CHAPTER II
LITERATURE REVIEWS

2.1 Cholangiocarcinoma

2.1.1 Definition and classification

Cholangiocarcinoma (CCA) is the malignant tumor originated from bile duct epithelium at the biliary tree of both intrahepatic and extrahepatic portions except gall bladder and ampulla of Vater (Uttaravichien et al., 1999). Regarding the anatomical position of the tumor mass, CCA can be divided into intrahepatic and extrahepatic types. In northeastern Thai population, intrahepatic CCA was found around 70% whereas that of extrahepatic type was 30% (Uttaravichien, 1996).

2.1.1.1 Intrahepatic CCA (ICC)

ICC is developed from the intrahepatic biliary tree including the right and left hepatic ducts. It is topographically divided into two types: a central type which is related to a major intrahepatic bile duct and a peripheral or hilar type, in which tumor is located mainly at the hepatic hilum. Patients with peripheral type most commonly present with abdominal pain, weight loss and liver mass whereas the central type often shows the obstructive jaundice as the main symptom (Bhudhisawasdi, 1997).

2.1.1.2 Extrahepatic CCA

Extrahepatic CCA is developed in the extrahepatic bile duct including the common hepatic duct, cystic duct and common bile duct except parts of gall bladder and the ampulla of Vater. The gross pathology of this type including the upper third is usually scirrhous or infiltrative, however, papillary or nodular types may occur. The middle and the lower thirds mostly exhibit nodular and papillary types (Bhudhisawasdi, 1997).

For the growth and spreading pattern, CCA falls into three subtypes; the mass forming type, the periductal infiltrating type and the intraductal growing type which may be intrahepatic or extrahepatic (Fig 2-1). (1) The mass forming type is the most common type of CCA. The cancer forms central mass

around the bile duct. For this type, the tumor borders between the cancerous and non-cancerous portions are relatively distinct. (2) The periductal infiltrating type is usually associated with biliary stricture. The tumor exhibits diffuse infiltration along the portal pedicle. (3) The intraductal growth type is the less common type of CCA. The tumors are confined within the dilated part of intrahepatic large bile duct with no or mild tumorous extension beyond the bile duct walls (Nakanuma et al., 2003). However, advanced ICC often exhibits mixed pattern.

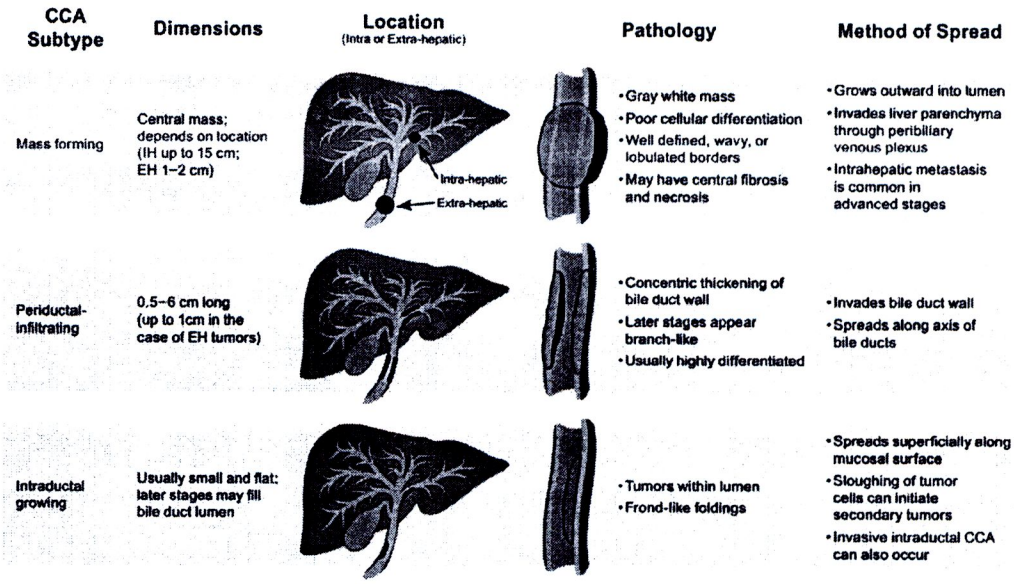

Figure 2-1 Characteristic of CCA. CCA can be divided into three subtypes: mass forming, periductal infiltrating, and intraductal growing. These patterns are independent of the location of tumor arising (intrahepatic: IH or extrahepatic: EH) (Sripa et al., 2007).

2.1.2 Histopathology and histologic classification

The most common histopathology of CCA is adenocarcinoma showing a glandular and/or papillary structure lined by cuboidal or columnar epithelium with a variable fibrous stroma. Several histological variants are also recognizable including squamous cell carcinoma, mucinous carcinoma, mucinous cystadenocarcinoma, sarcomatoid CCA, clear cell variant and signet ring cell carcinoma (Nakajima et al., 1988; Nakanuma, 1997). Histological feature of CCA can be graded into papillary,

well, moderately and poorly differentiated adenocarcinoma regarding to their shapes and differentiations. (1) Papillary adenocarcinoma shows a well differentiated, distinctive papillary growth pattern lining with columnar or cuboidal tumor cells around the fibrovascular stalks. (2) Well differentiated shows a uniform glandular structure lining with basally situated nuclei. (3) Moderately differentiated shows moderately distorted glandular or tubular patterns with cribriform formations and/or a cord-like pattern. (4) Poorly differentiated shows markedly distorted tubular structures with cellular pleomorphism (Vatanasapt et al., 1990).

2.1.3 Epidemiology and etiology

In Thailand, CCA was observed in a high percentage of liver cancers in northeastern part where the prevalence of *Opisthorchis viverrini* infection is the highest (Srivatanakul, 2001). Several surveys of Thai villagers showed a strong association between the prevalence of *O. viverrini* infection, parasite-specific antibody response, and abnormalities of the biliary tract, including suspected CCA (Elkins et al., 1990; Mairiang et al., 1992). The frequency of suspected CCA and *O. viverrini* faecal egg count was observed with an odds ratio of 14.1 in a group with elevated egg counts (Haswell-Elkins et al., 1994).

Moreover elevated anti-*O. viverrini* antibody titres increased the risk of CCA appearance up to 27-fold (Honjo et al., 2005). These data suggested the correlation between CCA and *O. viverrini* infection. Study from Sriamporn et al. in Thai people during 1990-2001 confirmed a strong positive correlation between the incidence of CCA with the prevalence of *O. viverrini* infection (Fig 2-2) (Sriamporn et al., 2004). In the North and the Northeast of the country, prevalence of *O. viverrrini* infection was the maximal value among all other areas together with the high CCA cases were found. It was reported that CCA in Khon Kaen province was 118.8 per 100,000 in average and believed to be the highest incidence among all over the world.

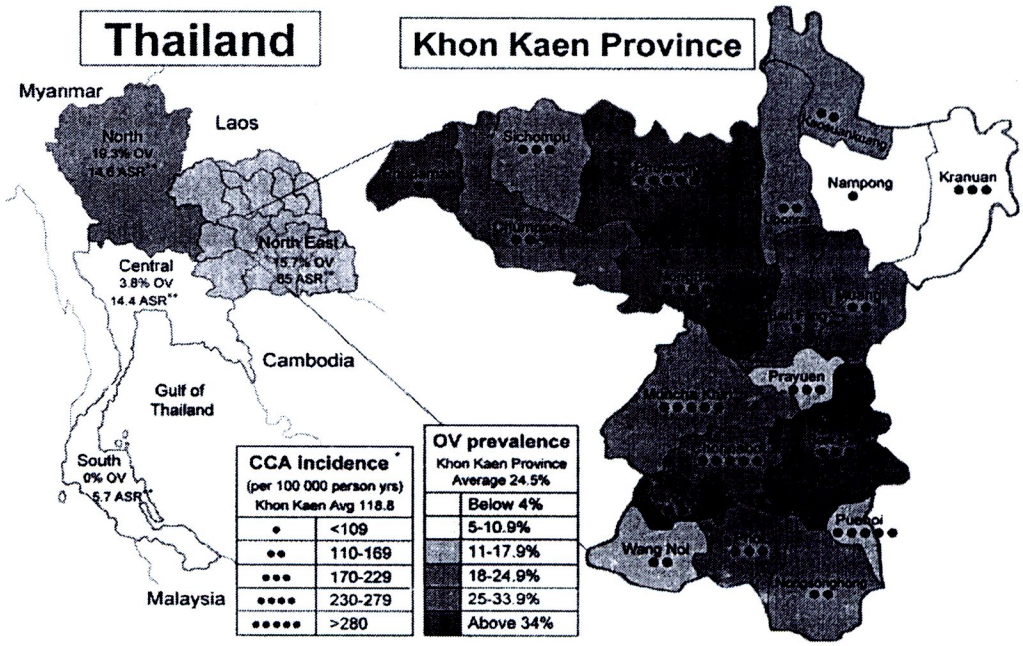

Figure 2-2 Incidence of CCA and *O. viverrini* in Thailand from 1990–2001. Increasing intensity of red represents increasing prevalence of *O. viverrini*, while increasing number of dots represents increasing cancer rates. *Truncated age-standardised incidence from 35–64 years. **Age-standardized incidence of CCA throughout registered regions (Sripa et al., 2007).

2.1.4 Risk factors

The etiology of CCA in Asian countries is mostly associated with liver fluke infection including *C. sinensis* and *O. viverrini* (Lim et al., 2006; Sriamporn et al., 2004). Infection with *C. sinensis* and *O. viverrini* are classified as "carcinogenic to humans" or Group 1 carcinogen by International Agency for Research on Cancer (IARC, 1994).

2.1.4.1 *C. sinensis* infection

Currently, the most prevalence of *C. sinensis* infection was detected in Korea, Japan, China and Taiwan. Hamsters infected with *C. sinensis* were increased susceptibility for developing CCA through dimethylnitrosamine (DMN)-induced or inflammation-mediated carcinogenesis (Lee, Rim, and Bak, 1993). Moreover, the proliferative effects of excretory/secretory products (ESP) from *C.*

sinensis and of DMN on human epithelial cells *in vitro* were examined (Kim et al., 2008). Human epithelial cells treated with *C. sinensis* ESP alone and simultaneously induced by DMN were increased cell proliferation and proportion of cells in the G2/M phase of the cell cycle. These results supported that exposure to *C. sinensis* and stimulation with some carcinogen such as DMN promoted cholangiocarcinogenesis.

2.1.4.2 *O. viverrini* infection

In Thailand where people eat unwell-cooked Cyprinoid fishes which act as the intermediate host of the parasites and contain *O. viverrini* metacercaria. The metacercaria can develop to be adult parasite in the human bile duct as the definite host. Chronic inflammation caused by the parasite infection through mechanical, biochemical and immunological processes is acting together to facilitate CCA formation. The detail of *O. viverrini*-induced cholangiocarcinogenesis is discussed in 2.1.5.

2.1.4.3 Other risk factors

The other risk factors for CCA including cirrhosis, chronic non-alcoholic liver diseases, bileduct adenoma, multiple biliary papillomatosis, choledochal cysts, congenital fibropolycystic liver, Caroli's disease (cystic dilatation of intrahepatic bile ducts) and exposure to the radiopaque medium thorium dioxide are also recognized (Khan, Toledano, and Taylor-Robinson, 2008). Moreover, bacterial infection and bile stasis are also demonstrable in virtually all patients underlie CCA development (Chen, 1999).

2.1.5 Cholangiocarcinogenesis

CCA is a result of chronic inflammation of the bile duct where in Thailand mainly from *O. viverrini* infection. Several mechanisms by which the parasite infection may involve in cholangiocarcinogenesis have been proposed and recently summarized in Sripa and Pairojkul, 2008 (Fig 2-3).

The pathogenesis of *O. viverrini*-mediated hepatobiliary changes may be due to mechanical irritation caused by the liver fluke suckers and/or its metabolic products (Bhamarapravati, Thammavit, and Vajrasthira, 1978; Sriamporn et al., 2004). During liver fluke infection, inflammation, periductal fibrosis, and proliferative responses, including epithelial hyperplasia, may represent predisposing lesions that enhance susceptibility of DNA to carcinogens (Flavell and Flavell, 1986;

Kim, 1984). The supportive evidences showed that the substances released from the parasites increased cell proliferation of mouse fibroblast cell line by stimulating the expression of phosphorylated retinoblastoma (pRB) and cyclin D1 to drive cell cycle progression (Thuwajit et al., 2004; Thuwajit et al., 2006). Moreover, endogenous nitrosation caused by *O. viverrini* infection and inflammation has been observed in animals and humans. Both exogenous and in situ nitrosamine formation may lead to DNA alkylation and deamination in predisposed and inflamed tissues (Sripa et al., 2007). Therefore, by hyperplasia responsiveness synchronized with DNA damage enhancement of the infected biliary epithelium may lead to cholangiocarcinogenesis.

However, the immunopathological process may also contribute to biliary epithelial damage in order to chronic inflammation. The inflammation around infected hamster bile ducts was a consequence of the host's cellular response to *O. viverrini* antigens. Marked infiltration of inflammatory cells at the periportal areas of infected hamster liver was associated with the presence of parasite antigens in the bile duct epithelium (Sripa and Kaewkes, 2000). Moreover, excess nitric oxide and other reactive oxygen intermediates produced by inflammatory cells during infection might exert direct cytotoxic and mutagenic effects and increase cell proliferation. With less apoptosis of the infected biliary epithelium after several successions of replication, genetic alterations may lead to malignant transformation.

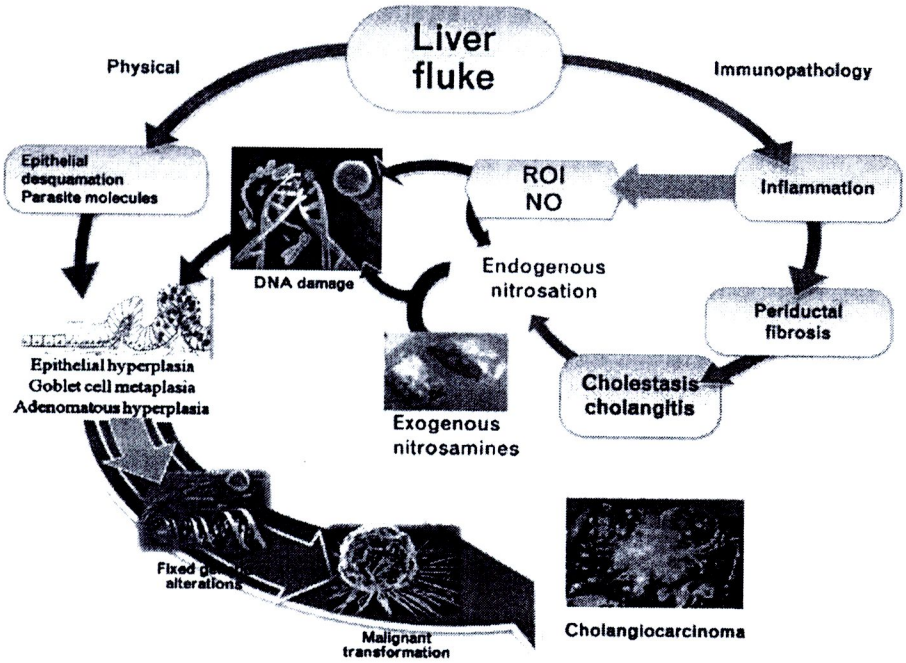

Figure 2-3 Proposed mechanism of cholangiocarcinogenesis induced by *O. viverrini* infection. The mechanical process caused by parasite sucker, excretory/secretory products and immunopathology to parasite antigens were proposed. Inflammation-induced oxidative DNA damage occurs concurrent with biliary epithelial proliferation driven by parasite molecules may lead to CCA (Sripa and Pairojkul, 2008).

2.1.6 Diagnosis and prognosis

Nowadays, the diagnosis of CCA is performed using clinical symptoms, ultrasonography, and tissue pathological result which the last one is the gold standard. Unluckily, almost the patients coming to see the doctor already contain late stage of the diseases. The diagnostic and prognostic markers are required to help the management of CCA patients.

Several researches on CCA have been performed to identify biomarkers with adequate diagnostic accuracy for CCA in serum or biological fluids (Nehls, Gregor, and Klump, 2004; Ramage et al., 1995), which could be useful for CCA screening. Unfortunately, up to now, none of these markers has reached adequate specificity for CCA (Gatto et al., 2010). Carbohydrate antigen (CA 19-9) is

the most widely used to be serum marker for CCA with 79% sensitivity and 98% specificity. However, it is also elevated in pancreatic cancer, gastric cancer, primary biliary cirrhosis. In the same direction, interleukin 6 (IL-6) has been provided a diagnostic with high sensitivity and specificity (Goydos et al., 1998). The increasing of IL-6 level has been correlated with tumor size in CCA, and interestingly, 1 month after treatment, the mean level was significantly decreased. Although these results seem to encourage IL-6 as a good diagnostic and poor prognostic marker for CCA patients but, serum IL-6 is also elevated in hepatocellular carcinoma, benign biliary disease and metastatic lesions. In particular, mucin 1 (MUC1) and MUC5AC are not expressed by hepatocellular carcinoma, suggesting a possible role in the differential diagnosis (Park et al., 2009). Moreover, CCA immunohistochemical staining for mucin, has provided evidenced that MUC1 expression in CCA closely related to dedifferentiation and infiltrative growth pattern, implying that the expression of MUC1 might be associated with the progression of CCA.

Increased expression and activity of various matrix metalloproteinases (MMPs), in particular, MMP-2, -7, and -9, are associated with tumor invasion and metastasis in malignant neoplasms, including intrahepatic CCA (Itatsu et al., 2008; Miwa et al., 2002; Shirabe et al., 1999). Relative to other MMPs, MMP-7 appears to have significant potential as a prognostic factor for poor survival in CCA patients after surgery (Itatsu et al., 2008; Miwa et al., 2002). CCA-associated stroma has also provided flavour condition for tumor cells by expressing of specific proteins as poor prognostic markers. Up-regulation of galectin-1 in CCA stroma was correlated with histologic dedifferentiation and significantly correlated with perineural and vascular invasion (Shimonishi et al., 2001). In addition, high expression of alpha-smooth muscle actin (α-SMA) in CCA-associated fibroblasts exhibited poorer survival times than those with low α-SMA expression (Chuaysri et al., 2009).

2.1.7 Treatment

Clinical treatment options for CCA are limited. The conventional chemotherapy and radiation therapy have been notably ineffective in improving long-term survival of which the useful treatment is complete resection of the tumor. Recently, several preclinical studies supporting the therapeutic potential of selected

targeting strategies against biliary tract cancer cells were proposed (Sirica, 2005) (Table 2-1).

Although the results of the preclinical therapeutic studies were obtained from *in vitro* experiments and from a limited number of *in vivo* studies involving tumor cell xenografts, the potential clinical value of novel therapies against CCA are based on selective molecular targeting. For example, cyclooxygenase (Cox)-2 specific inhibitor or celecoxib was evaluated the mechanism and inhibition effect on CCA cell proliferation *in vitro* (Wu et al., 2003). Celecoxib suppressed the production of Protaglandin E2 (PGE2), inhibited the growth of CCA cell lines with dose and time dependent fashion and significantly induced apoptosis of the cancer cells. This study indicates that inhibition of proliferation and induction of apoptosis in human CCA cells by celecoxib may involve in COX-dependent mechanisms and PGE2 pathway. Celecoxib as a chemopreventive and chemotherapeutic agent may be effective in COX-2-expressing CCA patients. Interestingly, tauroursodeoxychate (TUDCA), the agent for treatment of cholestatic liver diseases has been studied role in regulation of human CCA cell growth (Alpini et al., 2004). TUDCA inhibited the growth of CCA cell lines in concentration- and time-dependent manners. TUDCA regulated cell growth by up-regulation of protein kinase C (PKC)-α and increasing of intracellular Ca^{2+} concentration. Moreover, TUDCA could inhibit cell growth via mitogen-activated protein kinase (MAPK) p42/44 pathway but independent from Raf proteins and MAPK p38 and Jun amino-terminal kinase (JNK)/stress-activated protein kinases. Therefore, using TUDCA for treatment may improve clinical outcome and histological features of cholestatic liver diseases which is the important risk factor for CCA.

Until now CCA continues to be a challenging cancer that requires innovative approaches to early diagnosis, and in particular, the development of novel adjuvant therapies having potential for greatly improving long-term survival rates. The challenge is to target the complex interactive roles played by cancer cells and their microenvironment in affecting cancer cell growth, invasion, and angiogenesis signaling pathways as the powerful new molecular therapies for CCA patients (Sirica, 2005).

Table 2-1 Preclinical approaches supporting potential therapeutic of molecular targeting strategies against biliary tract cancer cells

Agent	Class	Experimental Condition	Biological effects on cancer cells	References
Anti-IL-6R	Neutralizing antibody	Cell culture	Attenuated cell growth	Park et al., 1999
HGF/NK4	HGF antagonist	Cell culture	Inhibited HGF-induced invasion	Date et al., 1998
		in vivo	Suppressed growth, invasion	
Celecoxib	COX-2 inhibitor	Cell culture	Induced dose-dependent growth inhibition and apoptosis	Zhang et al., 2004 Hayashi et al., 2001 Wu et al., 2004, Han et al., 2004
		in vivo	Suppressed growth	Wu et al., 2003
TNF-related apoptosis-inducing ligand (TRAIL)/Apo2L	TNF family cytokines	Cell culture	Induced significant apoptosis	Tanaka et al., 2000
		in vivo	Inhibited tumorigenic in nude mice	
Tautoursode-oxycholate	Bile acid conjugate	Cell culture	Inhibited CCA cell growth	Alpini et al., 2004

(Sirica, 2005)

2.2 Stromal-epithelial interaction in cancers

Cross-talk between mesenchyme and epithelium in normal situation has been described as a known driver of differentiation and development, with examples in prostate and ovary (Aboseif et al., 1999). Several studies have shown that changes in stromal behavior can promote epithelial transformation (Aboseif et al., 1999; Baskin, 2001; Olumi et al., 1999). Tumor stroma is the microenvironment of cancer cells and defined to contain both cell and non-cell components. The stromal cells are fibroblasts, immune cells, endothelial cells, and pericytes whereas proteins produced from these cells together with cancer cells are able to secrete out and then form the extracellular matrix (ECM). Among these cells, fibroblasts are the most abundant and can produce many substances involved in several carcinomas or cancers of the epithelial origin.

The stromal-epithelial interaction can influence cancer initiation and progression through many mediators. This interaction is mediated by soluble paracrine signals and ECM components secreted from developing mesenchyme that induce the adjacent epithelia to proliferate rapidly. In normal condition, as epithelial cells differentiate, so do adjacent stromal cells. These differentiated stromal cells generally express lower quantities of growth factors, and in the same time differentiated epithelial cells express cytokines for the maintenance of stromal differentiation. During tumorigenesis, however, the prevailing model suggests a process whereby pre-cancerous epithelial cells acquire multiple genetic mutations, and the associated-stroma becomes activated, including activated fibroblasts (Ronnov-Jessen, Petersen, and Bissell, 1996).

Molecular cross-talk between cancer fibroblast and epithelial cancer cell has been reviewed and summarized (Fig 2-4) (Liotta and Kohn, 2001). Fibroblasts produce several growth factors such as growth factor scatter factor/hepatocyte growth factor (SF/HGF), which can stimulate motility of tumor cells by binding to the Met receptor (c-Met). Tumor cells produce angiogenesis factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which bind to receptors on stromal vascular cells and cause increased vascular permeability and endothelial cell proliferation.

Fibroblasts and endothelial stromal cells elaborate latent enzymes, including MMPs and urokinase plasminogen activator (uPA), which dock on the surface of the carcinoma invadopodia and become activated, thereby degrading the ECM, and clearing a pathway for cancer cells to migrate. In addition, ECM degradation releases bound growth factors such as transforming growth factor-beta (TGF-β) and epidermal growth factor (EGF), which bind to the specific receptors TGF-βR and uPAR, respectively on the carcinoma cell leading to cell proliferation. ECM proteolysis also exposes cryptic Arg-Gly-Asp (RGD) sites, which are recognized by integrins (ITGs).

Moreover, cross-talk between signal pathways within the carcinoma cells causes motility, proliferation and pro-survival signals. For example, phosphorylation of focal adhesion kinase (FAK) through Met and ITG signaling transduces signals through Ras, phosphoinositol triphosphate kinase (PI3K), β-catenin and myosin light chain kinase (MLCK), causing cytoskeletal remodeling, extracellular signal-regulated

kinase (ERK) activation of mitogenesis, and sustainment of survival through phosphorylation of serine/threonine protein kinase (AKT), a downstream target of PI3K.

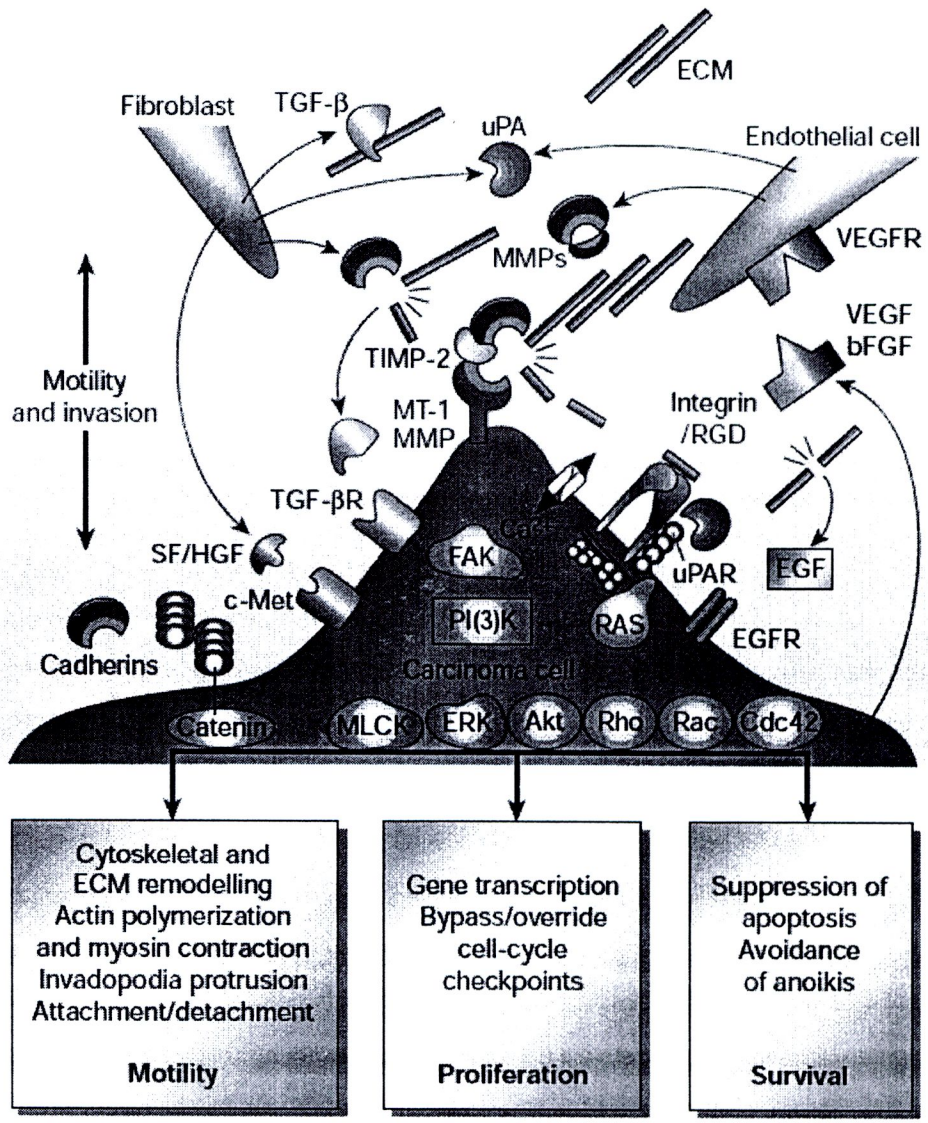

Figure 2-4 Molecular cross-talk between fibroblast and carcinoma cell. Example mediators are shown (Liotta and Kohn, 2001).

2.3 Cancer-associated fibroblasts

As mentioned above, the stroma or supportive base of the epithelial layer is composed of fibroblastic, smooth muscle, inflammatory, endothelial, and nerve cells. Changes in these stromal cells have been postulated to enhance several tumorigenic

phenotypes of the epithelial cells (Hong and Sporn, 1997; Rinehart and Torti, 1997; van den Hooff, 1983). The fibroblast is a major cell type of the stromal compartment, and as such is intimately involved in orchestrating the dialogue in tissue homeostasis. Moreover, modification of fibroblasts in the stroma immediately adjacent to transformed epithelial cells has been documented in several tumor systems (Chiquet-Ehrismann et al., 1986; Singer et al., 1995; Wright et al., 1994; Yee et al., 1991).

Activated fibroblasts are found in healing wounds and sclerosing tissues, and are also associated with cancers. They were first identified over 30 years ago in healing rat wounds with the different from normal fibroblasts by their capability to express fibroblast activation protein (FAP) and produce more secretary proteins (Gabbiani, Ryan, and Majne, 1971). Cancer-associated fibroblasts (CAFs) by the name are defined to fibroblasts that found in cancer tissues. Several lines of evidence support CAFs as myofibroblasts with smooth muscle phenotypic properties (Lazard et al., 1993) and typified by the expression of α-SMA *in vivo* and *in vitro* (Noel and Foidart, 1998). Thease fibroblasts may influence the propensity of luminal epithelial cells to undergo an epithelial-mesenchymal transition (EMT) and hence become malignant (De Wever and Mareel, 2003).

2.3.1 Characterization

CAFs differ from normal fibroblasts in their molecular marker expressions, expression of growth factors (Heffelfinger et al., 1999), and profiles of ECM molecules (Barsky et al., 1982; Matsumoto et al., 1999) (Figure 2-5). In addition to α-SMA, CAFs express fibroblast activation protein (FAP), a 93 kDa cell surface protein of reactive-tumor stromal cells that is not present in most normal human adult tissue. CAFs can increase secretion of ECM-degrading proteases such as MMP-2, MMP-3 and MMP-9, facilitating increased ECM turnover and altered ECM composition (Rodemann and Muller, 1991). CAFs are also increased secretion level of several growth factors (Bhowmick, Neilson, and Moses, 2004).

Normally, fibroblasts are constitutively expressed vimentin and fibroblast-specific protein 1 (FSP1) and appear as fusiform cells with a prominent actin cytoskeleton and vimentin intermediate filaments. When fibroblasts acquire the activation from environmental growth factors, ECM proteases and chemokines, they can enhance proliferative activity and secretion of ECM proteins. Furthermore, their

typical characteristic are converted to activated fibroblasts together with the increased α-SMA expression (Kalluri and Zeisberg, 2006).

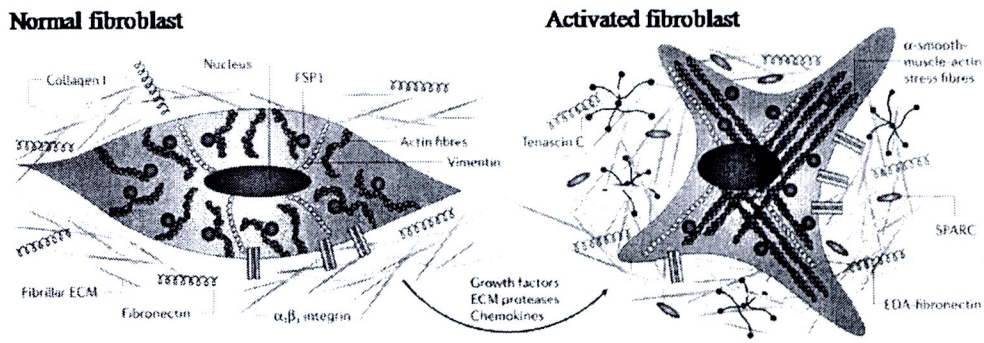

Figure 2-5 Differential characteristic of normal and activated fibroblasts. This figure shows phenotypic change from normal to activated fibroblasts induced by growth factors, ECM proteins and chemokines (Kalluri and Zeisberg, 2006).

2.3.2 Fibroblast-derived substances

Fibroblasts can produce stromal ECM proteins and secrete many growth factors and hormones. Several families of growth factors, implicated as autocrine and paracrine mediators of stromal-epithelial interactions, are involved in carcinoma initiation and progression. These include the fibroblast growth factor (FGF), insulin-like growth factor (IGF), EGF, HGF, TGF-β (Table 2-2). Most of these factors are predominantly stimulators of proliferation and can play a part in promoting the carcinogenic process. Platelet-derived growth factor (PDGF) expressed by immortalized skin keratinocytes induces the expression of fibroblast growth factor 7 (FGF7) by fibroblasts (Brauchle et al., 1994). FGF7 in turn has been shown to produce further epithelial proliferation and promote carcinogenesis. In the prostate, FGF7 and FGF10, produced by fibroblasts, stimulate the proliferation of adjacent epithelia (Lu et al., 1999).

Some growth factors were indicated the differential roles in prostate cancer (Russell, Bennett, and Stricker, 1998) such as the expression of TGF-β family has been shown to associate with cell migration and angiogenesis. Increased EGF expression appears to be associated with the invasive ability of prostate cancer cells

by induction of tumor proteases whereas IGF could promote metastasis in this cancer (Russell, Bennett, and Stricker, 1998). In addition, HGF secreted from gastric fibroblasts could stimulate not only tumor growth but also invasion in gastric cancer (Murakami, Koufuji, and Shirouzu, 2001). MMP-1 and MMP-7 are of fibroblastic origin and can induce increased susceptibility to mammary cancer when overexpressed in transgenic mice (Lynch and Matrisian, 2002).

These evidences suggest that substances derived from fibroblasts are the important mediators for tumorigenesis and cancer progression and play the various roles in several cancers.

Table 2-2 Fibroblast derived-growth factors and roles in cancers

No.	Soluble factors	Cell expressed	Responding cells	Possible role
1.	HGF and MSP	Fibroblasts	Epithelia	+ Proliferation +Transformation + Morphogenic
2.	IGF-1, IGF-2	Fibroblast	Epithelia (breast)	- Apoptosis + Proliferation
3.	EGF and TGF-α	Epithelia and fibroblasts	Epithelia	+ Proliferation + Morphogenic
4.	TGF-β1, TGF-β2, TGF-β3	Epithelia and fibroblasts	Epithelia and fibroblasts	+ Proliferation +/- Apoptosis + Morphogenic
5.	FGF7/ KGF	Fibroblast	Epithelia	+ Proliferation + Morphogenic
6.	IL6, LIF	Fibroblast	Epithelia (colonic)	+ Proliferation +Transformation
7.	FGF2	Fibroblast	Epithelia	+ Proliferation +Transformation
8.	FGF10	Fibroblast	Epithelia	+ Proliferation
9.	NGF	Fibroblast	Epithelia	+Transformation
10.	Stromal cell-derived factor 1α (CXCL12)	Fibroblast	Epithelia (glioblastoma)	+ Proliferation +Transformation
11.	Wnt1, Wnt3	Fibroblast	Epithelia	+ Proliferation +Transformation
12.	MMP-1, MMP-7	Fibroblast	ECM and growth-factor activation in stroma affect epithelia	+ Proliferation +/- Apoptosis + Morphogenic

MSP, macrophage stimulating protein; IL6, interleukin 6; LIF, leukemia inhibitory factor; NGF, nerve growth- factor.

(Bhowmick, Neilson, and Moses, 2004)

2.3.3 Roles of fibroblasts in human cancers

In several cancer diseases, fibroblasts are involved in cancer initiation and progression, through the synthesis, deposition and remodeling of the ECM and are also the main source of paracrine factors that influence cancer cell growth, invasion, metastasis, and angiogenesis. All these fibroblasts-induced tumorigenic effects are summarized herein as the roles of fibroblasts in the initiation and progression of cancers.

2.3.3.1 Role of CAFs in cancer initiation

Decades ago, CAFs enhanced tumor formation were investigated (Hodges, Hicks, and Spacey, 1977; Uchida et al., 1990). The urinary bladder carcinogen, N-butyl-N-(4-hydroxybutyl) nitrosamine (BHBN) was used to treat the rats. After 4 weeks, their bladders were removed and epithelial cells were completely eliminated. The bladders without epithelium (stroma bladder) were heterotopically transplanted to syngeneic recipients. The result showed a higher tumor incidence of carcinoma in BHBN-exposed stroma bladders transplanted rats compared to the control. Moreover, among the bladders that had received BHBN urothelial cells, carcinogen-exposed stroma bladders proved to be better soil for neoplastic cells to proliferate by determination of significantly increased of mean total tumor volume per bladder in the bladders transplanted with BHBN-exposed stroma compared those of control. These data suggested that carcinogen induced alterations in the stroma could provide carcinoma initiation and enhance neoplastic growth by interaction with carcinogen treated urothelium.

The effects of carcinogen-treatment on stromal cells have also been examined in murine mammary tissues (Barcellos-Hoff and Ravani, 2000). In this study, irradiation of epithelial cell-free mammary stroma facilitated tumorigenesis. In the irradiated stroma, the mammary epithelial cells developed tumors faster, more often and reached a greater size than the same cells transplanted into unirradiated stroma. These results indicated that carcinogens could affect the neoplastic process not only by inducing genetic changes in the epithelial cell, but also by altering the stromal cells.

2.3.3.2 Role of CAFs in cancer progression

An essential characteristic of cancer cells is their potential to proliferate and infiltrate surrounding normal tissue. In most cancers, lethality is the result of local invasion and the metastasis of neoplastic cells from the primary tumor to other tissues. Several lines of studies done *in vivo* (Camps et al., 1990) and *in vitro* (Grey et al., 1989; Picard, Rolland, and Poupon, 1986) indicated that the growth and invasive potentials of carcinoma cells were influent through interactions with host stromal cells. Moreover, *in vitro* culture and *in vivo* tissue recombination systems were used to study role of CAFs in prostate cancer (Olumi et al., 1999) and demonstrated that human prostatic CAFs grown with initiated human prostatic epithelial cells dramatically stimulated growth and altered histology of the epithelial population. This effect was not detected when normal prostatic fibroblasts were grown with the initiated epithelial cells under the same experimental conditions. In contrast, CAFs did not affect growth of normal human prostatic epithelial cells under identical conditions. The authors concluded that CAFs could direct tumor progression of an initiated prostate epithelial cell.

Moreover, *in vitro* growth and invasion of certain carcinoma cells was markedly accelerated when interact with stromal fibroblasts (Nakamura et al., 1997). They found that HGF-derived fibroblast could stimulate cell growth, scattering and invasion of cancer cells. Although these cancer cells did not produce biologically significant levels of HGF, but the HGF level was induced by their production of IL-1, bFGF and PDGF. When these carcinoma cells were co-cultured with fibroblasts, HGF levels in the co-culture system were much higher than the levels in fibroblasts alone, without co-cultured carcinoma cells. Together with the increase in HGF levels, the number of invasive cell was increased. The mutual interactions, as mediated by HGF and HGF inducers, may play a significant role in the occurrence of invasion and metastasis of carcinoma cells.

Additionally, CAFs-mediated angiogenesis of tumor cells were mentioned (Crawford et al., 2009). They found that CAFs isolated from anti-VEGF-resistant tumors could stimulate tumor angiogenesis *in vivo*. They compared gene expression profile of these CAFs versus normal skin fibroblasts and focused on platelet-derived growth factor C (PDGF-C) which was one of up-regulated angiogenic

gene. Interestingly, blocking PDGF-C using specific antibodies reduces the growth of tumors that are resistant to anti-VEGF treatment and exhibits additive effects with anti-VEGF therapies. Hence, these data suggest that some tumors may overcome inhibition of VEGF-mediated angiogenesis through up-regulation of PDGF-C.

In conclusion, local and mutual interactions between carcinoma cells and stromal fibroblasts are of particular importance in regulating the growth, invasion, metastasis and angiogenesis of tumor cells. However, the molecular mechanisms of fibroblasts leading to the tumor progression are still unclear. Hence, several studies in the last years, using whole gene expression study, tried to explain the possible molecular mechanisms of CAFs in cancer.

2.4 Gene expression profile of fibroblasts in human cancers

Though the evidence that gene expression pattern of fibroblasts from different organs revealed unique profile (Fromigue et al., 2003; Mercier et al., 2008; Nakagawa et al., 2004; Rosenthal et al., 2004; Wang et al., 2010), it is possible to hypothesize that CAFs in different cancers may have different gene expression pattern. In addition, several lines of studies have confirmed the impact of CAFs in cancers, many research groups have worked on expression analysis of CAFs with the aim to better understand the role of CAFs in cancers. In human basal cell carcinoma, CAFs and normal skin fibroblasts were isolated by laser microdissection and preceded to microarray analysis (Rosenthal et al., 2004). The results revealed 415 up-regulated and 458 down-regulated genes. These were genes involved in growth regulation including amphiregulin, SDF-1, IGF-1, TGFβ-3; angiogenesis such as angiopoietin 2, and matrix remodeling including MMP-5, -11, TIMP-4. The microarray approach has been extended to different types of solid tumors including breast, colon, lung and prostate cancers.

CAFs and their matched normal mammary fibroblasts isolated from breast cancer tissues were studied the differential gene expression profiles using DNA microarray (Mercier et al., 2008). By comparing three pairs of normal and CAFs, the microarray result showed 118 common up-regulated and 66 common down-regulated genes in CAFs with fold change more than 3. Among 118 common up-regulated genes, MET and its co-receptor CD44 are both up-regulated. Moreover, cell cycle

progression related genes were also up-regulated in CAFs such as cell division cycle 2 (CDC2), cell division cycle associated 3 (CDCA3), CDCA5 and CDCA8. In contrast, the down-regulated genes in CAFs exhibited only a weak enrichment for genes involved in extracellular matrix biology and adhesion. The authors suggested that the activation of HGF/MET signaling and up-regulation of cell cycle progression related genes in CAFs might support the hyperproliferative characteristic of breast cancer-derived fibroblasts.

CAFs in metastatic colon cancer were subjected to explore their expression profile compared to those of uninvolved fibroblasts (Nakagawa et al., 2004). The result showed that 170 genes among the approximately of 22,000 total genes were 3-fold and more up-regulated specifically in CAFs compared to skin fibroblasts. The up-regulated genes included many adhesion molecules such as vascular cell adhesion molecule-1 (VCAM1), intracellular cell adhesion molecule 1 (ICAM1), desmoglein 2, K-cadherin, N-cadherin; extracellular matrix/extracellular matrix remodeling molecules for example proteoglycan1, collagen type 4 alpha 1 (COL4A1), collagen type 6 alpha 2 (COL6A2), procollagen-lysin hydrogenise; and proteases/protease inhibitors including tissue plasminogen activator, plasminogen activator inhibitor type 1, tissue factor. Importantly, multiple paracrine/autocrine growth factors including PDGFA, FGF1, insulin-like growth factor binding protein-7 (IGFBP7), IGFBP5, connective tissue transforming growth factor beta (CTGF), prostate differentiation factor (PLAB), VEGF, TGF-β2; survival cytokines, and COX2 were also up-regulated. In parallel, 203 genes were down-regulated in CAFs. These genes included many extracellular matrix and adhesion molecules, but not growth factors or cytokines. These results suggest that CAFs in metastatic colorectal cancers enhance the growth of cancer cells, and CAFs may be essential in the establishment of cancer metastasis by altering the microenvironment and promoting the cross- talking of many autocrine/paracrine factors and cell adhesion molecules between cancer cells and stromal components.

Gene expression profile of CAFs following tumor-fibroblast interaction was performed in non-smallcell lung cancer (Fromigue et al., 2003). After co-culture with cancer cells, the fibroblasts were analyzed their gene expression using DNA filter array. The most highest up-regulated genes coded for secreted proteins in ECM

including secreted protein acidic cysteine-rich (SPARC), angiogenic factors (VEGF, IL-8), growth factors and growth factor-binding proteins including inhibin beta A/activin A/AB, follistatin, IGFBP5. The result suggested that the fibroblasts potentially affected the regulation of matrix degradation, angiogenesis and promoted invasion, cell growth and survival of cancer cells.

Relatively, gene expression profile of CAFs in prostate tumors was analyzed (Wang et al., 2010). The result revealed strong up-regulation of stromal represented genes in CAFs including α-SMA, COL4A1 and vimentin. Furthermore, phosphoglycerate kinase-1 (PGK1), an ATP-generating glycolytic enzyme was the most up-regulated gene and was highlighted the role in tumor progression. They found that CAFs with up-regulated of PGK1 displayed a high proliferative index and contributed prostate tumor cell invasion. This study indicated that PGK1 played role to support fibroblast cell proliferation by autocrine manner together with promoted cancer progression via tumor-stromal interaction.

In conclusion, the comparison of gene lists in CAFs and normal fibroblasts reveal differentially regulated genes, and thus a unique gene signature for both cell types. Some of these explicit candidates were already evaluated in *in vitro* and *in vivo* studies and some are now under investigated their roles in tumor growth and progression with the ultimate goal to utilize CAFs as targets for novel anti-cancer therapies.

2.5 Stromal therapy as a new strategy in cancer treatment

With regard to accumulation evidence that the cancer-stroma cross-talk affects the behavior of malignant cells, stromal therapy could emerge as a viable approach to cancer prevention and intervention. Similar to successful anti-angiogenic approaches by targeting the tumor vasculature, fibroblasts can be considered as genetically stable cells that do not undergo rapid mutational evolution and are therefore, less capable to develop drug resistance (Liotta and Kohn, 2001).

Several agents currently under clinical study as stromal therapies fall into several categories: (1) enzyme and protease inhibitors (2) anti-adhesive molecules (3) signal modulators and (4) anti-fibrotic drugs (Table 2-3) (Liotta and Kohn, 2001). ITG-targeting agents, such as antibodies or peptides that block ITG αvβ3 do alter

motile and survival functions of responsive stromal and endothelial cells (Bonfoco et al., 2000; Brooks et al., 1995; Gutheil et al., 2000; Lode et al., 1999). Several agents currently under investigation have been shown to disrupt cell adhesion, or the downstream signals propagated through ITGs. Anti-αvβ3 antibody has been implicated in human neuroblastoma (Bonfoco et al., 2000). The results indicated that anti-αvβ3 could rapidly trigger an apoptotic pathway in neuroblastoma cells which involves cytochrome c and caspases-9 and -3. So far, it has not reached clinical trial yet.

MMP inhibitors have been used for the treatment of many cancers such as ovarian and colorectal carcinomas (Brown and Giavazzi, 1995). The result showed that MMP inhibitor therapy had the potential to arrest tumor growth and spread. Moreover, they have been used under phase I clinical trial (Baker et al., 2000).

Signal pathway modulators have been proposed in many fibroblast-activated signal transduction pathways. Anti-EGFR monoclonal antibody (C225) was used in human transitional cell carcinoma and showed effective inhibition *in vitro* in animal models (Perrotte et al., 1999). However, in human, this agent has been tested in phase I clinical trial. Anti-VEGF (squalamine) was successfully used to block angiogenesis and growth of human ovarian cancer (Sills et al., 1998). PI3K inhibitor (LY294002) could decrease proliferation and increase apoptosis in ovarian cancer (Shayesteh et al., 1999) but, this result was observed only in *in vitro* study. CAI (carboxyamido-triazole), a modulator of transmembrane calcium uptake, was shown to regulate phosphorylation of FAK and could reduce migration, adhesion and matrix-survival signals resulted in a reduction in invasive capacity (Ilic et al., 1995; Kohn et al., 1995; Masiero et al., 1999).

Pirfenidone, an anti-fibrotic drug and an experimental agent to suppress bleomycin-induced pulmonary fibrosis, has been shown to reduce the influx of activated macrophages and inflammatory cells and to down-regulate the overexpression of TGF-β, event that precede the ECM changes associated with fibrosis (Iyer, Gurujeyalakshmi, and Giri, 1999).

Extracellular growth factor and cytokine ligands constitute targets for stromal therapy. Ongoing strategies for inhibiting tumor angiogenesis are aimed at blocking extracellular angiogenesis factors for example VEGF and bFGF which stimulate

vascular permeability, growth and stromal invasion (Fang et al., 2000). New extracellular mediators are being identified that offer fresh approaches to stromal therapy.

However, up to now, there have been the difficulties to translate positive results of stromal therapy from preclinical studies into successful clinical therapies. Further detailed understanding of the complex nature of tumor-stroma interaction seems to be imperative, in order to design meaningful studies.

Table 2-3 Therapeutics targeting stroma-tumor interaction

Target	Example agent	Comments
Adhesion/ Attachment	- RGD-toxin construct and RGD-targeted gene therapy	- Have not reached clinical trials
	- Anti-αvβ3 monoclonal Antibody (Vitaxin, Medi522)	- Cytostasis in patients; anti-tumor and anti-angiogenic in animal model
Proteolysis	- Matrix metalloproteinase inhibitors	- Cytostasis in patients; rare occurrence of tumor partial regression; stromal fibrosis; activity seen in multiple animal models and in combination with chemotherapy
Signaling pathway	- Squalamine (NHE-3 small molecule inhibitor)	- Selective to endothelial cells
PDGF and EGF pathway	- PDGFR, KDR and EGFR small molecule inhibitors	- Active *in vitro* in animal model; preclinical activity in combinations; phase I trials completed of several agents, some tumor stabilization some tumor stabilization or regression
	- Anti-EGFR monoclonal antibody (C225)	- Neutralizing antibody; active *in vitro* in animal models; phase I trial ongoing
VEGF pathway	- Anti-VEGF antibody	- Blocking antibody; active *in vitro* in animal models; preclinical activity; phase I-III trial ongoing
PI3K/AKT pathway	- PI(3)K inhibitors; LY294002	- Reversible inhibition; clinical trials have no report
	- Wortmannin	- Irreversible inhibition; clinical trials have no report
	- AKT inhibitors; Perifosine	- Inhibit translocation of AKT; phase I and II clinical trial
FAK Phosphorylation	- CAI (nonvoltage-gated Ca^{2+} uptake inhibitor)	- Preclinical activity in combination; phase I trial of single reagents and combinations some tumor stabilization or regression
Extracellular matrix	- Pirfenidone	- Suppress inflammatory cell by stromal expression of TGF-β; phase I trial for pulmonary fibrosis

(Liotta and Kohn, 2001)

2.6 Cancer-associated fibroblasts and CCA

CCA has been proven to have abundant intratumoral fibrosis. The most prominent histological feature of opisthorchiasis-associated CCA is periportal and periductal fibrosis (Bhamarapravati, Thammavit, and Vajrasthira, 1978). This fibrosis correlated with a marked increased in synthesis of type I and III collagen in long term *O. viverrini*-infected hamsters. In human, fibrosis usually found within CCA tissues, is variable and more abundant in poor differentiated adenocarcinoma type (Nakanuma and Terada, 1997). In addition, almost of stromal fibroblasts in CCA tissues were α-SMA positive (Chuaysri et al., 2009; Terada et al., 1996). The immunohistochemistry was carried out for α-SMA staining in CCA, hepatocellular carcinoma and metastatic liver carcinoma tissues (Terada et al., 1996). The result showed that α-SMA positive stromal cells were divisible into peritumoral α-SMA-positive perisinusoidal cells and intratumoral α-SMA-positive stromal cells. In addition, both types of α-SMA positive cells were abundant in CCA and metastatic liver carcinomas, but much more scanty in hepatocellular carcinomas. The number of both types of fibroblasts showed a significant positive correlation with the degree of tumor fibrosis. The authors suggested that in CCA and metastatic liver carcinomas, peritumoral α-SMA-positive perisinusoidal cells transformed into activated fibroblasts or myofibroblasts, were incorporated into the tumor and could produce extracellular matrix proteins that may lead to tumor fibrosis.

The recent work from our research group confirmed the previous finding that CCA stromal fibroblasts were α-SMA positive and importantly the presence of α-SMA positive fibroblasts in CCA stroma correlated with poor patient survival (Chuaysri et al., 2009). In addition, using *in vitro* primary culture fibroblasts, the results demonstrated that CCA-derived fibroblasts could induce cell proliferation of both immortalized non-tumorigenic and tumorigenic human biliary epithelial cell lines with statistical significance compared to normal fibroblasts by activating cells into the active stage of cell cycle. To our knowledge, no study regarding mechanism of CCA-associated fibroblasts in cancer progression has been reported.

2.7 Periostin

Perisotin (PN) or osteoblast-specific factor 2 was firstly identified as a protein expressed in mouse osteoblastic cell line (Takeshita et al., 1993). Later it has been found preferentially expressed in periosteum and periodontal ligament *in vivo* (Horiuchi et al., 1999). In normal condition, PN has been shown the involving in development of embryonic teeth (Kruzynska-Frejtag et al., 2004) and heart valves (Kuhn et al., 2007) and re-expressed in adults after myocardial, vascular and skeletal muscle injuries including bone fracture (Lindner et al., 2005).

Human *PN* gene is located on chromosome 13q. It has a total of 23 exons and transcribes as 4 splicing isoforms that differed in the length of the C-terminal domain (Fig 2-6) (Kim et al., 2008a). Isoform 1 is a full length PN composed of 836 amino acids. The C-terminal domain of isoform 1 is the longest containing 204 amino acids (633-836) encoded from exons 15-23. Different exons are spliced out in different isoforms including exons 17, 18 and 21 in isoform 2; exons 17 and 21 in isoform 3; and exons 17 and 18 in isoform 4.

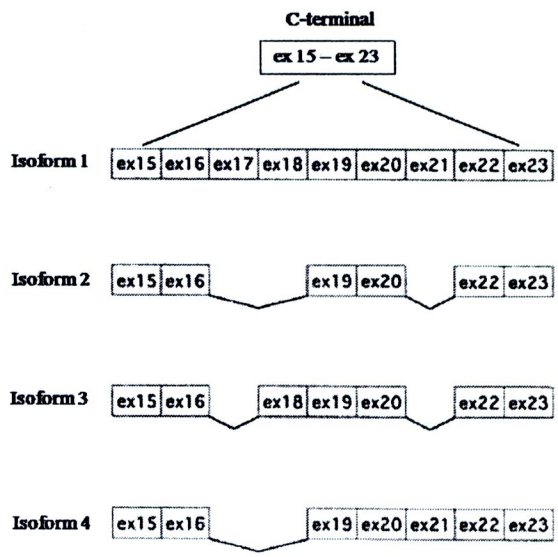

Figure 2-6 Different splicing isoforms at C-terminal part of transcribed PN (Modified from Kim et al., 2008a).

PN is a disulfide linked 90-kDa secreted protein with signal peptide (Horiuchi et al., 1999). It is a unique ECM protein which shares structural similarity with *Drosophila* Fasciclin 1 (FASI) (Takeshita et al., 1993). Therefore, it has been included into fasciclin family which composes of TGF-β induced gene clone 3 (βIG-H3), stabilin 1 and 2 and periostin-like factor (PLF) (Litvin et al., 2005). PN contains NH_2- terminal secretory signal peptide, followed by EMILIN (EMI) domain which is a small cystein-rich module of around 75 amino acids, four internal FASI repeats and a COOH-terminal hydrophilic domain (Horiuchi et al., 1999; Takeshita et al., 1993) (Fig 2-7). Among these domains, four FASI repeated domains are believed to confer PN activity by binding to integrin receptor (Kudo et al., 2007).

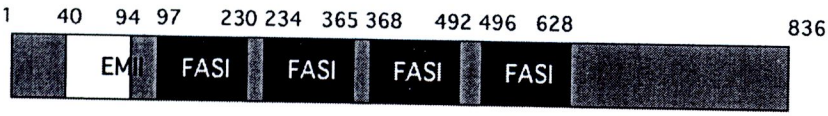

Figure 2-7 Schematic structural domain of PN (Kudo et al., 2007).

2.7.1 Expression of periostin in normal tissues and cancers

PN was detected in a wide range of normal tissues and serum. Interestingly, it was found to be up-regulated gene in several human cancers (Tischler et al., 2010) and predominantly expressed in different cell types in cancerous tissues including prostate (Tsunoda et al., 2009), head and neck (Kudo et al., 2006; Siriwardena et al., 2006), ovary (Gillan et al., 2002) and colon (Bao et al., 2004; Kikuchi et al., 2008; Tai, Dai, and Chen, 2005) (Table 2-4). Although PN was produced from different cellular sources, most of the reports confirmed roles of PN to promote cancer progression. Additionally, increasing level of PN in serum was investigated in some cancers including lung (Hong et al., 2009; Ouyang et al., 2009; Sasaki et al., 2001a; Sasaki et al., 2001b; Takanami, Abiko, and Koizumi, 2008), breast (Puglisi et al., 2008; Sasaki et al., 2003; Shao et al., 2004) and pancreas (Baril et al., 2007; Erkan et al., 2007; Fukushima et al., 2008; Kanno et al., 2008) which highlighted the applicable measurement of PN in cancer patients (Table 2-4).

Table 2-4 Expression of PN in normal and cancer tissues and serum

Cancers	Tissues			Serum	
	Normal	Cancer		Normal	Cancer
		Cancer cells	Stromal cells		
Prostate [1]	+	++	++	ns	ns
Lung [2]	+	++	ns	+	++
Breast [3]	+	++	++	+	++
Head and neck [4]	+	++	ns	ns	ns
Ovary [5]	+	++	ns	ns	ns
Colon [6]	+	–	++	ns	ns
Pancreas [7]	+	–	++	+	++

ns = not study, - : no expression, + : low expression, ++ : high expression

Reference 1: Tsunoda et al., 2009

2: Hong et al., 2009; Ouyang et al., 2009; Sasaki et al., 2001a; Sasaki et al., 2001b; Takanami, Abiko, and Koizumi, 2008

3: Puglisi et al., 2008; Sasaki et al., 2003; Shao et al., 2004

4: Kudo et al., 2006 and Siriwardena et al., 2006

5: Gillan et al., 2002

6: Bao et al., 2004; Kikuchi et al., 2008; Tai, Dai, and Chen, 2005

7: Baril et al., 2007; Erkan et al., 2007; Fukushima et al., 2008; Kanno et al., 2008

2.7.2 Roles of periostin in cancers

PN has been proposed as a marker-associated cancer aggressiveness in some cancers such as pancreatic cancer (Erkan et al., 2007; Fukushima et al., 2008), gastric cancer (Li et al., 2007), breast cancer (Puglisi et al., 2008), thyroid carcinoma (Puppin et al., 2008) and non-small cell lung cancer (Takanami, Abiko, and Koizumi, 2008). The potential role of PN in regulating at each step of the transformation of normal into malignant cells and metastatic tumors has recently been well concluded (Fig 2-8) (Ruan, Bao, and Ouyang, 2009). Current reports have revealed that PN contributes tumor progression mainly by induction of tumor growth, preventing apoptosis and promoting angiogenesis, invasion and metastasis. Several reports have demonstrated different roles of PN in various cancers which were summarized (Table 2-5).

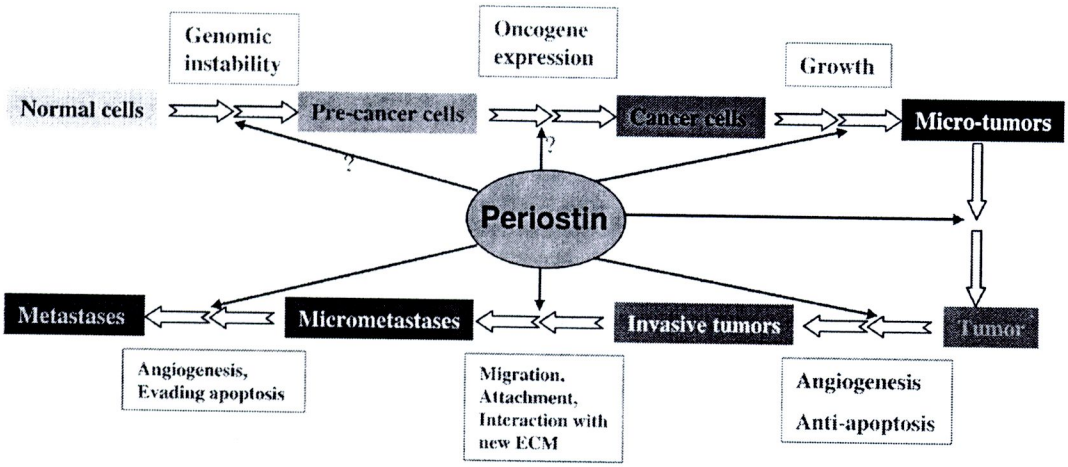

Figure 2-8 Tumorigenic roles of PN at different step of cancer formation and progression. Induction of cancer cell growth and migration, angiogenesis, evasion of apoptosis and anti-apoptosis is shown. However, some roles of PN including inductions of genomic instability and oncogene expression have not been clearly explained (Ruan, Bao, and Ouyang, 2009).

Table 2-5 Different roles of PN in human cancers

Roles of periostin	Cancer types	References
Anti-apoptosis	Colon cancer	Bao et al., 2004
	Pancreatic cancer	Baril et al., 2007
	Non-small cell lung cancer	Ouyang et al., 2009
Cell growth/ proliferation	Colon cancer	Bao et al., 2004 Tai et al., 2005 Kikuchi et al., 2008
	Head and neck cancer	Kudo et al., 2006
Invasion	Head and neck cancer	Kudo et al., 2006
	Oral cancer	Siriwardena et al., 2006
	Pancreatic cancer	Baril et al., 2007
Migration	Ovarian cancer	Gillan et al., 2002
	Pancreatic cancer	Kanno et al., 2008
Metastasis	Colon cancer	Bao et al., 2004
	Melanoma	Tilman et al., 2007
	Gastric cancer	Li et al., 2007
Angiogenesis	Colon cancer	Bao et al., 2004
	Oral cancer	Siriwardena et al., 2006

2.7.2.1 Anti-apoptosis

In contrast to normal cells, cancer cells can achieve cell death under hypoxia condition by producing anti-apoptotic substances to promote cell survival. PN has been proposed as an anti-apoptotic factor in several cancers. In colon cancer, PN was up-regulated in colon carcinoma in both primary and metastatic site when compared to those of normal tissues (Bao et al., 2004). PN was transfected into colon cancer cell line and transplanted into the nude mice. The metastatic organs were collected by autopsy of the mice after transplantation. The result showed that overexpression of PN could reduce apoptosis in the metastatic tumor *in vivo*. Moreover, PN could promote cancer survival under conditions of hypoxia and nutrient deprivation with serum depletion *in vitro*. The potent anti-apoptosis of PN by promoting cell survival under hypoxia condition was also found in pancreatic and

non-small cell lung cancers (Baril et al., 2007; Ouyang et al., 2009). In pancreatic cancer, they found that the cancer cells could survive and delaying apoptosis under hypoxic condition when exposed to recombinant PN (Baril et al., 2007). Additionally, lung cancer cell line could response to the stress of chemical-mimic hypoxia by up-regulation of PN (Ouyang et al., 2009). PN therefore proposed as a potent factor to contribute survival of cancer cells.

2.7.2.2 Cell growth and proliferation

Cell growth and proliferation are induced by signaling of growth factors to drive cell cycle progression. Cancer cells have proliferation capacity higher than those of normal cell as a result of the enrichment of mitogenic substances in cancer microenvironment over normal condition. PN as an ECM protein can act as a growth factor in some cancers. It was revealed to promote tumor growth *in vivo* by increasing of tumor volume in colon and head and neck cancers (Bao et al., 2004; Kudo et al., 2006). Moreover, PN could promote anchorage-independent growth of head and neck squamous cell carcinoma *in vitro* (Kudo et al., 2006). Supportive data was found in colon cancer by another group showed that the cancer cell lines which were exposed to recombinant PN was increased cell proliferation and could be inhibited by anti-PN antibody (Tai, Dai, and Chen, 2005).

Moreover, Kikuchi et al. found that PN was exclusively expressed in colon cancer-associated fibroblasts but not cancer cells (Kikuchi et al., 2008). This fibroblast-derived PN could induce cancer cell growth *in vivo* and enhanced colon cancer cell proliferation by increasing size and number of colony forming.

Conversely, growth suppression manner of PN was proposed in some cancers. In tumor initiation step, PN+/+ mice that were injected with sarcoma, lung and melanoma cell lines showed decreasing of tumor mass by induce capsule formation (Shimazaki and Kudo, 2008). Moreover, PN isoform 2 which predominantly expressed may be essential for suppression of tumor growth. In addition, growth suppression effect of PN in bladder cancer and osteosarcoma were demonstrated (Yoshioka et al., 2002). The bladder cancer and osteosarcoma cell lines which reduced endogenous PN were transfected with PN and showed reduction of anchorage-independent growth. Mutational analysis revealed that the C-terminal

region of PN is required for growth suppression in these cell lines. Moreover, C-terminal part which is a variable region among 4 PN isoforms has selective expression in different organs (Kim et al., 2008a). These data imply that the function of PN to promote or suppress tumor growth is tissue dependent fashion.

2.7.2.3 Invasion and migration

Cell invasion and migration are the important steps for cancer metastasis. PN enhanced invasion of cancer cells *in vitro* was demonstrated in the cancers of head and neck (Kudo et al., 2006), oral cavity (Siriwardena et al., 2006) and pancreas (Baril et al., 2007). Interestingly, PN promotes the invasiveness of pancreatic cancer cells by increasing the motility of cells without inducing expression of proteases (Baril et al., 2007). Whereas, a PN-engineered non-metastatic cancer cell line was increased expression of MMP-9 and epithelial-to-mesenchymal (EMT)-associated markers together with enhanced cell invasion and migration (Yan and Shao, 2006). Role of PN in ovarian cancer was investigated using recombinant PN treated with cancer cell lines and observed cell motility under time-lapse microscope (Gillan et al., 2002). They found that PN promoted cancer cell motility by forming fewer stress fibers and increased motility when plated on PN compared with those plated on fibronectin.

However, PN plays a role as a suppressor of invasion was mentioned in human bladder cancer (Isono et al., 2009; Kim et al., 2005). The bladder cancer cell lines transfected with PN were decreased invasiveness capability. Moreover, they found that the C-terminal region of PN is sufficient to suppress the *in vitro* invasiveness of this cancer. Deep investigation of how PN suppress tumor invasion was proposed (Isono et al., 2009). They performed mass analysis of proteins co-precipitated with PN-tranfected 293T cells and identified TAB1 and TAK1 as PN-binding proteins. The author concluded that PN could suppress cell invasiveness via activation of TAB1/TAK1 signaling pathway in bladder cancer. These results suggest that organ influences the role of PN related to cancer invasion.

2.7.2.4 Metastasis and angiogenesis

In addition to promote metastasis by induce the capability of cancer cells to migrate and invade, PN is a potent angiogenic factor which can promote metastatic growth via induction of neo-vessels in colon cancer (Bao et al.,

2004). PN overexpressing colon cancer cells were dramatically enhanced metastatic growth and vascularization *in vivo*. PN promoting angiogenesis in oral cancer was explored (Siriwardena et al., 2006). They demonstrated that PN-positive oral cancer tissues had higher blood vessel density than those of PN-negative tissues. The human endothelial cells treated with different doses of recombinant PN were determined CD31 which is a human endothelial marker using immunocytochemistry. The result confirmed that PN could enhance capillary formation *in vitro* with a concentration-dependant manner.

In melanoma tissues, the average PN expression was not increased in primary tumors, whereas its overexpression was detected in about 60% of metastatic melanoma tumors in the liver or lymph nodes (Tilman et al., 2007). PN was also overexpressed in gastric cancer and lymph node metastases (Li et al., 2007). The data suggest that PN is associated to cancer metastasis and also functions as angiogenic factor to support metastatic tumor.

2.8 Integrin

Integrins (ITGs) are belonging to the large family of transmembrane glycoproteins that were initially identified as cation-dependent receptors for components of ECM (Hynes, 1987; Ruoslahti and Pierschbacher, 1987) that serve as fundamental components of an 'integral membrane complex' linking the cytoskeleton to ECM. ITGs are transmembrane heterodimers that consist of non-covalently bound α and β glycoprotein subunits. In human, there are 24 different ITGs, which arise from the association between one of each 18 α-subunits and 8 β-subunits. Importantly, some subunits can combine with several different partners and some can bind to only specific partner leading to different active ITGs on cell membrane (Fig 2-9).

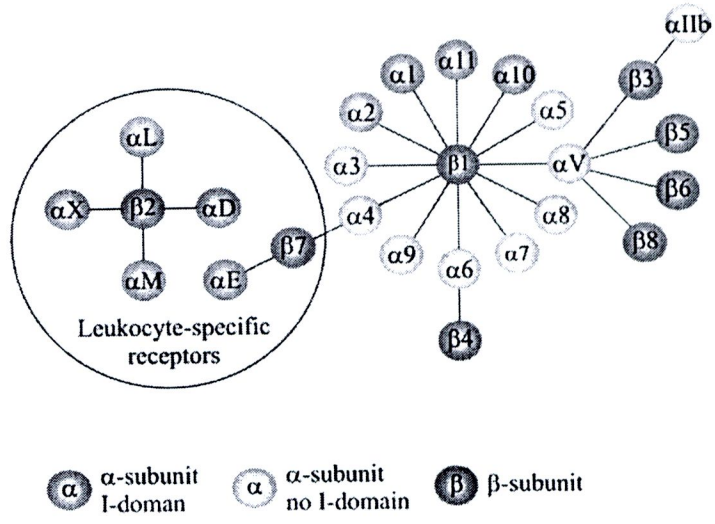

Figure 2-9 The integrin superfamily. Twenty-four different αβ integrin complexes are presented in humans (Gahmberg et al., 2009).

2.8.1 Integrin structure

Heterodimer form of α and β ITGs compose of 3 important domains; (1) the extracellular domain, (2) transmembrane domain and (3) cytoplasmic domain (Fig 2-10).

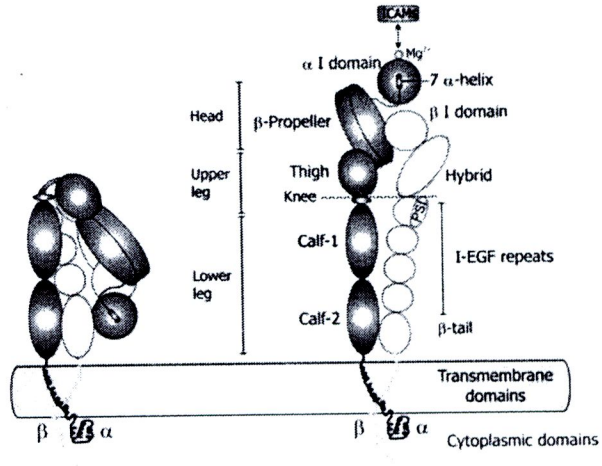

Figure 2-10 Structure of ITGs shows their extracellular, transmembrane and cytoplasmic domains. In the crystal structure, the ligand binding site is turned towards the membrane (left); to the right is shown the stretched-out intermediate affinity form (Gahmberg et al., 2009).

2.8.2 Integrin signal transduction pathway and biological functions

ITGs regulate various aspects of normal cell behavior and contribute to cancer progression in multiple biological functions (Fig 2-11). In normal cells, ITGs mediate activation of FAK-Src signals and control cell survival, proliferation and cell motility. FAK is a key signaling molecule which can influent cell survival and cell proliferation signals through kinase-dependent signaling such as PI3K/AKT which affects cyclin D1 expression (Cary and Guan, 1999) or kinase-independent effects on p53 activity (Golubovskaya, Finch, and Cance, 2005). The regulation of cell motility involve in the activation of Rac and Rho GTPases, as well as the targeting of the calpain protease to focal contact sites (Schlaepfer and Mitra, 2004). In transformed cells, FAK-Src activity can be enhanced by oncogenes such as *erbB-2* and *v-Crk* independent of ITG input and can result in enhanced anchorage-independent cell growth (Benlimame et al., 2005). However, ITG signaling can regulate angiogenesis, invasion or metastasis processes of cancer cells by promoting gene expression changes in VEGF and proteases such as MMPs and uPA (Guo and Giancotti, 2004).

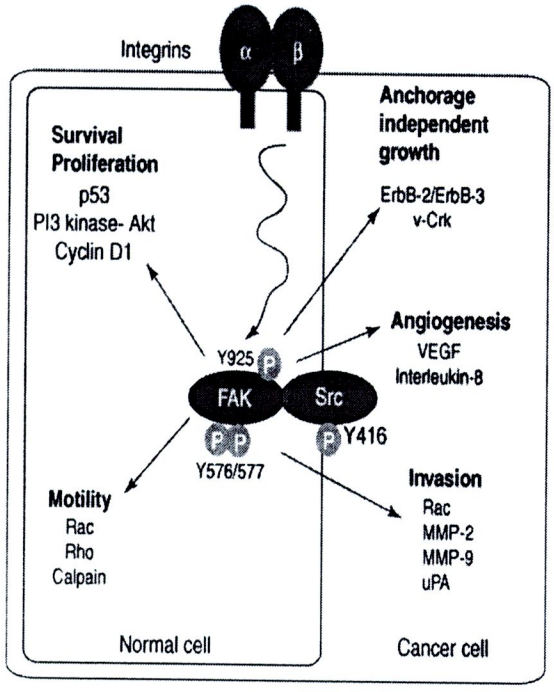

Figure 2-11 Integrin pathways regulated biological functions in normal and cancer (Mitra and Schlaepfer, 2006).

2.8.3 Integrins and cancers

Different types of ITGs expressed in tumor cells contribute to tumor progression and metastasis by increasing tumor cell migration, invasion, proliferation and survival (Table 2-6). ITG-mediated migration generally requires FAK and FAK-Src family kinase (SFK) signaling. For example, in neuroblastoma cells, although the ITGα5β1 used FAK-mediated activation of SrC, ITGα4β1 activated SrC through a FAK-independent mechanism (Wu et al., 2008). Some ITGs inhibit tumor cell motility, as *ITGβ1* gene deletion increased tumor cell dissemination in a mouse model of spontaneous pancreatic islet cancer (Kren et al., 2007).

Regulation of ITG recycling is also crucial for tumor cell invasion. Rab GTPases direct ITGs to the leading edge of invading tumor cells (Caswell et al., 2008) and coordinately regulate ITG and growth factor receptor recycling, resulting in enhanced growth factor signaling. ITGs also regulate cell proliferation by controlling the expression of key cell cycle proteins, including cyclin D1 (Fournier et al., 2008) and the cyclin-dependent kinase inhibitor family which regulate entry into the S phase of the cell cycle (Carrano and Pagano, 2001). However, the variety of ITGs expression and their roles in cancers are different depend on cancer types.

Table 2-6 Integrin expression and cancer biology

Cancers	Integrins expression	Associated phenotypes	References
Melanoma	αvβ3 and α5β1	Vertical growth phase	Danen E. H. et al., 1994, Albelda S.M. et al., 1990
		Lymph node metastasis	Nip J. et al., 1992
Breast	α6β4 and αvβ3	Increased tumour size, grade and decreased survival (α6β4)	Diaz L. et al., 2005 Friederichs et al., 1995
		Increased bone metastasis (αvβ3)	Sloan E. et al., 2006
Prostate	αvβ3	Increased bone metastasis	McCabe N. et al., 2007
Pancreas	αvβ3	Lymph node metastasis	Hosotani R. et al., 2002
Ovary	α4β1 and	Increased peritoneal	Slack-Davis J. K. et al., 2009
	αvβ3	metastasis (α4β1) and tumor proliferation (αvβ3)	Landen C. N. et al., 2008
Cervix	αvβ3 and αvβ6	Decreased patient survival	MacDonald T. et al., 2001 Hazelbag S. et al., 2007
Non-small-cell lung	α5β1	Decreased survival in patients with lymph node- negative tumors	Adachi M. et al., 2000
Colon	αvβ6	Reduced patient survival	Bates R. et al., 2005

2.8.4 Integrin signaling and cancer progression

ITGs regulate signal transduction pathways which important for a wide variety of functions in tumor cells including adhesion, proliferation, migration, invasion, survival and angiogenesis. ITG signaling functions are mediated by signaling complexes that link a variety of intracellular proteins with its cytoplasmic domains (Liu, Calderwood, and Ginsberg, 2000). These protein complexes are capable of undergoing rapid formation and dissolution, resulting in reorganization of

the cytoskeleton and modulation of multiple downstream signaling pathways (Hynes, 2002; Liu, Calderwood, and Ginsberg, 2000). Actin-binding proteins such as FAK, integrin-linked kinase (ILK), talin, α-actinin, and filamin link ITGs directly to the actin cytoskeleton in many cell types (Hannigan, Troussard, and Dedhar, 2005; Schaller et al., 1995) (Fig 2-12).

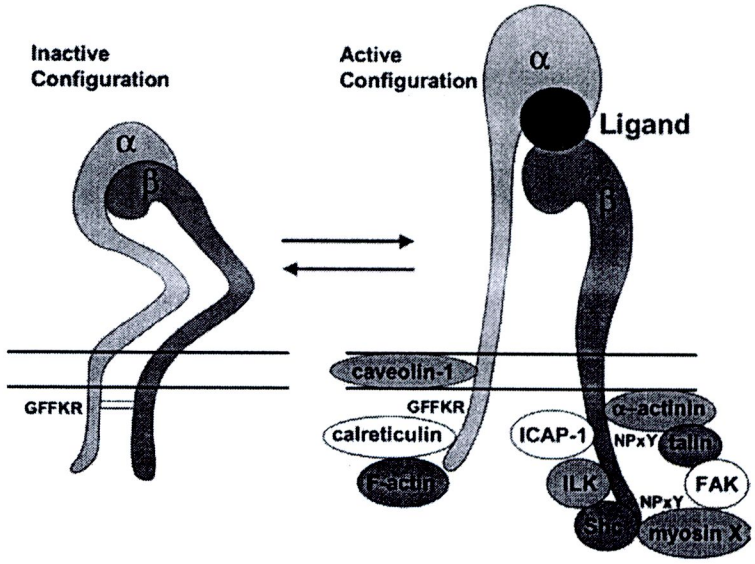

Figure 2-12 Schematic diagram of ITG binding proteins activated by specific ligand. GFFKR and NPxY domains depict the cytoplasmic domain of α and β-subunit, respectively (Gilcrease, 2007).

As well as activating signaling from PI3K to AKT/protein kinase B (PKB) through phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P_3], FAK functions as a phosphorylation-regulated scaffold to recruit Src to focal adhesions. Here, Src phosphorylates p130CAS and paxillin, which recruits the Crk–DOCK180 complex and thereby results in the activation of Rac which then leads to the activation of p21-activated kinase (PAK), JNK, and nuclear factor κB (NF-κB) (Parsons and Parsons, 1997). FAK also activates ERK/MAPK by recruiting the RAP1 Guanine Nucleotide-Exchange Factor (GEF) C3G through Crk. RAP1 then activates ERK/MAPK through B-Raf. Alternatively, FAK can activate ERK/MAPK by recruiting the growth-factor-receptor-bound-2 (GRB2) and son-of-sevenless (SOS) complex. These downstream

signaling pathways are festinated to cellular functions such as cell proliferation, survival and migration depend on type of ITGs and extracellular ligands (Fig 2-13).

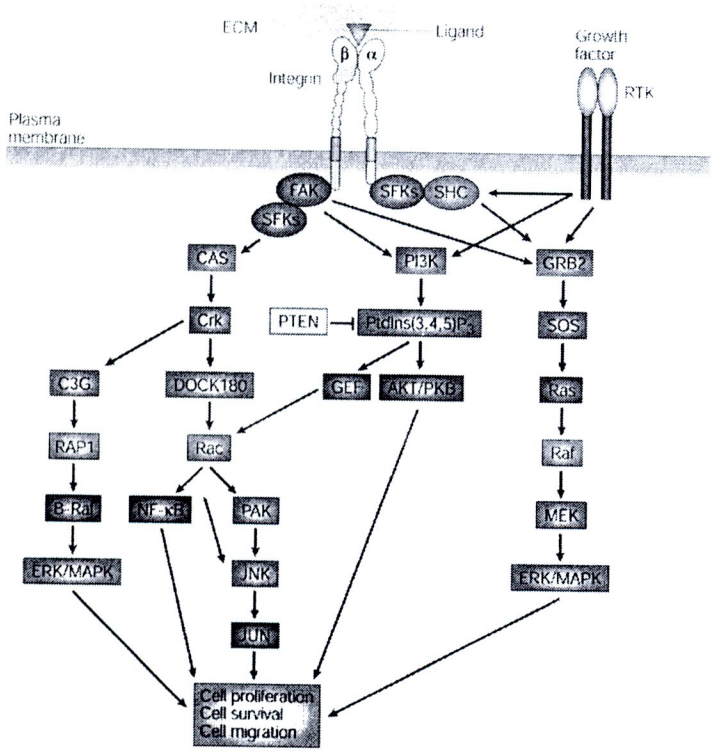

Figure 2-13 Signaling pathways in cancer cells induced by binding of the ligand to integrin receptor and activating cell proliferation, survival and migration (Guo and Giancotti, 2004).

2.8.4.1 Integrin signaling in tumor growth and proliferation

In tumor growth and proliferation, ITGs and receptor tyrosine kinases (RTKs) exert a joint control on survival and mitogenic pathways (Giancotti and Tarone, 2003). ITGs have been hypothesized that they can cooperate with RTKs to enhance mitogenic and survival signaling in cancer cells. For example, the expressions of α6β4 or αvβ3 are up-regulated in certain cancers. The α6β4 ITG cooperates with the receptor for EGF, ERBB2, and Met and so it is likely to promote the growth of carcinomas with activating mutations or amplifications of the genes that encode these RTKs (Mercurio and Rabinovitz, 2001; Trusolino, Bertotti, and Comoglio, 2001). Similarly, αvβ3 might cooperate with the PDGF receptor to

enhance the growth of gliomas that secrete large amounts of PDGF (Schneller, Vuori, and Ruoslahti, 1997). Conversely, the expressions of ITGs that induce growth-inhibitory signals should be down-regulated for tumors to grow efficiently (Giancotti and Tarone, 2003).

2.8.4.2 Integrin signaling in tumor metastasis

To metastasize to a distant organ, neoplastic cells have to transverse several basement membranes, migrate through a variety of interstitial matrices and invade the ECM. The mechanisms by which integrin signals orchestrate cell migration and invasion are incompletely understood, but at least three main pathways are involved (Fig 2-14).

FAK is important for cell migration and invasion by acts as an ITG-regulated scaffold that recruits Src family kinases (SFKs) to focal adhesions and positions them close to target-effectors that are crucial for cell migration and invasion through several pathways which are Ras, Rac, Rho and expression of non–receptor tyrosine kinase (ETK) pathway. These signals exert their effect by orchestrating changes in the cytoskeleton and by inducing gene expression. Both Rac and Cdc42 activate Wiskott–Aldrich syndrome protein (WASP)-family proteins and PAK, which then activate the ARP2/3 complex and LIM kinase (LIMK), respectively to induce actin polymerization. MLCK and the Rho effectors Rho kinase (ROCK) and mammalian diaphanous (mDIA), regulate bundling and contraction of actomyosin fibers.

PAR6 and protein kinase C (PKC)ζ function downstream of Cdc42 to control cell polarity during migration. JNK and ERK/MAPK which can be activated by SHC or FAK, promote cell migration by activating activator protein-1 (AP-1)-dependent gene expression. Signaling through Ras ERK/MAPK also cooperates with TGF-β/SMAD signaling to induce EMT. Finally, the activation by FAK of ETK tyrosine kinase is also important for cell migration (Guo and Giancotti, 2004). In addition, specific ligand-integrin binding activates both ERK and the PI3K/AKT pathways, which results in AP-1 binding activity and c-Fos expression (Han et al., 2006; Thant et al., 2000; Yang et al., 2005). This, in turn, stimulates the expression of MMP-9 which play role in cancer invasion and metastasis

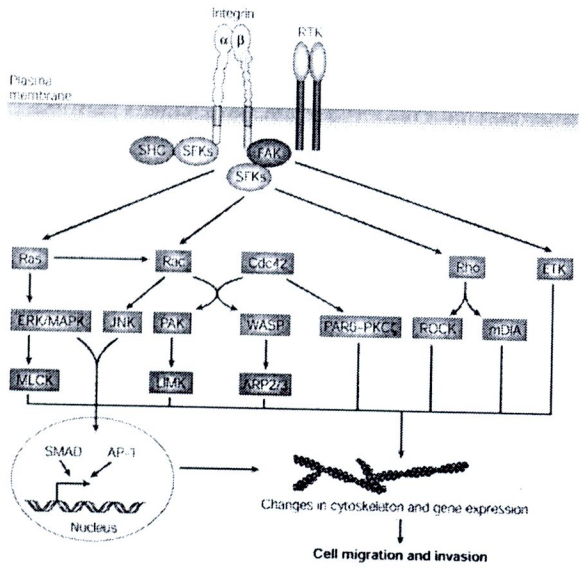

Figure 2-14 Integrin and RTK signaling induce cell migration and invasion (Guo and Giancotti, 2004).

2.8.5 Periostin mediates cancer progression via integrin signaling pathway

Up to date, several evidences have indicated that PN mediates progression of many cancers through ITG receptors. Purified recombinant PN supported the adhesion of ovarian cancer cells that could be inhibited by monoclonal antibodies against αvβ3 or αvβ5 ITGs, but not by anti-β1 ITG antibody (Gillan et al., 2002). Furthermore, αvβ3 ITG, but not β1 ITGs, co-localized to the focal adhesion plaques formed on PN. Cells plated on PN form fewer stress fibers and were more motile compared with those plated on fibronectin. So, PN has been proposed as a ligand for αvβ3 and αvβ5 ITGs to support adhesion and migration of ovarian cancer cells.

The signaling pathway activated by PN was deeply explored in various cancers. Since PN bind to αβ ITG complex, the intracellular signaling cascade involving tumor progression is then activated (Fig 2-15). For example, PN prevented stress-induced apoptosis in the colon cancer cells and augment endothelial cell survival to promote angiogenesis via ITGαvβ3 and activates AKT/PKB signaling pathway (Bao et al., 2004). In breast cancer, PN enhanced angiogenesis in part from

the up-regulation of the VEGF receptor Flk-1/KDR by endothelial cells through ITG αvβ3-FAK-mediated signaling pathway (Shao et al., 2004).

In contrast to pancreatic cancer, ITGα6β4 complex acts as the cellular receptor for PN and is shown to promote the invasiveness of pancreatic cancer cells through phosphorylation of FAK and PI3k/AKT kinase pathway (Baril et al., 2007). In addition, PN could induce EMT characteristic resulting tumor metastasis by requirement of the cross-talk between αvβ5 ITG and EGFR signaling pathways in tumorigenic but non-metastatic 293T cells (Yan and Shao, 2006).

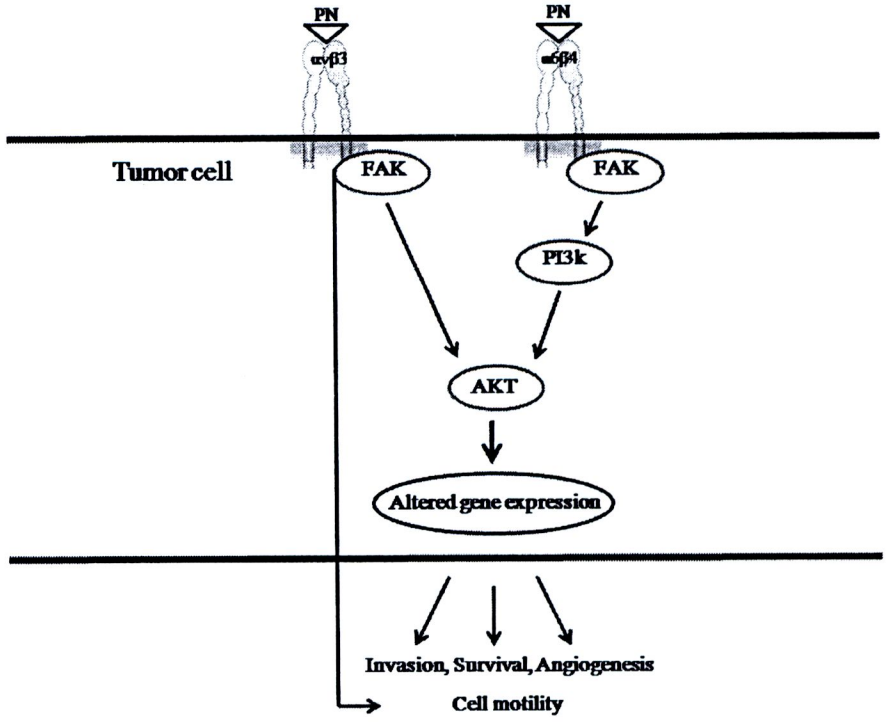

Figure 2-15 Collective signaling pathways of PN-induced cancer progression via ITGs. This figure depicts binding of PN to different ITG receptors depending on cancer types and then activates intracellular signaling cascade leading to tumor progression.

2.8.6 Integrins expressions in CCA

So far, various types of ITGs were observed as the specific receptors expressed on bile duct cancer cells. ITGα2, α3, α6, β4, αvβ3 and αvβ5 were expressed in CCA tissues but, these ITGs were also found in normal bile duct epithelial cells (Volpes, van den Oord, and Desmet, 1993). Moreover, ITGβ4 subunit was highlighted as the cellular phenotype of CCA distinguished from hepatocellular carcinoma (Volpes, van den Oord, and Desmet, 1993). Interestingly, ITGαvβ6 was strongly expressed in human CCA but not in hepatocellular carcinoma tissues and could be considered as a specific immunohistochemical marker for CCA (Patsenker et al., 2010).

However, the signaling pathway activated by PN through certain ITGs has never been noted in CCA. ITGα5β1 is one of the most interest since being a known receptor of fibronectin (Robinson et al., 2003) which is the most abundant ECM in CCA (Chen et al., 2003). It can be hypothesized that CCA cells may produce ITGα5β1 for responding to extracellular activation not only from fibronectin but also from PN.