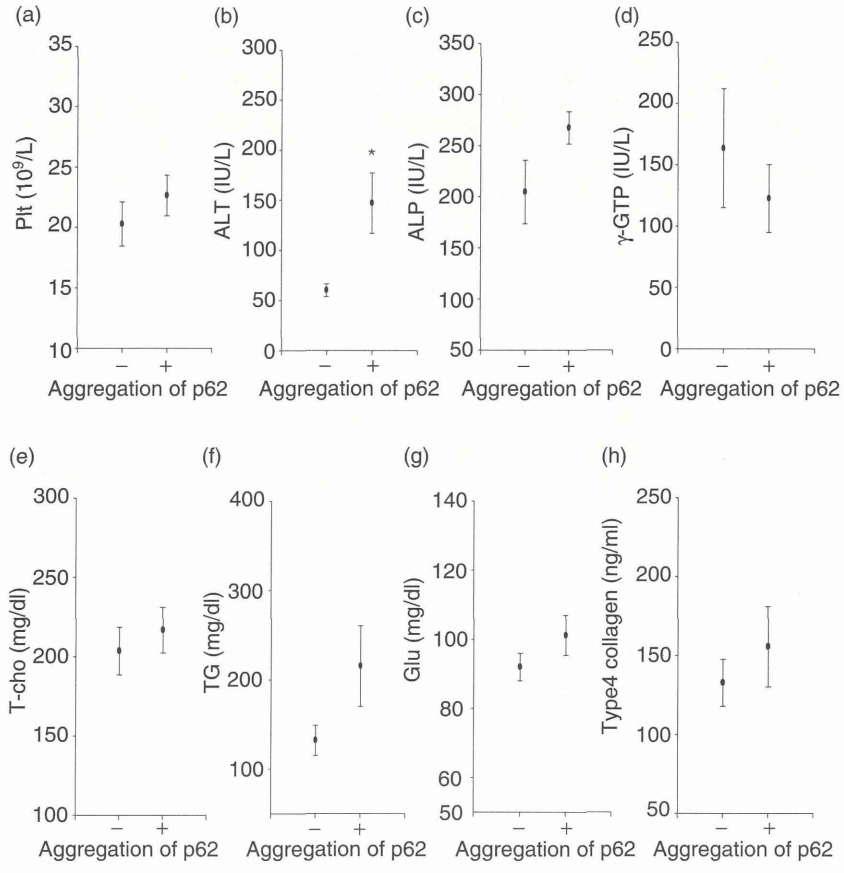

Figure 4 Relationship between aggregation of p62 and laboratory values in NAFLD group. For all of the following parameters in NAFLD patients, comparisons were made of those patients with p62 aggregation ($n = 7$). (a) Plt, (b) ALT value (IU/L), (c) ALP (IU/L), (d) γ-GTP (IU/L), (e) T-cho, (f) TG (mg/dL), (g) Glu (mg/dL), (h) type 4 collagen (ng/mL). Data are presented as means ± standard error ($^*P < 0.05$ compared to NAFLD patients without p62 aggregation by Mann–Whitney *U*-test). γ-GTP, γ-glutamyltranspeptidase; ALP, alkaline phosphatase; ALT, alanine aminotransferase; Glu, glucose; NAFLD, nonalcoholic fatty liver disease; Plt, platelets; T-cho, total cholesterol; TG, triglycerides.

formation of p62 aggregation significantly (Fig. 5c). However, we could not identify the statistical correlation between p62 aggregation and hepatic steatosis (Fig. 5b). Correspondingly, hepatic fibrosis and hepatocyte ballooning were not associated with the formation of p62 aggregation significantly (Fig. 5d,e).

DISCUSSION

THIS STUDY DEMONSTRATED that the number of autophagic vesicles is increased in both CHC and NAFLD (Fig. 1). The present data have been obtained using electron microscopy, which allows for identifying and quantifying autophagic vesicles. The results of this study were consistent with several recent reports of *in vitro* studies using a HCV replicon system.[18] ER stress caused by HCV infection plays a pivotal role in the induction autophagosome by analysis using HCV replicon system. Moreover, a previous study disclosed the data that proteolytic activity of autophagy is not disturbed by HCV infection.[18] In the present study, accumulation of p62 is hardly detected and cathepsin B and L expression are not altered in CHC patients (Figs 2a,3). On the other hand, cathepsin D is enhanced in the liver from CHC patients (Fig. 4). In previous analysis, proteome profiling and DNA microarray analysis revealed that cathepsin D mRNA and cathepsin D protein activity are upregulated in hepatoma cells transfected with HCV replicon. HCV core protein seems to be a key molecule to upregulation of cathepsin D.[22,23] Importantly, HCV infection is likely to elicit autophagy induction and does not suppress autophagic proteolysis.

Accumulation of autophagic vesicles was also observed in NAFLD patients (Fig. 1). Interestingly, the aggregation of p62 is highly detected in the liver from NAFLD patients but not CHC (Fig. 2a). These findings suggested that autolysosomes increased by hepatic steatosis are not able to enhance autophagic protein degradation. This phenomenon is in line with the results of recent work using an obese mouse model which reported that lipid accumulation of hepatocytes enhances the number of autolysosomes via the suppres-

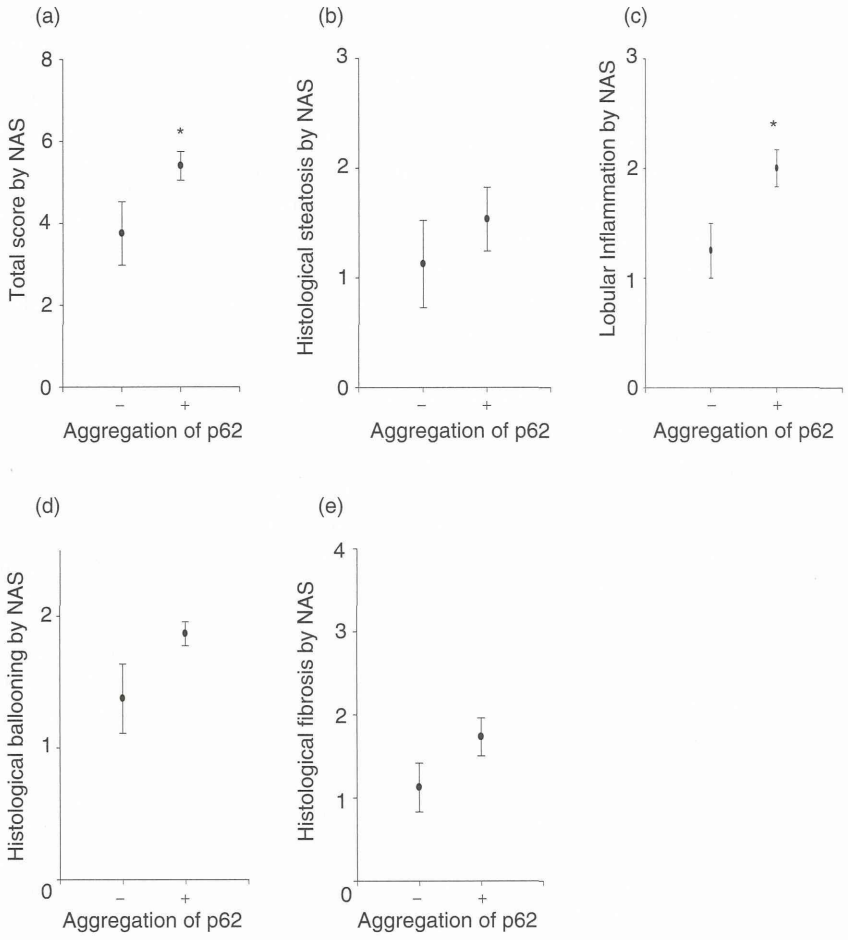

Figure 5 Relationship between aggregation of p62 and NAS in NAFLD group. For histological NAFLD activity score in NAFLD patients, comparisons were made of those patients with p62 aggregation ($n = 15$) and were compared to those patients without p62 aggregation ($n = 7$). (a) Total score using NAS, (b) hepatic steatosis by using NAS, (c) lobular inflammation by using NAS, (d) hepatocyte ballooning using NAS, (e) hepatic fibrosis by using NAS. Data are presented as means ± standard error ($^*P < 0.05$ compared to NAFLD patients without p62 aggregation by Mann–Whitney *U*-test). NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score.

sion of autolysosome clearance due to decreases in cathepsin B and L expression.[24] Cathepsins undergo proteolytic processing during their transit from the Golgi complex to lysosomes to become a mature active enzyme.[25–28] The lysosomal enzymes cathepsin B and L were similarly reduced in models of pancreatitis, and inhibitors of these enzymes increased the number of autophagic vacuoles in acinar cells.[29,30] Moreover, Ueno *et al.* proposed that autophagic membrane protein LC3-II and GABARAP-II were efficiently accumulated by cathepsin L-specific inhibitors or genetic cathepsin L deficiency, even though autophagic protein degradation was blunted.[31,32] These results indicate that decreased cathepsin activity impairs degradation of proteins incorporated by autophagosome and autophagic membrane. Therefore, the present data supports the hypothesis that proteolytic activity of autophagy is inhibited through suppression of lysosomal enzyme activity in NAFLD patients similarly to the obesity animal model.

Previous investigations showed the accumulation of p62 due to autophagy-deficiency accelerates liver damage, which leads to the development of hepatic cancer via persistent activation of Nrf2.[14,15] Although the mechanisms by which NAFLD progresses into hepatocellular carcinoma are still unknown, these findings suggest that aggregation of p62 due to hepatic steatosis promotes hepatic carcinogenesis.

Another important finding in this study was that p62 aggregation was connected to the histological score of NAFLD (Fig. 5b). Especially, lobular inflammation was potently correlated with appearance of p62 aggregation in hepatocytes. These findings are consistent with the recent evidences that autophagy participates in the inflammatory response. It has been shown that loss of autophagy by conditional Atg16L1, LC3 or Atg7 deficiency facilitates the production of pro-inflammatory cytokines in sepsis models.[33–35] Moreover, it was reported that the suppression of autophagy observed in

Kupffer cells isolated from an obese mice model enhanced both p62 expression and tumor necrosis factor-α production after LPS treatment.[35] This report indicated that hepatic steatosis suppresses the function of autophagy in both hepatocytes and Kupffer cells. Although we could not identify any Kupffer cells with p62 aggregation in this study, weak suppression of autophagy in hepatic immune cells may be involved in NAFLD with p62 aggregation and facilitate hepatic inflammation. On the other hand, the elevation of serum transaminases was associated with detection of p62 aggregation in NAFLD. The increases in serum ALT value are thought to reflect hepatic inflammation. The evaluation of hepatic inflammation by measurement of serum transaminases and histological analysis may be useful to evaluate the risk of development of liver cancer from NAFLD through autophagic dysfunction. Taken together, these findings suggest that the suppression of autophagic proteolysis by hepatic steatosis is involved in the progression of NAFLD.

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Review

EXPERT OPINION

Emerging drugs for biliary cancer

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Introduction: The number of biliary cancer patients is small and it is often complicated by serious adverse events making it difficult for clinical studies to be performed on this cancer. However, researches for clarification of the mechanisms of onset and proliferation of biliary cancer and of the effects of drugs suppressing these mechanisms have been initiated recently, with the goal of establishing effective treatments.

Areas covered: This review will cover epidemiological and biological features of biliary cancer, the efficacy and limitations of the existing methods of treatment, and current goals for the development of new treatment methods. Furthermore, the findings of pre-clinical studies on promising treatment targets and ongoing clinical studies are also reviewed, and perspectives for the future of treatment are discussed.

Expert opinion: Following the recent increase in the interest in drug development, attempts at clarifying the molecular mechanisms underlying the onset and proliferation have been made proactively, accompanied by clinical studies on various molecular-targeted drugs for the treatment of this cancer. To demonstrate the efficacy of these drugs, it is essential to establish a system for efficient screening of patients carrying the molecular targets and to devise an excellent clinical study design.

Keywords: biliary cancer, chemotherapy, cholangiocarcinoma, molecular-targeted drugs

Expert Opin. Emerging Drugs [Early Online]

1. Background

The biliary tract consists of the intra-hepatic bile duct, the extra-hepatic bile duct, the gallbladder and the ampulla of Vater. 'Biliary cancer' is a collective term for cancers arising from these organs. According to the UICC Classification, biliary cancer includes extra-hepatic biliary cancer, gallbladder cancer and cancer of the ampulla of Vater, while intra-hepatic biliary cancer is classified as primary liver cancer [1]. This classification is useful in the debate about the appropriate surgical procedure or extent of surgical resection. In terms of the histopathological characteristics, that is, carcinogenesis in the bile duct epithelium and predominance of adenocarcinoma, and clinical features, that is, likelihood of early lymph node metastasis or distant metastasis, intra-hepatic bile duct is akin to biliary cancer rather than to liver cancer. For this reason, intra-hepatic biliary cancer is often counted as biliary cancer when considering the appropriate drug therapy. In practice, many of the clinical studies on chemotherapy for biliary cancer have included intra-hepatic biliary cancer as one of the target diseases. Histologically, biliary cancer has been classified as adenocarcinoma, adenosquamous cell carcinoma, squamous cell carcinoma, small cell carcinoma, adenoendocrine cell carcinoma, etc. Among all cases of biliary cancer, adenocarcinoma accounts for the overwhelming majority (over 90%), followed by adenosquamous cell carcinoma (about 2%) [2].

The incidence of biliary cancer is particularly high in Chile and Japan, followed by Western Asian countries and India. This cancer occurs at a relatively low incidence in Europe and the USA. There are many countries in which the precise statistics about

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Table 1. Randomized controlled studies evaluating cytotoxic agents in advanced biliary cancer.

Regimens	Stage of development	Number of patients	Response rate (%)	Median survival (months)	p	Authors
5-FU	ND	30	10	NA	NA	Falkson *et al.* [91]
5-FU + STZ		26	13	NA		
5-FU + MeCCNU		31	10	NA		
5-FU	ND	18	0	NA	n.s.	Takada *et al.* [92]
5-FU + ADR + MMC		18	0	NA		
BSC	ND	19	NA	2.5	0.1	Glimelius *et al.* [93]
5-FU + FA + etoposide		18	NA	6.5		
GEM + MMC	rII	25	20	6.7	NA	Kornek *et al.* [94]
Capecitabine + MMC		26	31	9.3		
5-FU	rII	29	7	5	NA	Ducreux *et al.* [95]
5-FU + FA + CDDP		29	19	8		
5-FU + FA + etoposide	III	27	15	12	0.2	Rao *et al.* [96]
5-FU + EPI + CDDP		27	19	9		
GEM	rII	44	15	-	NA	Valle *et al.* [8]
GEM + CDDP		42	24	-		
BSC	ND	27	0	4.5	0.039	Sharma *et al.* [11]
5-FU + FA		28	14	4.6		
GEM + OX		26	31	9.5		
GEM	III	206	16	8.3	< 0.001	Valle *et al.* [9]
GEM + CDDP		204	26	11.7		
GEM	rII	42	12	7.7	NA	Okusaka *et al.* [10]
GEM + CDDP		41	20	11.2		
S-1	rII	50	17	9	0.52	Morizane *et al.* [97]
GEM + S-1		51	36	12.5		
GEM	rII	32	9	9.2	NA	Sasaki *et al.* [98]
GEM + S-1		30	20	8.9		

BSC: Best supportive care; FA: Folic acid; MeCCNU: Methyl-CCNU; NA: Not available; ND: Not described; n.s.: Not significant; rII: Randomized Phase II study; STZ: Streptozosin; III: Phase III study.

patients with biliary cancer are unavailable. The age-adjusted incidence of biliary cancer, excluding intra-hepatic biliary cancer, (per 100,000 population) varies greatly among countries: 10.4 in Chile, 4.9 in Japan and 1.7 in the USA [3]. The incidence of intra-hepatic biliary cancer is markedly high in the Khon Kaen District of Thailand (age-adjusted incidence per 100000 population: 71.3 for males and 34.6 for females). The reported causes of biliary cancer include parasites (at Khon Kaen District), exposure to Thorotrast (thorium dioxide), large gallstones and inflammatory bowel disease [4]. Biliary diseases such as primary sclerosing cholangitis (PSC), cirrhosis, hepato/chole/choledocholithiasis, chronic cholecystitis, chronic non-alcoholic liver disease, and hepatic C virus (HCV) infection are all known to be pre-disposing factors for neoplastic transformation. In most cases of biliary cancer, however, the exact cause is unknown and the large regional variances have not been explained sufficiently.

For biliary cancer, surgical resection is the only modality for radical treatment; however, the percentage of patients undergoing radical resection is not sufficiently high: 68.3% for intra-hepatic biliary cancer, 47.3% for gallbladder cancer, 46.7% for extra-hepatic biliary cancer and 86.6% for cancer of the ampulla of Vater, according to the registries in Japan [5,6]. The prognosis of patients undergoing surgical resection is poor, with reported 5-year survival rates after surgery of 32.7% for intra-hepatic biliary cancer, 41.6% for gallbladder cancer, 33.1% for extra-hepatic biliary cancer and 52.8% for cancer of the ampulla of Vater; these results suggest that complete cure is difficult in patients with this cancer [5,6]. For inoperable cases receiving chemotherapy, the median survival period has recently been reported to be about 8 – 12 months. Thus, the prognosis of patients with biliary cancer is still quite poor at present.

2. Medical need

It is not uncommon for a biliary cancer to be already at an advanced stage at the time of diagnosis. Even in resectable cases, biliary cancer often recurs soon after the surgery. Thus, patients with biliary cancer still have a poor prognosis at present, and development of effective non-surgical therapies is strongly needed. Clinical studies on non-surgical therapies conducted to date are confined to those involving only one group or small-scale comparisons, and there are scarcely any reports of large-scale Phase III trials (**Table 1**). Thus, no non-surgical therapies with satisfactory outcomes had been established until recently. Some of the possible reasons for this status include: i) there are very few known anticancer agents that may be expected to yield high response rates among patients with this cancer; ii) chemotherapy is not

always easy for patients with this cancer, as their general condition is often unstable, which increases their susceptibility to infection or sepsis; iii) large-scale clinical studies are difficult to perform because the number of patients with this cancer is limited; iv) global interest in the development of new treatment methods for this cancer has been limited. Recently, some large-scale Phase III studies were carried out for the first time, demonstrating the effect of some chemotherapies in prolonging the survival period of these patients, and development of molecular-targeted drugs has been successful in patients with non-biliary solid cancers, for which few valid drugs were available until recently. Thus, the interest in the development of new drugs for biliary cancer has recently begun to rise sharply.

3. Existing treatments

Of the chemotherapeutic regimens, single-drug chemotherapy using a drug of the fluoropyrimidine family or gemcitabine has yielded relatively favorable outcomes. As a result, these drugs are now often used as key drugs for the treatment of biliary cancer. Among others, gemcitabine has been authorized in several countries as a drug for insurance-covered treatment of biliary cancer on the basis of the results of Phase II trial [7], and has been extensively used in clinical practice in these countries.

Recently, a Phase III trial comparing gemcitabine monotherapy with combined gemcitabine + cisplatin (GC) therapy was carried out in the United Kingdom, which yielded a more favorable outcome of GC therapy [8,9]. A randomized Phase II trial using the same regimen carried out in Japan also yielded a similar outcome [10]. On the basis of these results, GC therapy is now positioned as a standard therapy for advanced biliary cancer. Combined gemcitabine + oxaliplatin (GEMOX) therapy was adopted as the control therapy in some Phase III trials, on the ground that oxaliplatin is classified as an anticancer drug of the platinum family to which cisplatin also belongs, and that a randomized comparative trial revealed a longer survival period following this therapy than following symptomatic therapy or combined 5-FU + folinic acid therapy [11]. However, the randomized study evaluating the survival-prolonging effects of GEMOX therapy was a small-scale study involving only patients with gallbladder cancer (n = 88), and there is no published randomized study comparing this therapy with gemcitabine monotherapy, that is a community standard in the past, or GC therapy, that is the current standard therapy. Even at present, with the availability of an established standard therapy, the median survival period of patients with advanced biliary cancer is quite short, < 1 year. Thus, development of a more effective treatment method is keenly desired.

As far as second- and subsequent-line treatments are concerned, no randomized comparative studies have been made, and no treatment method has been established yet as standard second- or subsequent-line therapy. Also no standard adjuvant therapies with established usefulness from randomized studies have been reported yet for surgically treated cases.

4. Current research goals

As stated above, standard therapy for biliary cancer was established for the first time only recently, although the history of drug development for biliary cancer is quite short as compared to that for other types of cancer. Other than the key drugs, that is, gemcitabine, drugs of the platinum family and drugs of the fluoropyrimidine family, there is no drug that has been fully recognized to be effective against biliary cancer. Moreover, the effects of these key drugs against biliary cancer are also limited. Thus, the most important goal at present is to develop new drugs that would improve prognosis in patients with this cancer.

The study comparing GEMOX + erlotinib combination therapy with GEMOX therapy in patients with advanced biliary cancer is the only Phase III trial reported after GC therapy was prolong the survival period and was positioned as a standard therapy [12]. That study adopted survival period as the primary endpoint and was designed to examine whether or not the addition of erlotinib to GEMOX would contribute to survival prolongation. Unfortunately, the median survival period was 7.5 months in both groups, without any significant inter-group difference. Phase III trials now under way include: i) a study in Korea designed to compare GEMOX therapy with capecitabine + oxaliplatin (XELOX) therapy (NCT01470443) and ii) a study in Japan designed to compare GC therapy with gemcitabine + S-1 therapy (UMIN000010667). The primary endpoint is progression-free survival in the former study, and overall survival in the latter. Phase III studies of post-operative adjuvant therapy now under way are: i) a study in the United Kingdom designed to compare the outcome of surgery alone with that of patients receiving capecitabine as post-operative adjuvant therapy, with the 2-year survival rate adopted as the primary endpoint (NCT00363584), ii) a study in Japan designed to compare the outcome of surgery alone with that of patients receiving S-1 as post-operative adjuvant therapy, with overall survival adopted as the primary endpoint (UMIN000011688), iii) a study in France designed to compare the outcome of surgery alone with that of patients receiving GEMOX therapy as post-operative adjuvant therapy, with recurrence-free survival adopted as the primary endpoint (NCT01313377), and iv) a study in Japan designed to compare the outcome of surgery alone with that of patients receiving gemcitabine as post-operative adjuvant therapy involving only patients with extra-hepatic biliary cancer, with overall survival adopted as the primary endpoint (UMIN000000820).

5. Scientific rationale

Biliary carcinogenesis is considered to follow the sequence of dysplasia followed by hyperplasia of the bile duct epithelium; however, these processes have not yet been fully clarified. To

Table 2. Overexpression frequencies of growth factors and their receptors in biliary cancer.

Target	Positive rate %	
	Extra-hepatic biliary cancer*	Intra-hepatic biliary cancer
EGFR [15-18]	0 – 19.2	10.7 – 81.3
HER2 [15-20]	5.1 – 15.7	0 – 81.3
VEGF [15,39,40,50]	31.4 – 59.2	53.8 – 100
c-Met [16,19,49-51]	0 – 80.8	21.4 – 57.7
HGF [50]	0 – 7.7	-

Reproduced from Ojima [21].
*Extra-hepatic biliary cancer, including gallbladder cancer and carcinoma of ampulla of Vater.

date, the involvement of several molecular pathways in the onset and proliferation of biliary cancer has been suggested. These pathways are expected to serve as potential targets for the treatment of biliary cancer (Table 2).

Epidermal growth factor receptor (EGFR) and human EGFR-2 (HER2) are members of the ErbB receptor tyrosine kinase family. The binding of ligands, such as epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α), to their extracellular ligand-binding domains initiates intracellular signaling cascades, leading to the progression, proliferation, migration and survival of cancer cells [13,14]. The proportion of previously reported EGFR-positive and HER2-positive cases has varied from 0 – 81.3% [15-18] and 0 – 81.8% [15-20], respectively, in biliary cancer. These values vary depending on the number of cases, the locations of the tumors and the antibodies that were used [21]. Yoshikawa *et al.* reported that EGFR overexpression was a significant prognostic factor and also a risk factor for tumor recurrence in intra-hepatic biliary cancer [15]. Ito *et al.* reported that EGFR expression was related to lymph node metastasis, aberrant p53 expression, proliferative activity and carcinoma differentiation [18]. Therefore, EGFR contributes greatly to biliary cancer progression. In contrast to EGFR, studies have reported that HER2 is significantly expressed in well-differentiated, non-invasive cancers [15], and is found in proliferative biliary epithelium and atypical epithelium in patients with hepatolithiasis, a risk factor for biliary cancer [19]. These findings suggest that HER2 and EGFR expression in biliary cancer is distinctively associated with biliary cancer. Yoshikawa *et al.* also showed that the TKKK cell line, which exhibits EGFR gene amplification, was sensitive to vandetanib, a multi-tyrosine kinase inhibitor that targets EGFR and vascular endothelial growth factor receptor-2 (VEGFR-2), whereas the OZ and HuCCTI cell lines, which harbor KRAS mutations, were resistant. These results suggest that EGFR gene amplification could be a predictive biomarker for anti-EGFR therapy, and even if EGFR phosphorylation is inhibited, KRAS mutations result in the constitutive activation of the downstream RAS/RAF/Grb2-Ras-mitogen-activated protein kinase (MAPK) signaling pathway, leading to treatment resistance (Table 3).

The RAS/RAF/extracellular-regulated kinase (ERK) signaling pathway plays a central role in the regulation of many cellular processes, including proliferation, survival, differentiation, apoptosis, motility and metabolism [22-24]. This pathway is activated by a diverse group of extracellular signals, including growth factor receptors like EGFR. Mutation and constitutive activation of the oncogene KRAS have also been reported in about 10 – 50% of biliary cancers [25-33]. Relatively higher percentages of KRAS mutation have been reported in East Asian countries, and lower percentages have been seen in Western countries, a result that might be explained by geographical differences in etiology or ethnicity. B-RAF mutation has been recognized in 8.1 – 33% of patients with biliary cancer [25,33-35]. Activated RAS triggers the phosphorylation and activation of RAF kinase, which then phosphorylates mitogen-activated ERK1 (MEK1) and MEK2 on two serine residues [27,36]. Activated MEK phosphorylates its only known substrates, ERK1 and ERK2. Phosphorylated ERK (pERK) dimerizes and translocates to the nucleus [37], where it is involved in several important cellular functions [27]. A pre-clinical study in a murine orthotopic model using a human gallbladder cell line harboring a KRAS mutation exhibited constitutive MAPK activation and the progression of gallbladder cancer; a MEK inhibitor (U0126), significantly prolonged the survival of the mice, compared with untreated controls [38].

VEGF plays a key role in tumor-associated neo-angiogenesis, which contributes to providing tumors with oxygen, nutrition and a route for metastasis. The proportion of previously reported VEGF-positive cases has varied from 31.4 to 100% for biliary cancer [15,39-41]. A high microvessel density (MVD) is reportedly a prognostic factor in biliary cancer [42,43] and is associated with VEGF expression in intra-hepatic biliary cancer [39]. Indeed, VEGF expression is significantly associated with intra-hepatic metastasis in intra-hepatic biliary cancer [15]. These facts suggest that VEGF plays an important role in the process of biliary cancer metastasis by promoting angiogenesis.

Hepatocyte growth factor (HGF) is a cytokine that acts as a growth factor in biliary cancer, and cancer cells themselves produce HGF to activate their proliferation [44,45]. The effects of HGF are transmitted through its receptor, c-Met, and the activation of HGF/c-Met signaling initiates cell invasiveness and triggers metastasis through the direct involvement of tumor angiogenesis [46]. Upon ligand binding, c-Met activates multiple downstream signal transduction pathways, including the MAPK cascade, the phosphatidylinositol-3 kinase (PI3K) pathway, and the signal transducer and activator of transcription (STAT) pathway [47,48]. The proportion of previously reported c-Met-positive cases has varied from 0 to 80.8% (Table 3) [16,19,49-51]. Immunohistochemical c-Met overexpression has been reported in hyperplastic as well as dysplastic epithelial cells of human hepatic bile ducts [19,51].

Table 3. Clinical trials evaluating molecular-targeted agents in advanced biliary cancer.

Compound	Targets	Regimen	Stage of development	Indication	No.	RR (%)	MST (months)	p	Authors
Erlotinib	EGFR	Erlotinib	II	1st/2nd	42	8	7.5	-	Philip *et al.* [73]
		GEM + OX	III	1st	133	16	9.5	0.61	Lee *et al.* [12]
		GEM + OX + erlotinib			135	30	9.5		
Cetuximab	EGFR	Cetuximab	Case series	Any	5	80	NA	-	Chang *et al.* [74]
		GEM + OX + cetuximab	II	1st	30	63	15.2	-	Gruenberger *et al.* [26]
		GEM + OX	rII	1st	74	29	12.4	0.19	Malka *et al.* [75]
		GEM + OX + cetuximab			76	23	11		
Panitumumab	EGFR	GEM + OX + CAP + panitumumab	II	1st	46	33	10	-	Jensen *et al.* [76]
Lapatinib	EGFR1, ErbB2	Lapatinib	II	1st/2nd	17	0	5.2	-	Ramanathan *et al.* [77]
		Lapatinib	II	1st/2nd	9	0	5.1	-	Peck *et al.* [78]
Bevacizumab	VEGF	GEM + OX + bevacizumab	II	1st	35	40	12.7	-	Zhu *et al.* [79]
		Erlotinib + bevacizumab	II	1st	49	18	9.9	-	Lubner *et al.* [80]
Sorafenib	VEGFR, PDGFR, c-KIT, Flt-3, RET	Sorafenib	II	Any	46	2	4.4	-	Bengala *et al.* [81]
		Sorafenib	II	1st	36	0	9	-	El-Khoueiry *et al.* [82]
		Sorafenib + erlotinib	II	1st	32	7	6	-	El-Khoueiry *et al.* [83]
		CAP + OX + sorafenib	I	1st/2nd	16	13	-	-	LoConte *et al.* [99]
Sunitinib	VEGFR, PDGFR, KIT, Flt-3, RET	Sunitinib	II	Any	56	9	13	-	Yi *et al.* [86]
Selumetinib	MEK1/2	Selumetinib	II	1st/2nd	28	12	9.8	-	Bekaii-Saab *et al.* [27]
ARRY-438162	MEK1/2	ARRY-438162	I	2nd/later	28	7	-	-	Finn *et al.* [100]
Bortezomib	NF-κB	Bortezomib	II	Any	20	5	9.3	-	Costello *et al.* [87]
Imatinib	Bcr-Abl, v-abl, c-abl, PDGFR	Imatinib	II	2nd	9	0	4.9	-	Roth *et al.* [88]

1st: First line; 2nd: Second line; CAP: Capecitabine; CDDP: Cisplatin; GEM: Gemcitabine; MST: Median survival time; NA: Not available; No.: Number of patients; OX: Oxaliplatin; RR: Response rate.

Furthermore, positive immunostaining for c-Met is highest among well-differentiated intraductal tumors and is relatively low in poorly differentiated invasive tumors [51]. Miyamoto *et al.* reported that a high c-Met expression level was significantly correlated with EGFR expression and the overall 5-year survival rate for patients with biliary cancer who had undergone curative surgery [49].

Transforming growth factor-beta (TGF-β) acts as a potent growth inhibitor for normal biliary epithelial cells. However, in many malignant tumors including biliary cancer, abnormalities reportedly occur at some steps of the TGF-β signal transduction pathway, resulting in cancer cell proliferation and progression [52,53]. Zen *et al.* showed that TGF-β1 was expressed as a diffuse and granular cytoplasmic staining pattern in hyperplastic biliary epithelium (70%), dysplastic epithelium (100%), intraductal papillary neoplasm of the bile duct (100%) and intra-hepatic biliary cancer with dysplasia (89%) or intraductal papillary neoplasm of the bile duct (86%), and the majority of these epithelial cells were positive, when detected. However, its expression was not observed in normal bile ducts [54]. Benckert *et al.* showed that both TGF-β1 and VEGF were overexpressed, suggesting that TGF-β1 can stimulate VEGF gene transcription in malignant cholangiocytes in a paracrine and/or autocrine manner through a Sp1-dependent mechanism [40]. In addition, the inhibition of interleukin-6 (IL-6) signaling [55] and cyclin D1 expression [54] using siRNA resulted in the disappearance of the biliary cancer growth-promoting effect of TGF-β, suggesting that IL-6 and cyclin D1 are closely involved in the promotion of cancer cell growth by TGF-β.

Isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2) are metabolic enzymes that, when altered, promote carcinogenesis. IDH1 and IDH2 are $NADP^+$-dependent enzymes that catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG) [56-64]. Somatic mutations in IDH1/2 result in proteins with neomorphic enzyme activity that allows α-KG to be more effectively converted to 2-hydroxyglutarate (2-HG). Increased levels of 2-HG are thought to promote carcinogenesis by competitively inhibiting enzymes that use α-KG as a cofactor [56,65-68]. Borger *et al.* reported that mutations in IDH1 and IDH2 were found only in intra-hepatic biliary cancer (9 of 40, 23%) and in none of extra-hepatic biliary and gallbladder cancers [69]. Kipp *et al.* showed that the mutations were identified in 21 (22%) of 94 biliary cancer specimens; they were more frequently observed in intra-hepatic biliary cancer compared with extra-hepatic cancer (28 vs 7%, respectively; $p = 0.03$) [56]. These findings may provide new insights into pathogenesis and therapeutic targeting for this disease.

Recently, Wu *et al.* and Arai *et al.* reported that some patients with biliary cancer harboring gene rearrangements of FGFR2 have been identified [70,71]. Cells harboring FGFR fusions showed enhanced sensitivity to FGFR inhibitors, suggesting that patients with cancer with FGFR fusions may benefit from targeted FGFR kinase inhibition. Gu *et al.* confirmed the presence of ROS kinase fusions in 8.7% (2 out of 23) of intra-hepatic biliary cancer patients [72]. The expression of ROS fusions in 3T3 cells confers a transforming ability both *in vitro* and *in vivo* and is responsive to its kinase inhibitor. These studies have suggested that FGFR and ROS kinases are new promising candidates for therapeutic targets in biliary cancer and suggest that other 'actionable' therapeutic targets may be identified in patients with biliary cancer in the near future.

6. Competitive environment

Studies have been conducted to clarify the mechanism underlying the onset and proliferation of biliary cancer, accompanied by efforts directed at the development of molecular-targeted drugs for the treatment of this cancer. To date, however, no molecular-targeted drug that can be positioned as standard therapy has been developed yet (**Table 3**). In many regions, the number of patients with biliary cancer is small and there has not been sufficient interest in conducting clinical studies aimed at developing new drugs for this cancer. Recently, however, a standard therapy has been established on the basis of the results of large-scale studies, which has stimulated the development of better treatment methods for biliary cancer. Furthermore, the systems for clinical trials have recently been improved in Asian and South American countries in which biliary cancer occurs at a elatively high incidence. Under such circumstances, many programs for the development of new drugs of the molecular-targeted drug category have been started (**Table 4**).

6.1 Drugs primarily targeting EGFR or HER2

6.1.1 Erlotinib

Biliary cancer has long been reported to show high expression levels of EGFR and its ligand, suggesting that EGFR inhibitors may exert efficacy against biliary cancer. Erlotinib, an EGFR tyrosine kinase inhibitor, is the first drug that has been shown, when administered in combination with gemcitabine, to prolong the survival period of patients with inoperative pancreatic cancer significantly than gemcitabine monotherapy. A clinical study of this molecular-targeted drug in patients with biliary cancer has been started earlier than such a study of any other drug of this category.

In regard to erlotinib monotherapy, a Phase II trial was carried out in 42 patients with inoperative biliary cancer (including 57% with a history of prior treatment), which yielded 3 responders (8%) and a median survival period of 7.5 months, suggesting the necessity of using this drug in combination with some other drug [73].

In Korea, a Phase III trial was carried out for evaluating the effects of addition of erlotinib to GEMOX therapy through comparison of the GEMOX + erlotinib group (n = 135) and the GEMOX group (n = 133). The results of this study were reported in 2012, and the response rate was significantly higher in the GEMOX + erlotinib group (30 vs 16%,