by OPRT in the presence of 5-phosphoribosyl-1-pyrophosphate (PRPP) as a cosubstrate; (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) with ribose-1-phosphate (Rib-1-P) as the cosubstrate; (3) indirectly to FdUMP by 2′-deoxy-5-fluorouridine (FUdR) catalyzed by TP with deoxy-Rib-1-P (dRib-1-P). Among these pathways, the direct pathway converting 5-FU to FUMP has been shown to be predominant in tumor tissues (Peters, 1991), indicating that OPRT activity is mainly

the rate limiting step in the phosphorylation process of 5-FU. OPRT activity is therefore likely to be associated with the anticancer effects of 5-FU. Numerous basic and clinical evidence suggests that OPRT levels are associated with sensitivity to 5-FU. Increased sensitivity to 5-FU has been reported *in vitro* by the introduction of the *E.coli UPRT* gene that corresponds to the OPRT gene in humans in gastric cancer cell line (Inaba *et al.*, 1999).

Several reports indicated that expression of the OPRT gene was useful to predict the efficacy of fluoropyrimidine-based chemotherapy for various malignancies, Nio and colleagues reported that the expression of OPRT was also a beneficial predictor for uracil and tegafur (UFT)-based adjuvant chemotherapy (ACT), and in patients with OPRT (+) pancreatic cancer, the survival curve of the patients who received oral UFT-based ACT, was significantly higher than that of the surgery alone group (Nio *et al.*, 2007). In metastatic colorectal cancer, the mRNA expression of OPRT, which was measured using real-time quantitative RT-PCR assays of surgically resected materials from primary colorectal tumors, was very useful for predicting the effects of 5-FU-based hepatic artery injection chemotherapy for synchronous liver metastases in 22 patients (Matsuyama *et al.*, 2006). It was also reported that in 37 patients with metastatic colorectal cancer, who received oral treatment of UFT and leucovorin, the patients with high OPRT gene expression survived longer than those with low OPRT expression, and the authors emphasized that both expression of the OPRT gene and the OPRT/DPD ratio might be useful as predictive parameters for the efficacy of fluoropyrimidine-based chemotherapy for metastatic colorectal cancer (Ichikawa, 2003b).

In gastric cancer, it was reported that the combination expression of OPRT and TS in prechemotherapeutic fresh frozen samples obtained from primary tumors might predict the response to S-1 in 59 metastatic gastric cancer patients as a first line treatment (Ichikawa *et al.*, 2006). In resectable non-small cell lung cancers, the 5-year survival rate of patients with OPRT (+) stage II-III tumors was significantly higher than that of those with OPRT (-) stage II-III tumors (Nakano *et al.*, 2006). Recent clinical studies also demonstrated that increased expression of the OPRT gene is associated with an increased sensitivity to 5-FU (Isshi, 2002), suggesting that the expression of the OPRT gene is a useful predictor of sensitivity to

5-FU. It is therefore reasonable to conclude that upregulation of OPRT activity increases phosphorylation of 5-FU to FUMP, which is an active form of 5-FU causing inhibition of RNA synthesis.

2.2 Chemosensitivity assays

Many surgeons confront the problem of whether or which chemotherapy should be administered to patients following surgery (Yasuda *et al.*, 1998). Investigators working over the past 50 years have developed *in vitro* drug-response assay systems to determine the potential activity of chemotherapy agents for a given patient prior to their administration. The central hypothesis underlying this approach is that the drug-response profile for each individual patient will differ based on their intrinsic genetic diversity and the development of subclones within tumors that have divergent phenotypes (Goldie and Coldman, 1979). *In vitro* assays that identify individual differences in tumor drug response make it possible to design patient-specific regimens targeted against each patient's tumor characteristics. By eliminating ineffective agents, the patient is spared toxic treatment without benefit, while the selection of agents active *in vitro* may increase the probability of response (Fruehauf, 2002).Various chemosensitivity assays that have been used to date include the differential staining cytotoxicity (DiSC) assay, the collagen-gel droplet embedded-culture drug sensitivity test (CD-DST), the monolayer culture [3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl-2H tetrazolium bromide] (MTT) assay, the human tumor clonogenic assay (HTCA), the extreme drug resistance (EDR) assay and histoculture drug response assay (HDRA).

2.2.1 Differential staining cytotoxicity (DiSC) assay

The (DiSC) assay is well suited for analysis of *in vitro* resistance to chemotherapy. The assay is based on culturing malignant cells in suspension in the presence of various chemotherapeutic agents. The specimens are processed to separate malignant cells, isolated cells are combined with chemotherapeutic agents. After 4 day of drug exposure, cells are exposed to the vital stain which can divided between dead cells and living cells by conventional hematoxylin and eosin after that cells are spun down onto slides, the cytotoxic effects of chemotherapy are examined

by light microscopy as shown in Figure 4. Percent cell kill is determined by comparing targeted cell death with untreated controls.

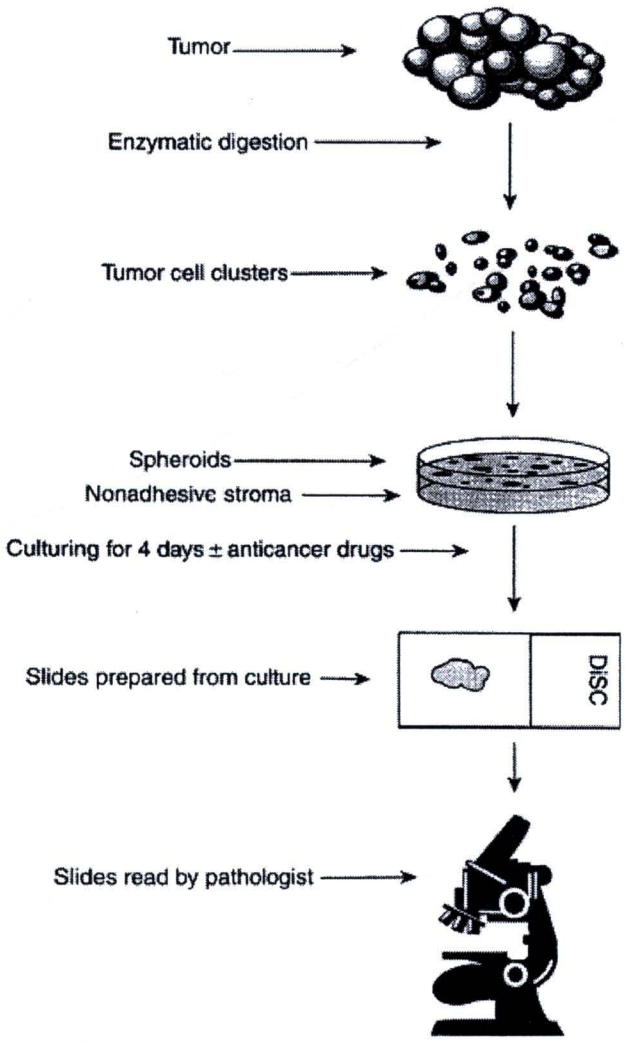


Figure 4 In vitro assay schema: DiSC assay (Fruehauf, 2002)

This method use agar-based culture systems, suppress cellular adherence to a growth surface, non-transformed cell can proliferate in adherence-based culture systems, results are adversely affected by proliferation of non-malignant cells that add 'noise' to the cancer cell growth signal (Campling *et al.*, 1991; Kitaoka *et al.*, 1997). Other authors reported that results from DiSC assay were correlated well with subsequent patient response in chronic lymphocytic leukaemia (Bosanquet *et al.*, 1999a; Bosanquet *et al.*, 1999b). However this assay take more time than other method such as ATP assay (Ann-Sofie Rhedin 1992).

2.2.2 Collagen-gel droplet embedded-culture drug sensitivity test (CD-DST)

The CD-DST is a chemosensitivity test, evaluate the efficacy of anticancer drug. The predominant of this assay is mimic *in vivo* condition by culturing in a three-dimensional (Yang *et al.*, 1979). *In vivo*, cells receive nutrients from tissue fluids and eliminate waste products in a stereospecific environment surrounded by an extracellular matrix. Collagen, the main component of the extracellular matrix, plays an important role *in vivo* in maintaining cellular differentiation, growth and function (Yasuda, 1998). Tumor cells should be cultured in an environment that closely relate to these *in vivo* surrounding as shown in Figure 5. In several researches used this method to investigated drug sensitivity test including in colorectal cancer (Nakahara *et al.*, 2004) ovarian cancer (Yabushita *et al.*, 2004) and breast cancer (Takamura *et al.*, 2002). This assay resolves some of the disadvantages of other available chemosensitivity assay including allows a reduction in the number of cells necessary for each assay. It has a high primary culture success rate with human tumor cells and maintains the original growth morphology of the cells, moreover, it can inhibits fibroblasts proliferation, however the weak points of this method are requirement for image analysis system and take a long time in step of culture (Yasuda, 1998).

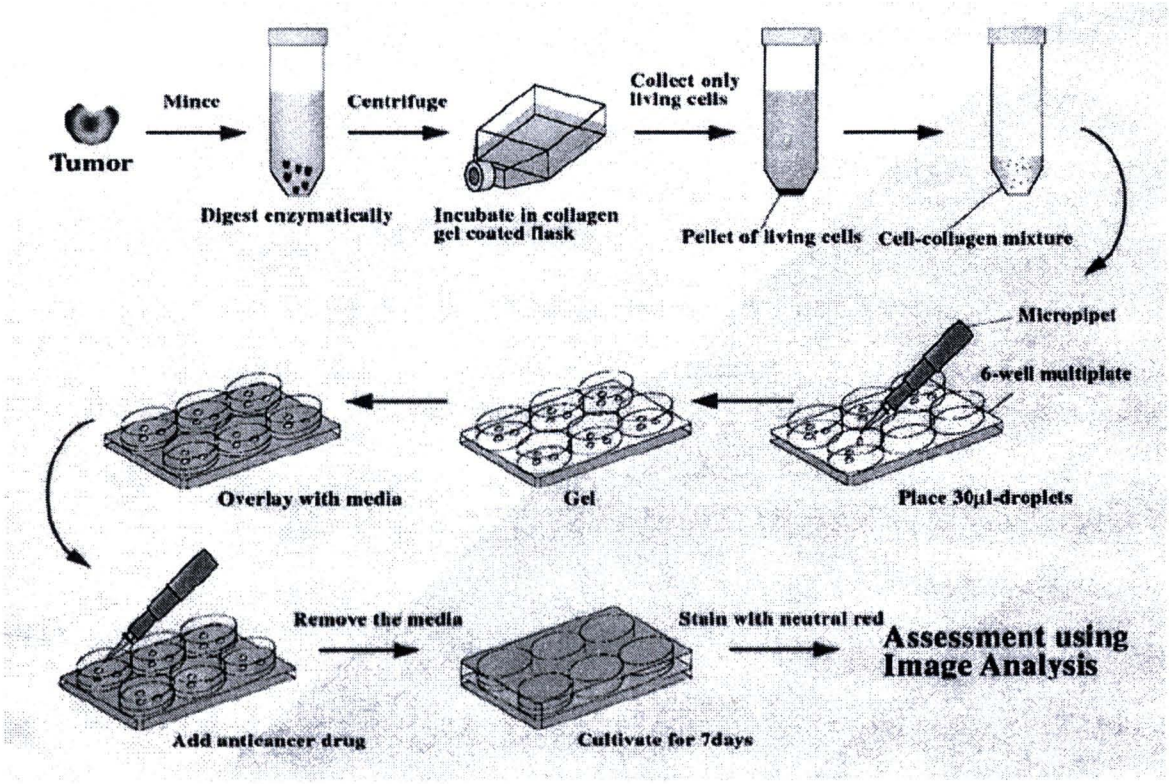


Figure 5 The CD-DST procedure (Yasuda, 1998)

2.2.3 Monolayer culture [3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl-2H tetrazolium bromide] (MTT) assay

The MTT assay is the method that used for drug sensitivity test in several cancers including hepatocellular carcinoma (Tam *et al.*, 2009), gastric cancer (Jung *et al.*, 2009) and brain tumors (Nikkhah *et al.*, 1992), they proposed that the MTT assay may be useful for the chemosensitivity prediction in the clinical trials. This assay based on the reduction of MTT by mitochondrial succinate dehydrogenase (SDH), resulting in the production of the colored compound formazan (Yasuda, 1998). MTT assay detect SDH activity as a determinant of mitochondrial function and cell viability. SDH is a component of the citric acid cycle, and generates FADH2 and fumarate from succinate and FAD. SDH activity resides on the mitochondrial inner membrane and requires a functioning electron transport system. SDH activity is therefore a measure of mitochondrial and cellular viability. Then SDH activity is measured by its capacity to convert MTT to a blue crystallized compound that is dissolved in dimethyl sulfoxide at the end of the assay. The amount of crystal

formation is determined by measuring the absorbance of the tissue culture well using a spectrophotometer (Fruehauf, 2002). This assay is a rapid, economical and most widely used (Iwahashi, 2005). However, this assay works well for cancer cell lines, but not for tumor tissues. The MTT assay signal source stems from all functional mitochondria, and therefore dose not distinguish between cancer cells and normal stromal cells in tumor tissues response to *in vitro* drug exposures (Fruehauf, 2002).

2.2.4 Human tumor clonogenic (HTCA) assay

The HTCA is a growth assay in which fibroblast proliferation is inhibited by using a double-layer soft agar medium (Yasuda, 1998). Agar is the growth substrate, mimics *in vivo* environment and suppresses the proliferation of non-transformed cells (Hamburger and Salmon, 1977; Puck and Marcus, 1955) as shown in Figure 6 (Fruehauf, 2002). After drug exposure, single cells are plated on an agar matrix. After 2-3 week period, colony counts is determined at the end point. The capacities of single malignant cells which divide and form colonies on an agar-based matrix were measures but malignant cells that have been killed by anticancer agents or have undergone damage causing cell cycle arrest fail to form colonies. Differential colony formation between untreated controls and treated cells represent drug sensitivity (Fruehauf, 2002).

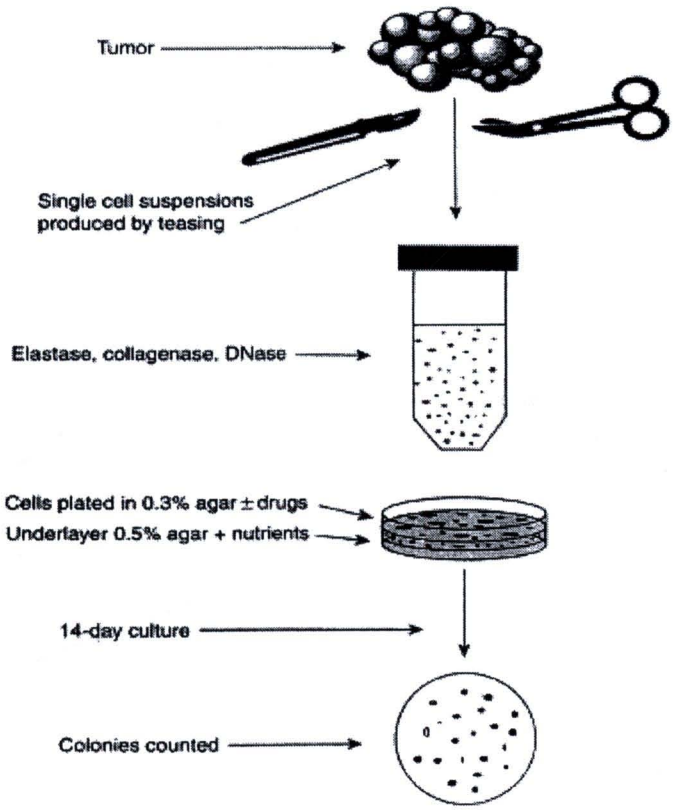


Figure 6 *In vitro* assay schema: HTCA (Fruehauf, 2002)

The major clinical studies for the HTCA were carried out by Von Hoff with results indicating that the HTCA was correlated with clinical drug resistance and drug sensitivity. A modified version of the HTCA developed by Kern and colleagues using (^{3}H) thymidine as an end point instead of colony formation was shown to be highly accurate in correlating to clinical drug resistance but less accurate in correlating to clinical sensitivity. Depending on the investigator, the various versions of the HTCA were shown to evaluate only 30-70% of the specimens attempted, thus limiting the usefulness of the HTCA (Furukawa, 1995). Moreover, the primary culture success rate of this assay is low, and many tumor cells are required (Yasuda, 1998).

2.2.5 Extreme drug resistance (EDR) assay

The EDR assay measures the effects of chemotherapeutic agents to inhibit proliferation of small tumor cell clumps suspended in a low density layer of agarose that overlays a solid layer of agarose (Sondak *et al.*, 1984). Cell cultures are incubated with drugs for 4 days and pulsed with ^{3}H-thymidine over the last 2 days of the 5-day culture. Radio labeled thymidine is incorporated into the replicating DNA

of dividing cells while non-proliferating cells and dead cells fail to incorporate the label. Cells are harvested onto glass fiber filters and lysed with deionized water. The filter traps the labeled macromolecular DNA, while unincorporated tritiated thymidine is washed through the filter. Filters and scintillation fluid are added to vials and radioactive decay is measured in a scintillation counter to determine the amount of DNA synthesis that took place in control and treated tumor cells as shown in Figure 7 (Elledge *et al.*, 1995). This EDR systems measure the growth of tumor clusters which can grow better compared with inform of single cell suspension, improving the assay success rate and shortening the time required for obtaining results. The EDR assay success rate is approximately 85% (Mehta *et al.*, 2001) and takes only 5 days for culture. In contrast HTCA, which requires that tumors be disaggregated to the single cell, has only 50% evaluation rate and takes 14 days or longer than with EDR assay (Clark GM 1984).

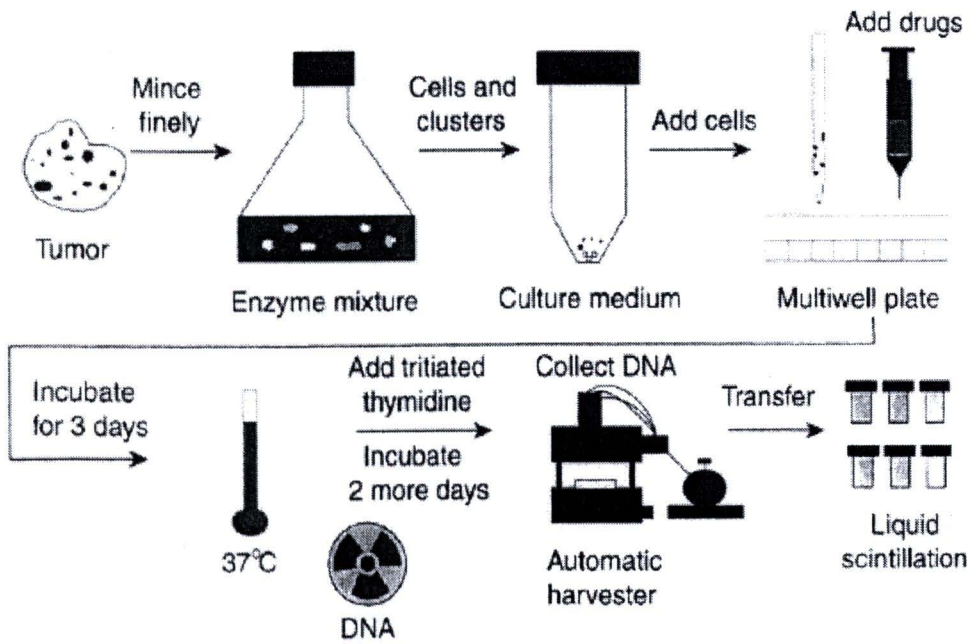


Figure 7 *Invitro assay* schema :EDR assay (Fruehauf, 2002)

2.2.6 Histoculture drug response assay (HDRA)

The HDRA is an *in vitro* chemosensitivity test. It was a tissue culture technique. The tissue sample were cut inform of fragments and placed into collagen sponges had been immersed in medium with exposure to anticancer agents, as shown

in Figure 8. The distinctive feature of this assay is its use of three-dimensional tumor tissue histology in culture instead of a culture of free cells. This assay maintains inter-cellular contact and interaction with stromal cells in a condition of native architecture thus this assay is closely related to the *in vivo* condition (Hasegawa, 2007). In addition to the mechanisms of resistance to anticancer drugs are present in three dimensional culture and absent when the same cells are cultivated as monolayers or cell suspensions (Desoize and Jardillier, 2000). The intracellular contact conditions a different expression of the enzymes implicated in cell division (e.g. topoisomerase II) as well as the emission of survival signals mediated by adhesion molecules. Apoptosis is induced in cells that lose contact with other cells. In solid tumors and tumoral spheroids, inhibition of apoptosis in cells that are in contact with others is one mechanism of multicellular resistance. Furthermore, the three dimensional structure is responsible for a reduced susceptibility to cytotoxicity as determined by the limited penetration of the drug (Tannock *et al.*, 2002) and by the existence of oxygen, nutrient, and tissue proliferation gradients (Desoize, 2000) and thus is deemed to assess the chemosensitivity of individual tumors in a state close to the *in vivo* condition.

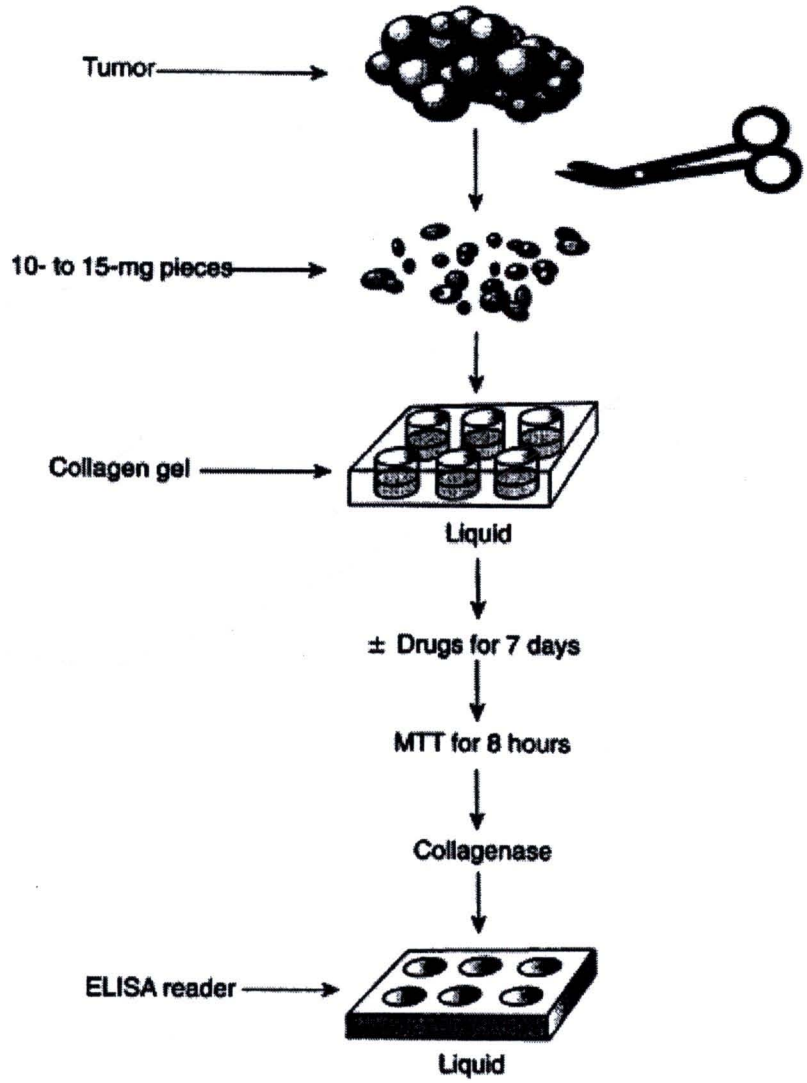


Figure 8 *In vitro* assay schema: HDRA (Fruehauf, 2002)

In several research, to investigate *in vitro* drug sensitivity test by using HDRA and find the association of HDRA sensitivity with clinical outcome in cancer of head and neck (Hasegawa, 2007), colon (Isshi, 2002), urinary tract (Hirano *et al.*, 2001), ovarian (Ohie *et al.*, 2000) and breast (Tanino *et al.*, 2001). Suda and colleagues investigate whether the effects of anticancer agent are able to be predicted by using HDRA compared with the results of preoperative chemotherapy in esophageal and gastric cancer or not. They found that the results of determinations by HDRA from the standpoint of clinical efficacy, sensitivity was found in 88%, and specificity was 80%. It was thus demonstrated that predictions of the effect of

anticancer agents could be made with considerable accuracy using HDRA (Suda, 2002). In squamous cell carcinoma in the oral cavity, a good correlation was obtained between the chemosensitivity test results and clinical response, so HDRA is a useful method for selection of an anticancer agent for individual oral cancer patients (Ariyoshi *et al.*, 2003). However, the HDRA uses a tissue culture technique to assess the response to anticancer agents, employing the MTT assay to determine cell viability in the end step of process, the problem of interference due to fibroblast cannot be eliminated. Therefore in this research we have also used the HDRA to assess the response of CCA to 5-FU with some modification of the end step instead of MTT assay by using formalin fix paraffin embedded assay to evaluate the viability of cancer cells by counting the number of viable cancer cells in the CCA tissue based on their morphology under microscope.