Gallbladder Cancer

Current Treatment Options

Vijay Kumar Shukla
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Foreword

Gallbladder cancer is one of the common neoplasms of the biliary tract, representing 80–95% of biliary tract cancers worldwide. It is among the top five malignancies in the gastrointestinal system. A satisfactory outcome depends on an early diagnosis and surgical resection. This tumour is regarded as a highly lethal disease with an overall 5-year survival of less than 5%.

Early gallbladder cancer symptoms, however, are typically nonspecific; hence disease has often been diagnosed at an advanced stage and cannot be resected. The methods of treatment are limited to palliative therapies, depending mostly on chemotherapy. ICMR also published a consensus document for the management of gallbladder cancer in 2014.

This book focuses on the recent progress in understanding the therapeutic targets for gallbladder cancer, which will provide opportunities for research and for developing innovative strategies that may enhance the benefit of conventional chemotherapy. The book is intended for clinicians, surgeons, scientists and academicians. I congratulate the editors for synchronizing with the global experts in the field to present the most viable options for treatment of gallbladder cancer that will ultimately be beneficial to humankind.

Balram Bhargava

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Foreword

Gallbladder cancer is an aggressive malignancy that disproportionately affects underprivileged populations in South Asia, Latin America and the Far East. Most patients are diagnosed at an advanced, unresectable stage in Asia, whereas incidental finding at cholecystectomy is often the mode of presentation in the Western world for whom cure is feasible with multimodality therapy.

Treatment goals in the majority of patients however include palliation of symptoms and prolongation of life, which is possible with systemic chemotherapy. Genetic profiling has identified potential targeted and immunological approaches.

The contents of the book focus on the recent progress in understanding the molecular underpinnings of gallbladder cancer, which will provide opportunities for research and innovative strategies that may result in incremental benefit over conventional chemotherapy. Furthermore, surgical and multidisciplinary care are highlighted throughout the book, stressing their critical role. This book is intended for clinicians, surgeons, scientists and academicians. I congratulate the editors for their efforts and the global experts who have contributed so generously towards this important effort.

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Milind Javle

Preface

Gallbladder Cancer (GBC) is characterized by an aggressive and extremely deadly cancer, which ranks fifth among the most common gastrointestinal tract cancers. Global mortality rates for people with gallbladder cancer have risen significantly with poor prognosis in recent decades.

With advances in our understanding of the GBC aetiology over the past decade, existing treatment options such as surgery, chemotherapy and radiotherapy are also associated with unacceptably poor survival rates. Even though surgery is the primary treatment available for early-stage GBC, most patients undergoing surgical resection generally suffer from a high risk of recurrence. Currently, the available treatments for GBC seem to be ineffective, as they only work to ease the symptoms but cannot prevent the disease's progression.

GBC entails different genetic factors including somatic events and germline predisposition. Apparently, the underlying basis for disease development and progression of this malignant tumour remains unclear. Indeed, despite the noteworthy achievements in the understanding of GBC mechanism, there has been little development in formulating new therapeutic strategies.

It is important to know the precise molecular or genetic changes occurring in the development of GBC to develop molecular targeted therapy for GBC. This book aims at current treatment options available for GBC and also the recent progress in understanding the therapeutic targets for GBC. Moreover, non-coding RNAs, such as miRNAs and lncRNAs, have also been discussed as potential therapeutic strategies in the regulation of gallbladder cancer development. This volume provides opportunities for advanced research and for developing innovative strategies that may enhance the benefit in gallbladder cancer management.

Varanasi, India — Vijay Kumar Shukla
Varanasi, India — Manoj Pandey
Varanasi, India — Ruhi Dixit

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Editors and Contributors

About the Editors

Vijay Kumar Shukla is Rector, Banaras Hindu University, and Professor in the Department of General Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi, UP, India. Dr. Shukla is internationally known for his research on cancer of the gallbladder and wound healing. He had been visiting professor at reputed international universities/medical schools. He has been honoured with several national and international awards including Life Time Achievement Award at the World Union of Wound Healing Societies at Toronto, Canada, in 2008 for his exemplary work in wound healing and research. He was awarded the Fellow of the College of Wound Care Specialist (FCWCS), Malaysia, in 2017 and fellowship of National Academy of Medical Sciences in 2007.

In his illustrious career, Prof. Shukla had been the President of Indian Society of Wound Management, Member of ICMR task force on gallbladder cancer, Examiner and Inspector of National Board of Examination, Vice-president, Hernia Society of India, Executive member, Association of Colon and Rectal Surgeons of India, Governing Council Member of ASI, and Member, Independent Innovators Expert panel of Ministry of Health and Family Welfare, Govt. of India. He has been member of 12 national and international professional bodies and has published 470 papers in International and National Journals.

Manoj Pandey is a Professor in the Department of Surgical Oncology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, and Visiting Professor in the Department of Surgical Oncology, AIIMS, Rishikesh, Uttarakhand. He has served as the Director of Bhopal Memorial Hospital and Research Centre and National Institute for Research in Environmental Health, Indian Council of Medical Research. He has expertise in gallbladder cancer research, and his aspiration for quality research can be reflected from his research publications. He has published more than 328 publications. He has been honoured with several awards including Dr. U C Gupta Memorial Award for Excellence in Surgery. Dr. Pandey has also been a member of task force of ICMR on review of cancer management.

Ruhi Dixit is working as Women Scientist in the Department of General Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. She has a keen interest in cancer research and has been working on gallbladder cancer. She has published in various journals of international reputation. Moreover, she has served as a reviewer of reputed double-blind referred international journals. To perform cutting-edge research, she has also participated in various hands-on trainings and workshops in related areas and has presented her novel findings in reputed international platforms. She is currently working for the development of diagnostic biomarkers for gallbladder cancer.

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Part I

Introduction

Gallbladder Cancer: Current Treatment Options and Therapeutics

1

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1.1 Introduction

The gallbladder, a pear-shaped structure, is located beneath the liver. The gallbladder and the liver are both located posterior to the lower right ribs. The gallbladder

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functions as a storage and concentrating organ that modifies the concentration of the bile prior to its release into the duodenum. The bile is initially synthesized by the liver and contributes to chemical digestion of foods. The bile enters the common bile duct (CBD) that is formed by joining cystic duct with the hepatic duct. CBD (common bile duct) combines later with the pancreatic duct (involving essential enzymes for digestion), which eventually releases its contents into the second duodenum through the ampulla of Vater. Some authors consider the gallbladder as a nonessential organ, and many patients undergoing cholecystectomy may have long healthy lives following their surgery [1].

The first valid gallbladder cancer description was published by Maximilian de Stoll, a surgeon from Vienna in 1777. In the previous two centuries, gallbladder cancer was considered a malignancy with unfavorable outcome in patients with advanced stage disease [2]. Gallbladder cancer ranks as the sixth frequent gastrointestinal tumors in the USA with an incidence rate of about 1.13 cases per 100,000 [3, 4]. However, since the 1960s, this incidence has diminished in some part of the world as an unexpected result of elevated cholecystectomy due to gallstones [5], however, there are areas of high incidence like Japan, Chile, Poland, and India. Most patients at early detection are in 60–70 years age group, and females have been diagnosed more than males, making it the only gastrointestinal tumor with female predominance [4]. New investigations have observed that progesterone and estrogen may contribute to the development of gallbladder carcinoma, and expression of estrogen or progesterone receptor has been associated with earlier disease stages and thus a better prognosis, whereas absence of estrogen or progesterone receptor expression carried a more advanced metastatic or nonoperable disease [6]. The most important risk factor for the development of gallbladder carcinoma is the presence of gallstones, which could be found in about 85% of gallbladder carcinoma patients [7, 8]. Interestingly, larger gallstones can increase the risk of gallbladder cancer progression; indeed stones >3 cm in diameter accelerate gallbladder cancer risk 10 times more than ones <3 cm [9, 10]. Gallstones can result in chronic irritation and inflammatory processes within the gallbladder mucosa, which may trigger the formation of gallbladder cancer. Risk factors that can be modified and thus alter the risk of carcinogenesis include unhealthy diet, obesity, and prolonged infections with *Helicobacter* or *Salmonella* species [11]. The distinct mechanism of action of these organisms in the carcinogenesis of gallbladder cancer remains poorly understood; however, it could be due to the destruction of bile elements mediated by bacterial activity or even changes in the expression of tumor-promotive genes or tumor suppressors [12].

Primary gallbladder carcinoma varies significantly in the incidence rate throughout the world. The incidence rate of gallbladder cancer in 2018 is high in Colombia, India, Bolivia, and Chile with approximately 20.1 per 100,000 [12]. Poland, the Czech Republic, and Hungary are eastern European countries which had high numbers of gallbladder cancer patients. The risk of gallbladder cancer incidence varies in different races. Gallbladder cancer occurs less commonly in black individuals of the USA, whereas its incidence is high among Alaska Native, American Indian, and Hispanic individuals [4]. Gallbladder carcinoma is 3–5 times more frequent in

Hispanic females in the United States compared to non-Hispanic females with the same geographical area [7]. Both genetic and environmental factors can be effective in gallbladder cancer pathogenesis, explaining this high dispersion of incidence rate among various ethnic and geographic. To date, early detection of gallstones and the subsequent cholecystectomy remain the only preventive approach in highly susceptible individuals. However, routine cholecystectomy should not be considered in individuals with low probability of disease progression due to possible morbidities of cholecystectomy and quite low incidence of gallbladder cancer. Nonsurgical treatments in patients with gallbladder carcinoma involve prominently of radiotherapy and chemotherapy. Treatment approaches have focused on immune therapy, specific target treatment, biotherapy, vaccine therapy, and nanoparticles, which were widely investigated in clinical studies and preclinical setting.

1.2 Surgical Treatment

Adenocarcinomas comprise the majority of gallbladder carcinomas (approximately 80%). The cancerous cells possess high ability to invade nearby structures such as the bile duct, parenchyma of the liver, blood vessels, local lymphatic tissue, and perineural structure that are usually involved. The American Joint Committee on Cancer classification has stratified the cancer extension [13] on the basis of imaging features, including computed tomography scanning (CT scan), ultrasonography (US), and magnetic resonance imaging (MRI). Potential roles of positron emission tomography (PET) scanning in diagnosis or classification of patients of gallbladder carcinoma were investigated in a scant number of studies. Inappropriate laparotomy may be minimized by performing staging laparoscopy [14]. Stage of the tumor determines the surgical procedure for the treatment of patients. Simple cholecystectomy has been performed to treat gallbladder cancers of T1a stage (tumor with invasion into lamina propria) and excision of local lymph nodes in conjunction with resection of the gallbladder bed comprise surgical treatment of T1b (tumor with further invasion into the muscle layer) cancers. Since the degree of resection of the liver is not correlated with prolonged overall survival, minimal hepatectomy must be performed to achieve a microscopically margin-negative resection in tumors with T2 (invasion into perimuscular connective tissue) or higher stages. In most patients, segments 5 and 4a are sufficiently resected; however, patients with advanced hepatic invasion or invasion into the blood vessels (including hepatic artery or right portal vein) may benefit from right hepatectomy. Hepaticojejunostomy in association with resection of bile duct is only reserved for patients with jaundice or in case of tumor invasion of the cystic duct [15, 16]. The regional lymph nodes located around the hepatic pedicle following the common hepatic artery and lymph nodes in the retropancreatic space are usually excised. At least six lymph nodes have to be obtained to achieve an appropriate tumor staging. Ultimately, hepatopancreaticoduodenectomy is performed in highly selective gallbladder cancer patients, as it is associated with high mortality and morbidity, and no added benefit on patient survival has been found [14, 15]. The estimated patient 5-year survival rates following surgical

resection of the tumor depend mainly on the tumor stage: with the highest survival rate for T1 tumors (90%), followed by T2 tumors (60%), T3 (tumor invasion into a nearby organ or the adjacent serosa) tumors (20–25%), and the least survival rate of approximately less than 10% is confined to T4 (invasion into hepatic artery, main portal vein or 2≥ nearby organs) cancers [17].

1.3 Chemotherapy and Radiotherapy of GBC

Chemotherapy has been widely employed in the management of various malignancies and comprises therapeutic targets which can non-specifically suppress malignant cell proliferation through abolishing synthesis of DNA. Two treatment approaches in gallbladder cancer have been suggested by National Comprehensive Cancer Network: chemotherapeutic regimen with single agent such as, gemcitabine or fluoropyrimidine, and regimen involving multiple chemotherapeutic agents, including cisplatin, oxaliplatin, and capecitabine [18–20]. The combined chemotherapeutic regimens including CAPOX (capecitabine and oxaliplatin), FOLFOX (5-fluorouracil and oxaliplatin), Gemox (gemcitabine and oxaliplatin), and GC (gemcitabine and cisplatin) are considered as the major chemotherapeutic regimens in clinical studies [21–24]. Several clinical studies found that combination chemotherapeutic regimens with FOLFIRINOX (fluorouracil, leucovorin, irinotecan, and oxaliplatin) may offer valuable therapeutic benefits in BTC patients [25, 26]. However, no single combination chemotherapeutic regimen has been suggested, due to the extensive unintended side effects and resistance to therapy, inadequate response to treatment and systemic toxicity [27]. As a result, a substantial number of clinical and preclinical studies are currently performed to determine the potential advantages of treatment with drugs, even in the case of presence of unintended side effects, which could be managed at a low level. For instance, recently resected specimens of gallbladder from cancer patients were transplanted in mice mini-PDX (mini patient-derived xenograft) model to evaluate drug sensitivity and identify drugs with greater efficacy. Oxaliplatin, gemcitabine, nanoparticle albumin-bound nab-paclitaxel, 5-fluorouracil, and irinotecan were all evaluated following surgery, establishing that patients undergoing PDX chemotherapy had longer survival (median overall survival of; 18.6 months; 95% confidence interval (CI) 15.9–21.3 months) and prolonged disease-free survival (DFS 17.6 months; 95% CI 14.5–20.6 months) compared to patients undergoing traditional random selective chemotherapeutic treatment, with overall survival of 13.9 months (95% CI 11.7–16.2 months) and disease-free survival of 12.0 months (95% CI 9.7–14.4 months). Thus, the mini PDX model may establish chemotherapeutic regimens to enhance clinical outcomes and offer the optimal individualized treatment approaches [28, 29].

Although previous studies found that treatment with chemoradiotherapy (CRT) and chemotherapy enhanced patient overall survival with T2 or more advanced stages, current evidence from the National Cancer Data Base (NCDB)

demonstrated no improvement in survival rate of gallbladder cancer cases receiving adjuvant therapy. To date, less than 30% of individuals with gallbladder cancer undergo adjuvant therapies [30].

1.3.1 Adjuvant Chemotherapy in Resected Gallbladder Cancer

Chemotherapy or chemoradiotherapy following surgical resection of gallbladder cancer was advised by The National Comprehensive Cancer Network (NCCN) [31]. However, there is not enough evidence in favor of adjuvant therapy, and no beneficial chemotherapeutic regimen was found in cohort studies. Personalized benefits and risks should be considered prior to adjuvant therapy. Horgan et al. [32] performed a meta-analysis in 1960–2010 over 6712 patients with cholangiocarcinoma or gallbladder cancer with surgical resection of the tumor. Overall, a small, unremarkable advantages were reported in overall survival of patients, particularly ones with lymph node involvement or R1 resection had more favorable outcomes. Ma et al. [33] performed a meta-analysis showed that positive lymph node involvements, combined stages of II/III or candidates for R1 resection may have good response from adjuvant therapy. Wang et al. [34] demonstrated that adjuvant chemoradiotherapy promotes survival of patients with T3, N1, or higher disease stages with the Surveillance, Epidemiology, and End Results Program (SEER) [35]. Using the NCBD, Mantripragada et al. [36] performed an analysis over 4775 gallbladder cancer patients undergoing resection. In the study, 4708 patients were studied to evaluate the final outcome among those receiving adjuvant therapy along with surgical resection and patients undergoing surgical resection alone. Patients receiving adjuvant therapy did not show better outcome, except for those with lymph node involvement receiving chemoradiotherapy for 3 months following surgery or patients with T3 disease stage. These patients had increased survival of about 3 months within the 5-year-follow up after surgery [36]. Another NCDB study [37] suggested combination of adjuvant chemotherapy with surgery in patients with lymph-node involvement may be associated with prolonged average overall survival of about 20 months vs. 8.6 months in patients underwent surgical resection alone. Nevertheless, in the current clinical setting, approximately 22% of patients with lymph node involvement undergo chemotherapy following surgery [35, 37]. Finally, Ghidini et al. [38] performed an analysis over 22,499 patients of Asiatic and Western cohort studies (of whom only 3967 patients had surgical resection). Their results showed enhanced survival of patients undergoing adjuvant therapy compared to surgery alone of only about 4.3 months [38].

1.3.2 Chemotherapy in Unresectable Gallbladder Cancer

Valle et al. in trial ABC-02 (phase 3), proposed adjuvant therapy of gemcitabine and cisplatin instead of gemcitabine alone in metastatic or advanced cholangiocarcinomas [39]. Overall survival of patients receiving combination regimen was higher

compared to that of single-agent regimen (11.7 vs. 8.1 months), even though two groups had unfavorable outcome in comparison of that in patients undergoing surgery [39]. However, 80% of patients had prognostic benefits following administration of gemcitabine along with cisplatin. Studies originally in 2010 suggest that risk factors that confer poor survival are higher levels of CA 19–9 at baseline, male gender, metastatic disease, decreased performance status, and Response Evaluation Criteria in Solid Tumors (RECIST) criteria [40]. Nevertheless, both therapeutic regimens provide a better overall survival in patients with metastatic gallbladder cancer compared to that in palliative care alone (35.6 vs. 13 weeks) [41].

1.3.3 Adjuvant Chemoradiotherapy in Resected Gallbladder Cancer

Despite the fact that ART (adjuvant radiotherapy) has been shown to improve the final outcome of patients in various cancers, its beneficial effects in gallbladder cancer need to be further elucidated due to the absence of robust data. Currently, there are no standardized adjuvant radiotherapy regimens and clinics commonly use a combination of chemotherapy and adjuvant radiotherapy. Promising findings have been demonstrated in a number of clinical settings [42]. A single-arm phase II study of the SWOG (Southwest Oncology Group) evaluated final outcomes of patients with gallbladder cancer and extrahepatic cholangiocarcinoma received adjuvant chemotherapy of gemcitabine plus capecitabin in conjunction with chemoradiotherapy (combined radiotherapy and capecitabine). In their study, the overall survival of patients at 2 years was about 65% in all patients (60% in patients had R1 resection and 67% in patients with R0 resection), while median overall survival was about 35 months. Moreover, only 14 of these patients experienced regional recurrences, indicating the effectiveness of administering chemotherapy in conjunction with chemoradiotherapy in conjunction with chemoradiotherapy in gallbladder cancer cases [43]. Combined radiotherapy regimen was tolerated well in these patients, and it was related to positive effects; however, more amounts of larger clinical trials are needed to prove the influence of combined radiotherapy in patients.

Adjuvant radiotherapy is able to enhance the survival of patients following R0 surgical resection [36, 44]. The impact of chemoradiotherapy as an adjuvant therapy on overall survival of patients following surgical resection was studied in a population of 78 patients with operable stages 2–4 gallbladder cancer [45]. In this study, patients treated with chemoradiotherapy received external beam radiotherapy along with double-agent chemotherapy (oxaliplatin-based) or single-agent chemotherapy (capecitabine). Patients underwent chemoradiotherapy had a prominently prolonged disease-free survival (23 months compared to 7 months) and overall survival (27 months compared to 13 months) [46]. Kim et al., [46] demonstrated that patients with gallbladder cancer undergoing R1 resection or with T2 disease stage are more likely to develop local relapses; therefore, these patients may benefit from administration of chemoradiotherapy prior to disease progression [47]. Another study performed by the National Cancer Database (NCDB) on patients with lymph node

involvement showed favorable overall survival in patients receiving adjuvant chemoradiotherapy in conjunction with R0 surgical resection [35, 48].

1.3.4 Neoadjuvant Chemoradiotherapy in Resectable Gallbladder Cancer

In gallbladder cancer patients with lymph node involvement or regionally advanced disease receiving either single-agent gemcitabine neoadjuvant regimen or gemcitabine in association with platinum combined regimen, 30% of patients (n = 22) were operable candidates, and about 14% had R0 surgical resection (n = 10). However, a significant discrepancy in overall survival was observed among the group with inoperable tumor and the group with operable disease (11 months compared to 51 months) [49]. According to this study, inoperable patients who are responsive to chemotherapeutic measures need to be reassessed by their clinician.

1.3.5 Chemoradiotherapy in Unresectable Gallbladder Cancer

Patients with inoperable gallbladder cancer may benefit from chemotherapy with gemcitabine+cisplatin (level 1 evidence in guidelines of NCCN, version 2.2018). The role of local therapy with radiotherapy in patients with inoperable tumor has not been evaluated in any randomized control trials [31, 35, 50]. Regional recurrences account for the majority of cancer-related deaths and are responsible for up to 85–90% of all recurrences following surgical resection [51]. NCDB performed an analysis in 2004–2013 in patients with inoperable gallbladder cancer without metastasis and evaluated the final outcome of patients undergoing chemoradiotherapy with patients receiving chemotherapy alone. In a cohort study of 1199 patients, 73% (872 patients) received chemotherapy and 27% (327 patients) had chemoradiotherapy. Further analysis demonstrated that overall survival in patients undergoing chemoradiotherapy was significantly higher compared to that of chemotherapy alone (12.9 months vs. 7.8 months) [50]. Collectively, beneficial effects of chemoradiotherapy in patients with nonmetastatic inoperable disease need to be further evaluated in prospective studies.

1.4 Chemoresistance and Gallbladder Cancer

Resistance to chemotherapeutic regimens is considered to be a crucial hindrance to chemotherapy in many human tumors. Previous studies have shown that resistance to chemotherapeutic drugs accounts for up to 90% of failure in treatment in cases of metastatic disease. Resistance to chemotherapeutic drugs may occur in two different phases; either primary prior to installation of drug or secondary, which occurs following the administration of anticancer drug [52]. Previous research verified that malignant cells in gallbladder cancer are chemoresistant [53, 54], thus inadequate

response to chemotherapeutic drugs remains of great concern in these patients [55]. Chemoresistance is closely correlated with unfavorable outcome in patients with gallbladder cancer [56]. Since aerobic glycolysis may function to the development of resistance to chemotherapy, measures with glycolysis targeting may be beneficial in enhancing chemotherapy [57, 58]. In vertebrates, UCP2 (uncoupling protein 2), which belongs to the family of mitochondrial-related uncoupling proteins, was detected to be widely expressed [59]. UCP2 was found to be deregulated in various malignancies, such as breast, lung, prostate, skin, pancreatic, and colorectal cancer [60–62]. Aberrantly elevated UCP2 expression in tumor cells regulates metabolic cycles including promotion of glycolysis from oxidative phosphorylation [63, 64]. Furthermore, UCP2 belongs also to the mitochondrial antioxidant family, as it functions in the ROS (electron transport chain-derived reactive oxygen species) [65, 66]. UCP2 suppression was found to promote the sensitivity of various tumor cells to anticancer drugs [56, 67]. Yu and his colleagues [68] demonstrated that prognosis and total survival rate of gallbladder cancer patients post-chemotherapy are inversely correlated with the levels of UCP2 expression, indicating that increased expression of UCP2 may result in resistance to chemotherapy in gallbladder cancer patients. UCP2 may enhance chemoresistance by the NF-κB/β-catenin axis. β-Catenin and NF-κB are the main regulators downstream genes expression resulting in chemotherapy resistance of tumors [69, 70]; however, this axis was not activated in UCP2 depleted cells, and cells with UCP2 depletion were more likely to respond to chemotherapy [68].

Previous studies found that abnormalities of redox hemostasis following administration of chemotherapeutic drugs may increase activity of antiredox system in tumor cells, leading to enhanced resistance to chemotherapy [71, 72]. NADPH oxidase 1 (NOX1) an enzyme bound to the membrane is responsible for catalyzing production of NADP+ from NADPH in the cytosol and contributes to the production of one proton and two electrons, which in turn gives rise to the production of a superoxide anion, an important reactive oxygen species (ROS) source [73, 74]. NOX1 was shown to be aberrantly upregulated in various malignancies such as ovarian and breast tumors, and it has crucial roles in tumor progression and metastasis [73, 75]. Silencing of NOX1 in HepG2 cell lines of hepatoblastoma resulted in attenuated cell proliferation through decreasing expression of EGFR and TGF-α in P38/MAPK/AKT axis [76]. NOX1 may contribute to both carcinogenesis and metastatic capability of tumor cells [77] as well as modulating resistance to chemotherapy in gallbladder cancer [78]. In a study by Zhan et al. [78], NOX1 expression was elevated in tissues of gallbladder cancer, and higher expression of NOX1 conferred chemosensitivity to cisplatin in cell lines of gallbladder cancer. Silencing of NOX1 enhanced the efficacy of GBC-SD cells to cisplatin, meanwhile promoted NOX1 expression was associated with reduced sensitivity to cisplatin in cell line SGC-996. NOX1 may contribute to the development of resistance to chemotherapy via various signaling pathways such as increased activity of HIF-1a/MDR1 pathway and accumulation of intracellular ROS. They have indicated that NOX1 might accelerate resistance of cells to chemotherapy in gallbladder cancer and thus may offer promising pharmacologic target in these patients [78].

CDDP (Cisplatin) is an antitumor cytotoxic agent that is commonly used in many cancers [79–81]. CDDP exerts its replication-mediated double-strand break (DSB) effects in tumor cells by the development of cross-links within the DNA strand or between two DNA strands after interacting with guanine N7-position [82, 83]. On the other hand, their curative capability is eliminated by chemoresistance. CDDP resistance, as well as resistance to many other chemotherapeutic drugs, is attributed in part to a variety of epigenetic and genetic modifications, leading to reduced cell survival [84–86]. Dual-Specificity Phosphatase 1 (DUSP1) belongs to the family of DUSP, comprising of 25 proteins. DUSP1 is expressed in various malignancies [87]. Expression level of DUSP1 was increased in numerous epithelial-related cancers such as non-small-cell lung cancer, pancreatic ductal adenocarcinoma (PDAC), gastric, ovarian, and breast cancer, as well as early stages of prostate cancer, whereas its expression was reduced in HCC [88–90]. Family of the dual-specificity phosphatase (DUSP) possess inhibitory activities, and they target mitogen-activated protein kinases (MAPKs) [91]. DUSP1 promotes chemoresistance to paclitaxel or doxorubicin in cell lines of osteosarcoma, breast cancer, and non-small-cell lung cancer through suppressing the activity of c-Jun N-terminal kinase (JNK) and p38 [90, 92, 93]. Nevertheless, only a few studies evaluate the correlation between the expression of DUSP1 and resistance to chemotherapy in gallbladder cancer. Fang and his colleagues [94] evaluated the level of expression of various genes associated with chemoresistance including MRP1, DUSP1, MDR1, and HIF-1α. They further evaluated the expression of these genes in GBC-SD cells, cisplatin-resistant SGC996 cell lines as well as normal SGC996 cell lines. The results showed that DUSP1 level of expression was elevated in cells resistant to cisplatin in comparison with healthy cells. Likewise, expression of DUSP1 was increased after the administration of cisplatin in subcutaneous tumors, proposing that DUSP1 may lead to chemoresistance and DUSP1 reduce p38 MAPK expression and DNA damage, prominently enhancing chemotherapy response [94]. DUSP1 reduces the cytotoxicity induced by gemcitabine in pancreatic cancer via JNK-MAPK signaling regulation [95]. Gemcitabine is almost utilized in the treatment of gallbladder cancer [96]. Overall, these findings suggest that DUSP1 may function as a promising pharmacologic target and facilitate chemotherapy effectiveness in gallbladder cancers.

Cyclin M belongs to the family of cyclin proteins, possessing major functions in modulating transcription and cell cycle progression [97]. Cyclin M was shown to bind to its related kinase, cyclin-dependent kinase 10 (CDK10) [98]. CDK10 was identified to play major role in fundamental cellular processes such as cell cycle progression and pathogenesis of many malignancies [99, 100]. Reportedly, CDK10 serves as an anti-tumor-promotive gene and modulates the viability of tumor cells in cells in biliary tract system such as gallbladder cancer [100]. Cyclin M has an important role in carcinogenesis by attaching to CDK10. Yu et al. [100] demonstrated that CDK10 may regulate the sensitivity of chemotherapy in tumor cells of gallbladder cancer and that cyclin M activates and attaches to CDK10. The influences of cyclin M or CDK10 high expression in sensitivity of tumor cells to chemotherapy was assessed. GBC-CDK10 and GBC-CDK10-GR cells both produced

elevated CDK10 levels, but GBC-CDK10-GR cells had resistance to gemcitabine comparable to that of GBC-Mock-GR cells, whereas GBC-CDK10 cells did not. This finding shows that level of expression of CDK10 did not have the capability to elucidate acquired resistance to gemcitabine in GBC-SD cells. On the other hand, GBC-Co and GBC-Co-GR cells, which had higher expression level of cyclin M and CDK10, conferred enhanced sensitivity to gemcitabine when compared to GBC-Mock and GBC-Mock-GR cells [100]. Moreover, cyclin M mRNA levels with the 3′UTR region were prominently reduced in subclones of GR in comparison with that of non-GR subclones. This finding demonstrates cyclin M is a regulator of acquired resistance to gemcitabine in tumor cells of gallbladder cancer.

miRNAs exert their regulatory function in the expression of various target genes through binding with the mRNA 3′UTRs region. Cyclin M 3′UTR was lowly expressed in subclones of GR and miR-433 that were further verified as cyclin M-targeting miRNA. The study found that secondary resistance to gemcitabine in tumor cells of gallbladder cancer was correlated with increased expression of miR-433. Likewise, serum level of miR-433 was increased in patients with inadequate response to chemotherapy, while miR-433 serum level was not altered in patients with favorable response to chemotherapy. Its findings showed that miR-433 may be used as a promising prognostic biologic marker to assess chemosensitivity in patients with gallbladder cancer. Taken together, the miR-433/cyclin M axis may contribute to the development of acquired chemoresistance in tumor cells of gallbladder cancer [101].

Chemoresistance of tumor cells may be mediated through a variety of regulators such as ineffective intracellular accumulation and uptake of drugs by cells, DNA repair accentuation, increased activity of antiapoptosis signaling pathway, elevated number of tumor stem cells, and promoted detoxification mediated by the activity of glutathione system antioxidant [102–106]. Diminished accumulation of drugs within cells was established as a key factor in the emergence of resistance to chemotherapy. Additionally, upregulation of pump-related proteins responsible for drug efflux including, multidrug resistance-related protein 1 (MRP1), the breast cancer resistance protein (ABCG2), and multidrug resistance-related protein 2 (MRP2) may lead to resistance to chemotherapy in cancerous cells [107–109]. In a study of Wang et al. [110], emodin, a herbal medicine used traditionally in China, inhibits MRP1 transcription in gallbladder cancer, thereby enhances cisplatin anti-oncogenic influence [110]. MiR-145, an anti-oncogene miRNA, was detected to be expressed lowly in several cancers, like bladder cancer, prostate cancer, and gallbladder cancer [111–113]. A study of Zhang et al. [114] found that mRNA expression of MRP1 and miR-145 was negatively correlated in tissues and cells of gallbladder cancer. Moreover, miR-145 was shown to be poorly expressed in tissues of gallbladder cancer and demonstrated that MRP1 may be directly modulated by miR-145, resulting in attenuated chemosensitivity to cisplatin both in vivo and in vitro. Finally, the results showed that mimic of miR-145 has the potential to be applied as therapeutic target, and it enhances chemosensitivity of cells of gallbladder cancer to cisplatin [114].

A group of noncoding genes carry fundamental functions in many cellular processes in tumors such as proliferation, formation, metastasis and formation of new

blood vessels [115, 116]. The lncRNAs have been widely applied as promising biologic markers of tumor diagnosis, prognosis, and therapeutic response considering the fact that these markers are detectable in bodily fluids including urine and blood. Growing evidence showed that lncRNAs may target various signaling pathways and thus play major roles in resistance to chemotherapy. Increased autophagy which occurs under hypoxia and starvation has been shown to convey a paradoxical effect on the development of chemosensitivity or chemoresistance in patients with malignancy [116]. For instance, lncRNA AC023115.3 was identified to diminish chemoresistance to cisplatin through suppressing autophagy in patients with glioblastoma. AC023115.3 was shown to attenuate miR-26a suppressive effects on glycogen synthase kinase 3 (GSK3) [117]. GSK3 resulted in the degradation of Mcl1, which belongs to the family of Bcl-2, with reduction in autophagy [117]. Cai and his colleagues [118] performed a microarray analysis to evaluate the expression level of various lncRNAs in tumor cells of gallbladder cancer resistant to doxorubicin. The results of their study showed that gallbladder cancer drug resistance-associated lncRNA1 (GBCDRlnc1) modulates resistance to chemotherapy through stimulating autophagy, meanwhile silencing of GBCDRlnc1 increased sensitivity of tumor cells of gallbladder cancer to doxorubicin through suppressing autophagy [118].

1.5 Future Directions in the GBC Treatment

According to the current guidelines, cytotoxic chemotherapy remains as the optimal treatment in terms of palliative therapy. Similar to other tumors, many efforts have been adopted worldwide to discover new agents for the management of gallbladder cancer patients; however, no distinct drug was established for the optimal management in routine clinical settings (see Table 1.1). Various clinical studies have combined tumors of the biliary tract together, such as gallbladder cancer, extra-hepatic cholangiocarcinoma, and intra-hepatic cholangiocarcinoma, rendering gallbladder cancer analysis impossible on its own. Here, we aim to explain a number of recent treatment strategies in patients with gallbladder cancer.

1.5.1 HER2 and EGFR Targeting Therapies

Treatments targeting the HER2/EGFR cascade have recently gotten attention in many malignancies of the gastrointestinal tract. About 16–64% of all gallbladder cancers have shown accentuated expression of the HER2 protein [119, 120]. Furthermore, increased expression and mutations of EGFR were found in 6% and 13.6%–15% of patients, respectively [121]. On the other hand, no clinical studies have found a prominent beneficial effect of drugs targeting the above-mentioned pathways solely. When administered with gemcitabine, lapatinib, which is a tyrosine kinase inhibitor suppressing both of the EGFR and HER2 signaling pathways, showed a synergistic effect in terms of tumor suppression in gallbladder cancer in in vitro [122]. Phase II clinical trials containing

Table 1.1 On-going clinical trials in progress for targeted therapeutic options for gallbladder cancer

Drug investigated	Molecular target	Regimen	Target population	Phase	Clinical trial ID
Durvalumab	PDL-1	Durvalumab/guadecitabine	GBC	Phase 1	NCT03257761
ADH-1	N-cadherin	ADH-1/cisplatin/gemcitabine	GBC	Phase 1	NCT01825603
Merestinib	Mesenchymal-epithelial transition (MET)	Merestinib/cisplatin/gemcitabine	GBC	Phase 1	NCT03027284
Pazopanib	VEGFR, cKIT, FGFR	Gemcitabine/pazobanib	GBC	Phase 2	NCT01855724
Selumetinib	MEK1–2	Selumetinib/cisplatin/gemcitabine	GBC	Phase 2	NCT02151084
Canlisib	PI3K	Copanlisib/cisplatin/gemcitabine	GBC	Phase 2	NCT02631590
Cetuximab, trastuzumab, gefitinib, lapatinib, everolimus, sorafenib, crizotinib	VEGF, HER2, EGFR, PDL-1, ALK/ROS1, mTOR	GEMOX + targeted therapy per proteomic/genetic profiling	GBC	Phase 2	NCT02836847
Regorafenib	VEGF	Regorafenib	GBC	Phase 2	NCT02115542
Pembrolizumab	PD-1	Pembrolizumab/cisplatin/gemcitabine	GBC	Phase 2	NCT03260712
Sorafenib	Multi-targeted TKIs	Sorafenib tosylate	GBC	Phase 2	NCT00238212
Sorafenib	Multi-targeted TKIs	Somatostatin, epirubicin, cisplatin, LV, 5-FU	GBC	Phase 3	NCT01053390
Apatinib	EGFR, HER2	Apatinib/tegafur	GBC	Phase 2	NCT03702491
Trastuzumab	HER2	Trastuzumab	Advanced or metastatic GBC	Phase 2	NCT00478140
Erlotinib	EGFR	Cetuximab/erlotinib hydrochloride	Solid tumors (GBC, etc.)	Phase 1	NCT00397384
KBP-5209	Multitargeted TKIs	KBP-5209	Solid tumors (GBC, etc.)	Phase 1	NCT02442414
Ceralasertib	PARP	Ceralasertib/olaparib	Solid tumors (GBC, etc.)	Phase 2	NCT03878095

CGX1321	PORCN	CGX1321	Solid tumors (GBC, etc.)	Phase 1	NCT03507998
Entinostat	HDAC	Entinostat	Solid tumors (GBC, etc.)	Phase 1	NCT00020579
FT-2102	IDH1	FT-2102/azacitidine/nivolumab/gemcitabine and cisplatin	Solid tumors (GBC, etc.)	Phase 1, 2	NCT03684811
Merestinib	MET	Merestinib/cisplatin/gemcitabine	Solid tumors (GBC, etc.)	Phase 1	NCT03027284
PSMA/PRAME	T cells	PSMA/PRAME	Solid tumors (GBC, etc.)	Phase 1	NCT00423254
ADH-1	N-cadherin	ADH-1/cisplatin/gemcitabine hydrochloride	Solid tumors (GBC, etc.)	Phase 1	NCT01825603
Recombinant EphB4-HSA fusion protein	HSA, EphB4	Cisplatin/docetaxel/paclitaxel albumin-stabilized nanoparticle formulation/ recombinant EphB4-HSA fusion protein	Solid tumors (GBC, etc.)	Phase 1	NCT02495896
CEA RNA-pulsed DC cancer vaccine	CEA	CEA RNA-pulsed DC cancer vaccine	Solid tumors (GBC, etc.)	Phase 1	NCT00004604
Recombinant interleukin-12	IL-12	Recombinant interleukin-12	Solid tumors (GBC, etc.)	Phase 1	NCT00003439
Recombinant interleukin-12	IL-12	Recombinant interleukin-12	Solid tumors (GBC, etc.)	Phase 1	NCT00003046
Avelumab	PD-L1	Avelumab/hypofractionated radiation therapy/ peposertib	Solid tumors (GBC, etc.)	Phase 1, 2	NCT04068194
Nivolumab, ipilimumab	CTLA-4, PD-1	Ipilimumab/nivolumab	Solid tumors (GBC, etc.)	Phase 2	NCT02834013
Everolimus	mTOR	Everolimus/gemcitabine hydrochloride/ cisplatin	Solid tumors (GBC, etc.)	Phase 1	NCT00949949

(continued)

Table 1.1 (continued)

Drug investigated	Molecular target	Regimen	Target population	Phase	Clinical trial ID
GSK1120212	MEK	GSK1120212/gemcitabine	Solid tumors (GBC, etc.)	Phase 1	NCT01324258
ARRY-438162	MEK	ARRY-438162 (MEK162)/MEK inhibitor	Solid tumors (GBC, etc.)	Phase 1	NCT00959127
Recombinant human IL-2	HER2	Recombinant human IL-2/HER2Bi-armed T cells	Solid tumors (GBC, etc.)	Phase 1	NCT02662348
Trastuzumab, IL-12	HER2, IL-12	Recombinant IL-12/ABI-007/carboplatin/trastuzumab	Solid tumors (GBC, etc.)	Phase 1	NCT00004074
Trastuzumab, R115777	HER2	Trastuzumab/tipifarnib	Solid tumors (GBC, etc.)	Phase 1	NCT00005842
Sorafenib	Multitargeted TKIs	Gemcitabine/oxaliplatin/sorafenib	BTC	Phase 1, 2	NCT00955721
Bevacizumab	EGFR, VEGFR	Erlotinib hydrochloride/bevacizumab	BTC	Phase 2	NCT00356889
DKN-01	DKK1	DKN-01/gemcitabine/cisplatin	BTC	Phase 1	NCT02375880
Glivec	KIT, ABL, PDGFR	Glivec	BTC	Phase 2	NCT01153750
CPI-613	α-KGDH, PDH	CPI 613/gemcitabine/cisplatin	BTC	Phase 1, 2	NCT04203160
MK-2206	AKT	Akt inhibitor MK2206	BTC	Phase 2	NCT01425879
Selumetinib	AKT	Selumetinib/Akt inhibitor MK2206	BTC	Phase 2	NCT01859182
Bintrafusp alfa	PD-L1, TGF-β	Bintrafusp alfa/gemcitabine/cisplatin/placebo	BTC	Phase 2, 3	NCT04066491
Durvalumab, tremelimumab	PD-L1, CTLA-4	Durvalumab/gemcitabine/cisplatin/tremelimumab	BTC	Phase 2	NCT03473574
Durvalumab	PD-L1	Durvalumab + gem/cis, gem/cis	BTC	Phase 2	NCT04308174
STI-3031	PD-L1	STI-3031	BTC	Phase 2	NCT03999658

Toripalimab plus lenvatinib	PD-1	Toripalimab plus lenvatinib	BTC	Phase 2	NCT04211168
M7824	PD-1	M7824	BTC	Phase 2	NCT03833661
Pembrolizumab	PD-1	Pembrolizumab/gemcitabine/cisplatin/placebo	BTC	Phase 3	NCT04003636
Pembrolizumab	PD-1	Pembrolizumab (MK-3475)/oxaliplatin/capecitabine	BTC	Phase 2	NCT03111732
Pembrolizumab	PD-1	Pembrolizumab (MK-3475)/oxaliplatin/capecitabine	BTC	Phase 2	NCT03260712
Nivolumab	PD-1	Gemcitabine/cisplatin/ipilimumab/nivolumab	BTC	Phase 2	NCT03101566
Nivolumab	PD-1	Nivolumab	BTC	Phase 2	NCT02829918
MEK162	MEK	MEK162 + capecitabine	BTC	Phase 1, 2	NCT02773459
MEK162	MEK	Gemcitabine/cisplatin/MEK162	BTC	Phase 1, 2	NCT01828034
MEK162	MEK	MEK 162/gemcitabine/oxaliplatin	BTC	Phase 1	NCT02105350
Trametinib	MEK	Trametinib (single tablet)/trametinib (multiple tablet)	BTC	Phase 2	NCT01943864
Trametinib	MEK	Capecitabine/fluorouracil/leucovorin/calcium/trametinib	BTC or GBC	Phase 2	NCT02042443
Atezolizumab	MEK	Atezolizumab/cobimetinib	BTC	Phase 2	NCT03201458
Selumetinib	MEK	Selumetinib/cisplatin/gemcitabine	BTC	Phase 2	NCT02151084
Selumetinib	MEK	Selumetinib/gemcitabine/cisplatin	BTC	Phase 1	NCT01242605
Panitumumab	Kras, BRAF	Panitumumab/oxaliplatin/gemcitabine	BTC	Phase 2	NCT01308840
Regorafenib	KIT, PDGFRβ, RET Raf-2, VEGFR1–3	Regorafenib	BTC	Phase 2	NCT02053376

(continued)

Table 1.1 (continued)

Drug investigated	Molecular target	Regimen	Target population	Phase	Clinical trial ID
Regorafenib	KIT, PDGFRβ, RET Raf-2, VEGFR1–3	Regorafenib	BTC	Phase 2	NCT02115542
Vandetanib	VEGFR2–3, RET, EGFR	ZD6474, vandetanib/gemcitabine/placebo matching ZD6474	BTC	Phase 2	NCT00753675
Pazopanib	c-Fms, PDGFRβ, VEGFR1–3, c-kit, FGFR1	Gemcitabine/pazopanib	BTC	Phase 2	NCT01855724
Ramucirumab, merestinib	c-MET, VEGFR2	Ramucirumab/merestinib/cisplatin/ gemcitabine/placebo oral/placebo IV	BTC	Phase 2	NCT02711553
Ramucirumab	VEGFR2	Ramucirumab	BTC	Phase 2	NCT02520141
Cediranib	VEGFR	Cediranib maleate/oxaliplatin/leucovorin calcium/fluorouracil	BTC	Phase 2	NCT01229111
Lapatinib	HER2	Lapatinib ditosylate	BTC	Phase 2	NCT00107536
Sorafenib	Multi-targeted TKIs	Gemcitabine/cisplatin/sorafenib	Extrahepatic bile duct cancer, GBC	Phase 2	NCT00919061

GBC gallbladder cancer, *Multi-targeted TKIs* multi-targeted tyrosine kinase inhibitors, *LV* leucovorin; *5-FU* 5-fluorouracil, *BTC* biliary tract cancer

single-agent lapatinib in biliary tract system tumors (such as gallbladder cancer) were conducted following these preclinical findings. These clinical trials had negative final outcome; meanwhile, the population of patients for this study was not chosen for the study population was not specified to the related HER2 proliferation [121]. In another case series of nine patients with gallbladder cancer who received HER2-targeted therapy including lapatinib, trastuzumab, or pertuzumab, either solely or coincident with installation of chemotherapy, one patient had complete response to therapy, three patients were stable, and four patients demonstrated partial response [123]. Among these, one patient received lapatinib solely, demonstrated a mixed outcome, which suggests the potential role of monotherapy with HER2. One phase II clinical trial examined the effectiveness of trastuzumab in patients with inoperable biliary tract tumors were abruptly banned (NCT00478140) (Table 1.1).

In the case of EGFR, a phase III clinical trial in cases of advanced biliary tract tumors assessed erlotinib effectiveness, which is an inhibitor of tyrosine kinase targeting EGFR, in conjunction with oxaliplatin and gemcitabine. The erlotinib group had a higher progression-free survival rate of 5.8 months versus 4.2 months; however, all groups had similar average survival and gallbladder cancer patients had no benefits in terms of long-term prognosis [124]. Various phase II clinical trials evaluating panitumumab (EGFR-targeted monoclonal antibody), in conjunction with chemotherapeutic regimens in biliary tract tumor patients, brought about various results, and no significant difference in overall survival was observed in the largest scale clinical trial [121]. Consequently, administration of routine chemotherapy combined with targeted therapies may provide promising therapeutic targets in gallbladder cancer.

1.5.2 Other Therapeutic Targets

Many clinical trial studies have researched the use of VEGF in GBC treatment for gallbladder cancer inhibition. Oxaliplatin and gemcitabine in association with bevacizumab (monoclonal antibody that inhibits VEGF-A) showed 63% 6 month PFS that was below the 70% target rate, in all biliary tract cancers in a phase II trial [124]. Cediranib, an oral VEGFR1, VEGFR2, and VEGF3 tyrosine kinase suppressor, failed to reach its primary endpoint of improved median PFS in another phase II trial [125], and also other researches had the same disappointing results with using antiangiogenic tyrosine kinase inhibitors sunitinib and sorafenib [120]. P53, KRAS, BRAF, and APC are some of the other pathways that have been studied for targeting purposes in GBC. In the same way, immunotherapy has shown promise in GBC. PD-L1 positivity was observed in 42% of patients with biliary tract cancer [120]. Several clinical trials are being carried out with immune check point inhibitors, including cancer. Several ongoing human studies are using immune checkpoint inhibitors such as subsets of biliary tract cancer. Research at Shanghai's Xinhua Hospital is of special interest because it aims to assign tailored treatment to biliary tract cancer patients based on relevant mutations (NCT02836847). A list of current clinical trials of targeted therapies for GBC can be found in Table 1.1.

1.6 Noncoding RNAs as Therapeutic Targets in Gallbladder Cancer

Most chemotherapeutic drugs influence DNA synthesis. Over the past decade, researchers have focused on RNA molecules that regulate tumor suppressor gene expression or oncogenes. In clear opposite to the conventional theory that noncoding RNAs are noisy and nonfunctional in modulation of gene expression, it seems that microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) serve as crucial factors to coordinately regulate multiple gene expressions underlying carcinogenesis. Several laboratories have demonstrated that lncRNAs and miRNAs regulate the invasion, proliferation, and GBC resistance to chemotherapy. Therefore, they can be used as new therapeutic targets for new approaches to GBC treatment [117, 126–134].

Brannan and his colleagues discovered the first long noncoding RNA, lncRNAH19, in 1990 [135]. Over 6700 lncRNA genes have been recognized in the human genome recently [136]. Long noncoding RNAs participate in different parts of pre- and posttranscription procedures such as imprinting, immunity, splicing, embryonic stem cells pluripotency, nuclear structure, nuclear import, cellular trafficking, and small RNA precursors [114]. The lncRNAs are implicated in cell cycle, differentiation, apoptosis, and proliferation and also affect and regulate cancer progression, development, and maintenance [137]. lncRNAs can play as onco-suppressors or oncogenic in GBC cells, onco-suppressive lncRNAs, such as LET, MEG, and GCASPC, but there is need for more research about their roles in GBC cells [138]. The lncRNA-GCASPC upregulation suppressed cell proliferation in vivo and vitro, but its downregulation increased cell proliferation. Pyruvate carboxylase was identified as a RNA-binding protein correlated with lncRNA-GCASPC. The pyruvate carboxylase activity level was downregulated by the miR-17-3p lncRNA-GCASP pathway. The miR-17-3p, lncRNA-GCASPC, and pyruvate carboxylase pathway is a novel mechanism that may help to define lncRNA-GCASPC function and pathophysiology [139]. Liu et al. showed that pcDNA-lncRNA MEG3 plasmid transfection downregulated the tumorigenic potential in QBC939, GBC-SD, and GBC human cell lines. The tumor size was smaller in 5-week-old male athymic BALB/c mice that were injected with GBC transfected DNA than in mice that received an empty vector injection. pcDNA-lncRNA MEG3 injection in GBC cells increased p53 level and reduced cyclin D1 gene expression. Thus, cell lines with GBC transfected DNA displayed aggregation of cells that arrested in G0-G1 phase have higher Caspase-3 expression level and lower ki-67 expression level. Taken together, these findings suggest that MEG3 plays an initial role in GBC apoptosis induction [140]. The lower level of O_2 in O_2 SGC-996 and GBC-EZ-GB2 cells was correlated with lncRNA LET downregulation. Under hypoxia, EZH2 overexpression reduced the invasive power of GBC cells, whereas LET knockdown increased the invasive potential of GBC cells. LET also inhibited GBC cell proliferation by inducing a G0/G1 pause in hypoxic environments, supporting a close relationship between hypoxia and LET impact [141].

Various types of lncRNA can be used as a biomarker to detect the oncogenic potential of GBC cells [138]. New research by Ma et al. assessed the expression of lncRNA AFAp1-AS1 by RT PCR in 40 gallbladder cancer tissues and their adjacent natural tissues [142]. The results proved that lncRNA AFP1-AS1 expression level was increased in GBC cell lines and also its level was correlated with tumor prognosis and tumor sizes. In the GBC_SD and NOZ cells, AFAP1_AS1 knockdown decreased cell growth, their invasion potential, and inhibited EMT by Twist1 (the transcription factor) downregulation, Vimentin and E-cadherin upregulation [142]. MALAT1 upregulated in GBC tissues compared to normal tissues [143] and its knockdown in NOZ and SGC-996 cell lines lead to inhibit cell line proliferation and metastasis in xenograft BALB/c nude mouse model of human GBC and in vitro. MALAT1 knockdown deactivated the ERK-MAPK pathway and led to reducing the JNK 1/2/3, ERK 1/2, and phosphorylated MEK1/2 proteins level, but MALT1 knockdown did not change their total level. Therefore, MALAT1 is a kind of lncRNA which acts as an oncogenic biomarker that cultivates GBC metastasis and its proliferation [143]. The role of MEG3 and lncRNA ANRIL were studied in GBC pathogenesis [140]. The authors examined 84 GBC patient tissues (GBC tissues and adjuvant natural tissues), using pcDNA-ANRIL and empty vectors, and transfected vectors into QBC939 and GBC-SD cells, respectively. The ANRIL expression was higher in pcDNA-ANRIL transfected cells as well as GBC cells compared to control samples and also ANRIL expression was correlated with GBC prognosis. The results showed that ANRIL expression might improve GBC proliferation and reduce apoptosis [140].

MicroRNAs (miRNAs) are noncoding RNAs that act as posttranscriptional regulators by linking to the 3'UTR of a target messenger-RNA (mRNA) completely or partially. The miRNAs specify the cell function in different situations such as pathological or physiological conditions [144, 145]. Several studies have assessed the roles of miRNAs in the pathogenesis of GBC. miRNAs are upregulated in cancer tissues and neoplastic cells and can also be used as an oncogene biomarker or tumor suppressors [138]. Some miRNAs have suppressive and oncogenic properties in GBC cells, such as miRNA-145 and miRNA-1, which were recently identified in a study that used significance analysis of microarrays (SAM) algorithm to identify the 36 miRNAs; some cellular miRNAs have been demonstrated to have oncosuppressive properties in GBC cells. miRNA-1 and miRNA-145 were analyzed in a study in which a significance analysis of microarrays (SAM) algorithm downregulated in GBC in comparison to normal tissues and the real-time PCR confirmed that miRNA-145 and miRNA-1 downregulated in GBC cells, statistically [112]. Ectopic expression of miRNA-1 and miRNA-145 in NOZ cells reduced colony formation and cell viability. The miRNA-1 downregulated oncogenes expression such as AXL receptor tyrosine kinase and vascular endothelial growth factor A (VEGF-A), thus these miRNAs could be used as tumor suppressors in gallbladder cancer [112, 138]. The results revealed that in cells which were transfected by miRNA-135a-3p mimetic, GBC cell proliferation and colony formation were reduced by G1-S cell phase arrest. Furthermore, miRNA-135a lentivirus-mediated overexpression leads to GBC cell proliferation decrease [146]. The other study revealed that

downregulation of miRNA-335 was associated with poor prognosis in GBC as well as it is correlated with positive lymph node metastasis, aggressive tumor behavior, and also high grade tumor histology [147]. Overexpression of miRNA-145b-5p leads to decreased malignant growth in GBC cells by G1 cell cycle arrest and promoting cell apoptosis. Furthermore, results showed that epidermal growth factor receptor (EGFR) acts as a mediator of the oncologic functions of miRNA-146b-5p in gallbladder cancer [148].

Several miRNAs lead to GBC development. For example, miRNA-20a participates in GBC cell progression and GBC poor survival via effecting the mothers in contrast to decapentaplegic homolog 7 (Smad7)-β-catenin axis [149]. The essential antagonist restored Smad7 expression and resulted in miRNA-20a downregulation in GBC cells in vivo and in vitro, as well as reduced transforming growth factor-β (TGF-β)-induced metastasis [150]. Downregulation of miRNA-182 led to reducing the GBC lung metastasis and its novel target, the cell adhesion molecule-1 (CADM-1) ectopic expression, and decreased tumor invasion [150].

Finally, advanced nanotechnology, which uses different engineered materials with optimal cargos to effectively deliver RNA molecules, has shown promise in the treatment of GBC. Chemotherapy and chloroquine, in conjunction with nanomaterial-induced photothermal therapy, decreased the proliferation of GBC cells, according to Cai and his colleagues [151, 152]. In the future, it will be important to evaluate the efficacy of this novel RNA-based delivery mechanism as an alternative therapy for GBC.

1.7 Conclusion

GBC is a relatively uncommon disorder with a weak prognosis, as well as a distinct regional spread and risk factor profile. Unfortunately, treatment choices for the disease are limited. GBC therapies include cytotoxic chemotherapy, radiation, and surgical resections, both of which have limited survival advantages. Understanding the molecular pathogenesis of GBC will help in the development of tailored therapeutic approaches. The tumor's rarity and the lack of robust fundamental science activities contribute to the difficulties in treating GBC. A major barrier to improved treatment modalities is the absence of freely available GBC animal models. Finally, many GBC patients have advanced disease and low performance status, making them unable to participate in clinical trials. GBC patients would benefit from increased attempts to improve preclinical studies with eventual translation to therapeutic clinical trials with the aim of increasing survival rates, given the high mortality and scarcity of therapeutic alternatives.

Chemoresistance is a significant issue in the care of cancer patients, when cancer cells develop resistance to the chemical compounds used in treatment, limiting the efficacy of chemotherapies. Drug tolerance in cancer is a complicated problem. As a result, for drug-resistant tumors, combined therapy is the smartest choice. After different mechanisms were evaluated in GBC drug tolerance, researchers have found that cancer cells can sensitize to chemotherapeutic drugs by gene therapy

methods such as siRNA/miRNA thus, by gene therapy, a new strategy; the chemotherapy drug resistance suppressed the drug resistance and sensitized the resisted tumoral cells to chemotherapy.

Furthermore, most chemotherapeutic agents interfere with DNA synthesis. There has been an increase in studies over the last decade based on RNA molecules such as noncoding RNAs (e.g., lncRNAs and miRNAs) that regulate oncogene or tumor suppressor expression of genes. Nonetheless, future efforts should focus on pathophysiological functions and translate them to diagnostic or prognostic indicators.

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Part II

Surgical Management, Adjuvant Chemotherapy and Palliative Chemotherapy in Gallbladder Cancer

Surgical Management of Gallbladder Cancer Patients

2

Marie Cappelle, Elise de Savornin Lohman, Philip de Reuver, and Bas Groot Koerkamp

2.1 Introduction

Gallbladder cancer (GBC) is the most prevalent biliary tract malignancy and the sixth most common gastrointestinal malignancy worldwide [1]. The global incidence is declining since the 1960s, which is probably a consequence of increased cholecystectomy rates secondary to gallstones [2]. Survival of GBC is poor, with overall 5-year survival across all stages of around 10% as most patients are diagnosed at an advanced stage [3]. GBC is rare and accounts for 1.2% of all cancers and 1.7% of all cancer mortality, respectively [4]. Best survival rates are obtained if GBC is diagnosed at an early stage and treated with complete (i.e., margin negative) resection.

2.1.1 Epidemiology and Risk Factors

GBC has a remarkable geographic distribution. The highest incidences are noted in Bolivia, Colombia, India, Chili, Eastern Europe (Poland, Hungary, Czech Republic), and among the American Indian, Alaska Native, and Hispanics. The incidence ranges from 12.3/100,000 for males and 27.3/100,000 for females in Chili, compared to 1/100,000 for males and 2/100,000 for females in the United States [4] (Fig. 2.1). The worldwide gender bias with a variable female-to-male incidence ratio of 5:1 is remarkable and attributed to the higher incidence of gallstone disease

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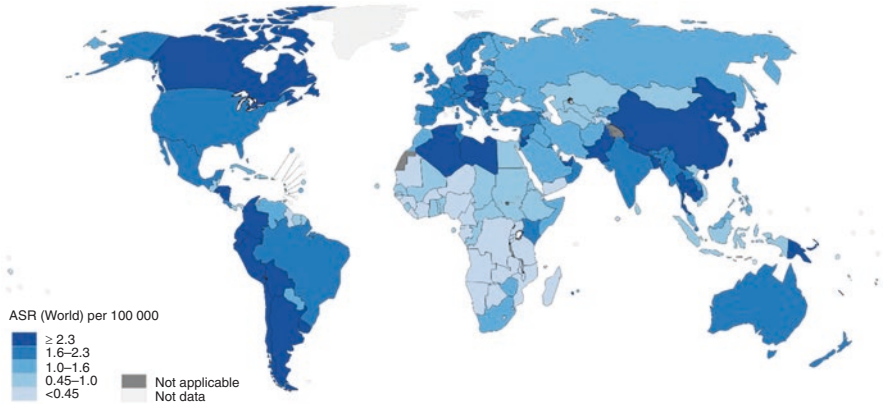

Fig. 2.1 Global incidence of gallbladder cancer. Estimated age-standardized gallbladder cancer incidence rates per 100,000 per year in 2018 for both sexes. (Reprinted by permission from Springer Nature: Gamboa et al. [5])

and presence of the female hormone estrogen [6]. GBC is a disease of advancing age with a mean age of diagnosis in the seventh decade [7].

Cholelithiasis is considered to be the primary risk factor for GBC and is present in 85% of patients [8]. Furthermore, risk of GBC is increased tenfold in patients with larger gallstones (>3 cm) compared to smaller stones [9]. Gallstones provoke chronic mucosal inflammation promoting epithelial dysplasia and adenocarcinoma formation in the gallbladder wall. Nevertheless, only about 1% of patients with cholelithiasis develop GBC [10].

Calcifications in the gallbladder seen on imaging have been considered a risk factor for malignancy. Therefore, a "porcelain" gallbladder is regarded as an indication for cholecystectomy [11]. A review reported a GBC incidence of 21% ($n = 72$) in porcelain gallbladders ($n = 340$) [12]. Though, in a subgroup analysis of these patients ($n = 124$) without selection bias, the incidence of GBC was only 6% compared to 1% in a matched cohort of patients without gallbladder wall calcification. The most recent and largest review confirmed a 6% ($n = 21$) GBC incidence in porcelain gallbladders ($n = 333$) in an overall cohort of 60,781 cholecystectomies [13]. Therefore, prophylactic cholecystectomy should be considered based on symptoms, and a nonoperative approach is justified in those with significant comorbidities. Nevertheless, the pattern of calcification may be predictive of malignancy; it appears that selective mucosal calcification may be predictive of GBC, whereas complete intramural calcification is not associated with GBC [14]. In conclusion, cholecystectomy should be considered particularly in patients with selective mucosal calcification on imaging.

Other factors associated with higher rates of GBC occurrence include obesity, chronic inflammation caused by anomalous pancreaticobiliary ductal junction, primary sclerosing cholangitis, and infection with *Salmonella typhi* or *Helicobacter* species.

2.2 Preoperative Planning

2.2.1 Clinical Presentation

GBC may present in two ways: incidentally (intra- or postoperatively during/after routine cholecystectomy for cholecystolithiasis or cholecystitis) or in symptomatic patients with findings suspicious for malignancy. The majority of GBC patients (60%) is diagnosed incidentally (iGBC), whereas 40% of patients present with symptomatic disease. Symptoms of GBC include right upper quadrant or epigastric pain, jaundice, nausea and vomiting, anorexia, and weight loss [15]. Most symptomatic patients have advanced disease at diagnosis since symptoms often only occur late in the disease course [16]. In a series of 162 patients, only eight patients (5%) with symptomatic disease had a tumor that was limited to the gallbladder wall. All other patients had tumors invading the liver or other organs [17]. There are no sensitive nor specific tumor markers for the diagnosis of GBC. CEA and CA19.9 can be considered at baseline assessment but have no diagnostic value [18].

2.2.2 Staging: Anatomy and Imaging

The American Joint Committee on Cancer (AJCC) published the eighth edition of the AJCC staging manual in 2017 [19]. GBC is staged according to the depth of tumor invasion (T), presence and number of lymph node metastases (N), and presence of distant metastases (M) (Table 2.1, Fig. 2.2).

2.2.2.1 Anatomy

The gallbladder is located in the inferior side between the right and quadrate lobe of the liver. The intraperitoneal part of the gallbladder is covered with peritoneum or serosa, whereas the extraperitoneal part, i.e., the part facing the liver, is covered by a perimuscular connective tissue called the cystic plate. Other organs, such as the stomach, duodenum, pancreas, or transverse colon, might be involved if cancerous cells extend beyond the peritoneal part. The tumor is located in the fundus in 60% of patients, in the body in 30%, and in the neck in 10% [20]. In case of neck involvement, inclusion of the biliary tree is more common because of the close relation to the right hepatic duct and biliary confluence [21]. In 98% of patients, GBC arises in the mucosal layer of the gallbladder. The majority of GBC are adenocarcinomas or their variants (adenosquamous, squamous) [22]. GBC's rare histologic variants include neuro-endocrine tumors, sarcomas, or metastases from other primary tumors such as melanoma. Subtypes have an infiltrative, nodular, or papillary growth pattern. Infiltrative GBC infiltrates the gallbladder in the subserosal plane, followed by invasion of the liver parenchyma and porta hepatis. Nodular GBC consists of circumscript lesions, whereas polypoid lesions characterize papillary GBC. Lymphatic flow from the gallbladder is primarily directed to the cystic duct node and the nodes around the bile duct, secondly to the hepatic vasculature and the posterior side of the pancreas, and finally to the aortocaval nodes near the left renal

Table 2.1 Eighth edition of American Joint Committee on Cancer (AJCC) TNM staging for gallbladder cancer. (Adapted by permission from Oxford University Press: SØreide et al. (2019))

	Description
T-stage	
Tis	Carcinoma in situ
T1a	Tumor limited to the lamina propria
T1b	Invades the muscle layer
T2	Invades the perimuscular connective tissue
T2a	On the peritoneal side
T2b	On the serosal side
T3	Perforates the serosa and/or directly invades the liver and/or other adjacent organs or structures
T4	Invades the main portal vein or hepatic artery or two or more extrahepatic organs or structures[a]
N-stage	
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1–3 regional lymph nodes
N2	Metastasis in 4 or more regional lymph nodes
M-stage	
M0	No distant metastases
M1	Distant metastases present

Stage	Tumor category	Node category	Metastasis category	Overall 5-year survival (%)
0	Tis	N0	M0	80–100
I	T1a/b	N0	M0	80–100
IIA	T2a	N0	M0	40–75
IIB	T2b	N0	M0	40–75
IIIA	T3	N0	M0	8–28
IIIB	T1–3	N1	M0	8
IVA	T4	N0–1	M0	7
IVB	Any T	N2	M0	4
	Any T	Any N	M1	0–2

[a] Extrahepatic organs or structures include the stomach, duodenum, colon, pancreas, omentum, and extrahepatic ducts

vein, and coeliac lymph nodes (LNs) (Fig. 2.3) [23]. Involvement of LNs beyond the hepatoduodenal ligament (i.e., aortocaval and/or coeliac LNs) is considered metastatic disease [19]. Distant spread takes place mainly through hematogenous dissemination, either directly or through invasion of the liver parenchyma [24].

2.2.2.2 Imaging

Imaging plays a vital role in detecting, staging, surgical planning, and evaluation of treatment in GBC. Imaging may show a focal or diffuse gallbladder wall thickening, an intraluminal mass, or a mass in the gallbladder fossa.

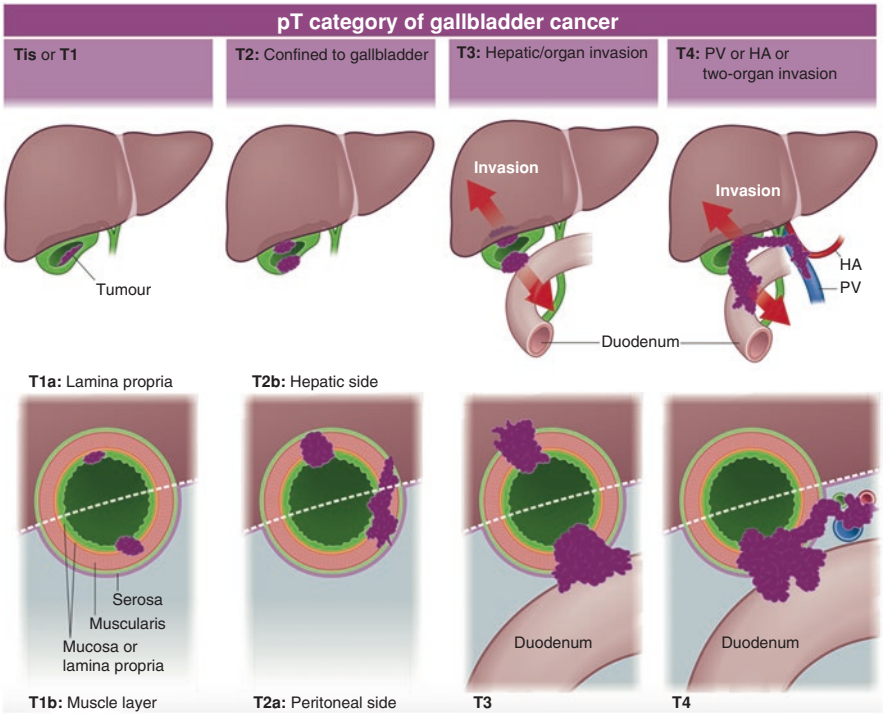

Fig. 2.2 Illustration of pT categories of the TNM system for gallbladder cancer. (Reprinted by permission from Oxford University Press: SØreide et al. (2019))

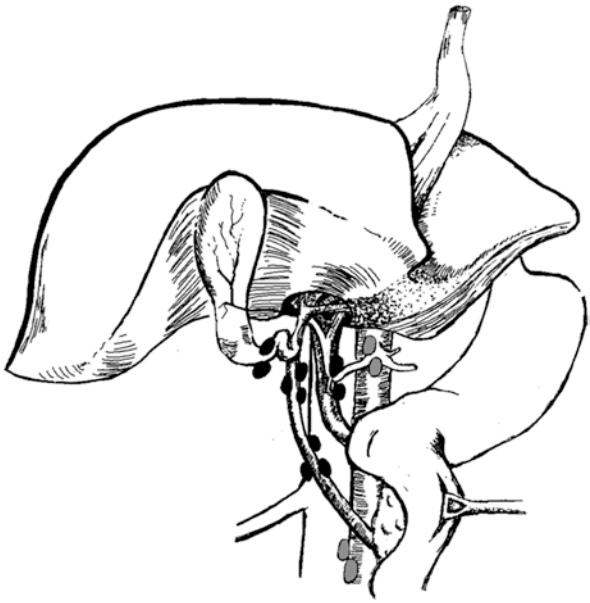

Fig. 2.3 Illustration of lymphatic nodes typically involved in patients with gallbladder cancer. Black labeled lymph nodes are considered loco-regional, gray labeled lymph nodes as metastatic. (Courtesy of Gavin Chekpui Lo (MD))

Ultrasonography

The primary modality by which GBC is detected is usually ultrasonography (US), as it is the initial imaging modality for evaluation of patients with abdominal pain or jaundice and has a high sensitivity to detect gallstones and gallbladder masses [16]. However, regular greyscale ultrasonography is limited in detecting early GBC, especially when attempting to differentiate GBC from gallbladder wall thickening due to cholecystitis [25, 26]. Evaluation of depth of invasion appears better in novel ultrasonography techniques such as endoscopic ultrasonography (EUS) and high-resolution ultrasonography (HRUS) [27–30]. Computed tomography (CT), however, has a similar sensitivity to detect malignant gallbladder lesions and is superior in detecting suspicious LNs or distant metastatic disease [27, 31]. The use of HRUS and EUS is therefore limited.

Computed Tomography (CT)

CT is the primary staging modality for GBC. Its sensitivity and diagnostic accuracy to detect malignant gallbladder wall thickening are 90% and 92%, respectively [32–34]. Since CT is a cross-sectional study, it may be better suited to detect subtle variations of the gallbladder wall which are not visible on US. Moreover, CT is less operator dependent than US. The diagnostic accuracy of CT for the assessment of T-stage is about 85%, with 100% sensitivity for discrimination for T4 lesions, and 79% for the discrimination between T1 and T2 lesions. Nevertheless, overstaging by CT is not a rare occurrence. In one of the included studies (Kalra et al.), 12 of 20 patients were deemed resectable by CT, whereas during explorative laparotomy only 11 of 20 patients underwent definitive resection [35]. Overstaging in this particular patient was primarily caused by duodenal infiltration on CT, which was not present during surgery. Understaging on CT is primarily caused by the low sensitivity of CT for peritoneal (30%) and distant LN metastases (20%) [35].

Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) has a higher soft-tissue contrast resolution compared to CT MRI with gadolinium-enhanced contrast can be helpful to differentiate between chronic cholecystitis and malignant gallbladder wall thickening, which is challenging using other imaging modalities [36, 37]. In a cohort of patients with PSC, MRI showed a 100% sensitivity for malignancy in gallbladder lesions of over 0.8 cm in size [38]. Precise assessment of the local extent of disease (i.e., involvement of adjacent liver, bile duct invasion, LN invasion, and vascular invasion) is important because it determines the resectability and extent of resection. Magnetic resonance cholangiopancreatography (MRCP) is a noninvasive technique providing projection images of the biliary tree. MRI combined with MRCP and MR-angiography (MRA) is superior to CT for assessing the local extent of disease with a sensitivity of 100% for liver and bile duct invasion and 92% for loco-regional LN involvement [39–41]. MRI with MRCP and MRA as part of preoperative staging should be considered in any patient with suspected GBC. It may affect clinical decision-making as it augments the diagnostic accuracy of CT.

Positron-Emission Tomography

CT and MRI have a low sensitivity for distant LN and peritoneal metastases [40, 42, 43]. Positron-emission tomography (PET) detects high glucose uptake of tumor cells and is combined with standard CT image. The sensitivity of PET-CT to detect distant and LN metastases is 85–100% and 67–71%, respectively, and it may alter management in 15–23% of preoperatively diagnosed GBC patients [44–47]. One study showed that the yield of PET-CT is lower in patients with iGBC, changing management in only 13% of patients [47]. This is probably caused by the earlier stage of iGBC compared to symptomatic GBC. Another study, in which PET-CT was conducted in patients with ≥pT1b disease before re-resection, showed that PET-CT changed the clinical stage in 38% of patients [48]. PET-CT detected in 50% of patients with pNx disease distant nodal and/or metastatic disease and in 30% of patients with pN1 disease. In summary, PET-CT can be a useful tool in patients who have a high risk for distant disease (i.e., cT3 stage or higher) or in patients with iGBC with positive or suspicious LNs.

2.2.3 Histopathological Diagnosis

Histopathological confirmation of GBC is not needed prior to surgery for patients who have potentially resectable disease on imaging and are operable. Nevertheless, if patients are eligible for palliative systemic chemotherapy, pathological confirmation is required. This is typically obtained with percutaneous biopsy of lesions that are suspicious for metastatic disease, or of the primary tumor in patients without metastatic disease for whom a resection is not considered. If GBC patients present with obstructive jaundice, endoscopic retrograde cholangiopancreatography (ERCP) can be used for the drainage procedure, and a brush cytology or biopsy can in the meantime be performed. One study investigated the role of EUS-guided fine needle aspiration (EUS-FNA) in 101 patients with gallbladder masses and biliary obstruction [49]. EUS-FNA confirmed malignancy in 89 out of 98 patients with GBC; sensitivity was 90.8% and the negative predictive value (NPV) was 10%. These outcomes reflect that EUS-FNA is a sensitive tool in this clinical setting. EUS-FNA can also aid in staging by sampling LNs beyond the hepatoduodenal ligament, in particular, aortocaval and coeliac LNs.

2.2.4 Staging Laparoscopy

Four studies investigated the role of staging laparoscopy (SL) in patients with GBC; three studies in patients with preoperatively diagnosed GBC [50–52], and one in patients with iGBC [53]. The yield of laparoscopic staging in preoperatively diagnosed GBC is about 23% [52]. Agarwal et al. showed that the benefit of laparoscopic staging was higher in patients with advanced (T3/T4, yield 25.2%) compared to early (T1/T2, yield 10.7%) GBC. The study in patients with iGBC demonstrated a yield of only 4.3%, which might be biased due to a low rate of staging laparoscopy

(46/136 patients, 33.8%) but also due to low prevalence of advanced disease [53]. However, the risk of disseminated disease was closely correlated to T-stage, with up to 26% of T3 patients having disseminated disease. Additionally, patients with a positive resection margin at index cholecystectomy, i.e., margin <1 mm and tumoral involvement of at least one resected LN, were five times more likely to show disseminated disease at re-exploration. In summary, staging laparoscopy should be strongly considered in all patients with suspected locally advanced disease (i.e., T3/4 or N1) on preoperative imaging, and in all iGBC patients with T3 disease or positive (cystic duct) margins.

2.3 Management of Stage I-III GBC

Treatment of stage I and II disease is surgical (Tables 2.1 and 2.2). Patients with stage III and IVa GBC have nonmetastatic locally advanced disease, and resection is only performed in selected patients with good performance status after multidisciplinary consideration [16]. Table 2.2 represents the recommended T stage-adjusted resection in GBC. Stage IVB disease is considered as disseminated disease and can be managed with palliative chemotherapy. The treatment for stage IV disease is discussed in Sect. 2.4 "Management of stage IV GBC."

2.3.1 T1a Disease

The majority of T1a gallbladder tumors is diagnosed after laparoscopic cholecystectomy (LC) for presumed benign gallbladder disease. In T1a GBC the tumor is limited to the lamina propria and is consequently considered as local disease (Table 2.1, Fig. 2.2). This is supported by the fact that prevalence of LN metastases in patients with T1a GBC is less than 2% and 5-year survival after LC is reported to approach 100% [54–57]. A systematic review including 706 patients with T1a GBC showed no significant differences in survival between patients that underwent

Table 2.2 T-stage-adjusted resection in gallbladder cancer

T stage	Recommendation
Tis/T1a	Simple cholecystectomy
T1b-T2	Extended cholecystectomy with regional lymphadenectomy: Cholecystectomy with nonanatomical 2-cm wedge resection of segments 4b and 5 and lymphadenectomy of the hepatoduodenal ligament
T3	Extended cholecystectomy as for T1b-T2, but GBC in the gallbladder neck or cystic duct may require right hepatectomy extended to segment 4b and/or bile duct resection with hepaticojejunostomy to obtain clear margins. Moreover, depending on involved organ: Wedge resection of duodenum or transverse colon. Only in patients with good performance status
T4	As for T3, palliative care if involvement of main portal vein or proper hepatic artery. Most patients in this category are unlikely to benefit from resection even if technically feasible. Only in patients with good performance status

simple versus extended cholecystectomy (EC), i.e., cholecystectomy with nonanatomical 2-cm wedge resection of segments 4b and 5 and lymphadenectomy of the hepatoduodenal ligament [55]. Therefore, the consensus is that a simple cholecystectomy suffices for the treatment of T1a GBC.

2.3.2 T1b Disease

Like T1a GBC, T1b GBC is typically diagnosed after LC for benign indications and is generally classified as early GBC. However, some argue that T1b GBC should be considered regional disease. There have been reports of loco-regional spread at presentation and LN metastases found in approximately 0–10% of T1b GBC patients [55, 58, 59]. Several retrospective cohort studies comparing survival after simple versus EC, i.e., cholecystectomy with nonanatomical 2-cm wedge resection of segments 4b and 5 and lymphadenectomy of the hepatoduodenal ligament, have been performed with conflicting outcomes [57–63]. Of three recently performed meta-analyses, two do not show prolonged survival after EC compared to simple cholecystectomy [57, 59]. The third meta-analysis did show favorable survival outcomes after EC (OR 2.75, 95% CI 1.13–6.69; $p = 0.03$). However, the authors considered the grade of evidence to be low as most included studies had serious limitations [64]. Nonetheless, several guidelines, including the National Comprehensive Cancer Network (NCCN) guideline, support EC as first-line treatment for T1b GBC [16, 18, 65]. The procedure is described in Sect. 2.5.2 "GBC suspicion before surgery."

2.3.3 T2/T3 Disease with or Without Lymphadenopathy

The standard of care treatment of T2 and T3 GBC is an EC, i.e., cholecystectomy with nonanatomical 2-cm wedge resection of segments 4b and 5 and lymphadenectomy of the hepatoduodenal ligament [16, 64, 66]. Though, currently, no consensus exists on the extent of liver resection. A 2010 study found superior survival in T2 and T3 GBC after anatomical segmentectomy of 4b and 5 versus nonanatomical 2-cm wedge resection [67]. However, another study of 485 T2/T3 patients with R0 resection reported no difference in survival between patients undergoing a nonanatomical 2-cm wedge resection compared to either anatomical segment 4b and 5 resection or extended right hepatectomy [68]. A similar study in patients with T2 disease showed a higher rate of postoperative complications after anatomical segment 4b and 5 resection compared to a nonanatomical 2-cm wedge resection, without significant survival differences between both groups [69]. Finally, a study in 16 patients with T3 disease showed no difference in survival in patients who underwent formal hepatectomy compared to a nonanatomical 2-cm wedge resection alone [70]. In summary, anatomical segmentectomy does not provide a survival benefit compared to a nonanatomical 2-cm wedge resection. More extended liver resections should only be performed if required to achieve R0 resection margins. For example, in GBC in the neck or cystic duct, the right hepatic artery might be involved necessitating a formal right hemihepatectomy.

2.3.4 Survival and Prognostic Factors After (Re-)resection

Overall, 5-year survival of GBC is estimated to be 80–100% in stage 0 and I disease, 40–75% in stage II disease, 8–28% in stage IIIA disease, 8% in stage IIIB disease, and 0–8% in stage IV disease (Table 2.1) [4, 16]. Estimated 5-year overall survival (OS) after potentially curative resection is 21% with occurrence rate of at least 50%. Although pT-stage, pN-stage, and positive resection margin are major prognostic factors, additional independent prognostic factors can further improve the prediction of survival after resection [71]. These prognostic factors include serum CA 19.9 levels, vascular invasion, perineural invasion, and differentiation grade [72–75]. Other prognostic factors include intraoperative bile spillage at index cholecystectomy and jaundice at presentation. Blakely et al. showed in a small subset of GBC patients ($n = 12$) that intraoperative bile spillage is associated with decreased progression-free survival (HR 5.5, 95% CI 2.63–32.3, $p = 0.0014$) [76]. Also, in a population-based study in Canada with 82 GBC patients, peritoneal carcinomatosis occurred more frequently in cases with bile spillage at the index cholecystectomy (24% vs. 4%, $p < 0.01$) [77]. These patients were also less likely to undergo complete re-resection (25% vs. 56%, $p < 0.01$) and to achieve R0 resection (OR 0.19, 95% CI 0.06–0.55). Therefore, bile spillage should be avoided at any time when GBC is suspected. Jaundice at presentation in GBC reflects T3/T4 disease and is associated with poor survival as well. Regardless of the implemented treatment, a median survival of 6 months was observed in jaundiced GBC patients with no survivors beyond 2 years after diagnosis [78].

The benefit of survival of re-resection is mainly determined by the presence and location of residual disease (RD) [58, 79]. In a group of 36 pT2 and pT3 iGBC, OS after re-resection was significantly worse if RD was present in the EBD and/or distant sites (5-year OS: 14.3%) compared to no RD (5-year OS: 88.7%) or RD in the gallbladder bed, stump of cystic duct and/or regional LNs (5-year OS: 55.6%) [80]. Also, Ramos et al. observed no improved OS of patients with regional or distant RD [80]. Therefore, creating a model to predict RD in iGBC might lead to better selection of patients that most likely benefit from surgery. Ethun et al. published a pathology-based GBC Risk Score, and also Creasy et al. developed a model to stratify high-risk patients [74, 81]. It seems that benefit in survival of re-resection is especially observed in pT2 and pT3 iGBC. However, in pT2 patients, it remains unclear whether the increase in survival in patients who received re-resection is a result of the procedure itself or whether the apparent survival benefit is attributable to the upstaging of these patients.

2.4 Management of Stage IV GBC

In T4 GBC disease invasion of the main portal vein or hepatic artery or two or more extrahepatic organs or structures is present. It remains unclear whether locally advanced invasion into the porta hepatis, duodenum, or pancreas necessitating extended surgery such as hepatopancreatoduodenectomy should be considered as

resectable disease, which also accounts for vascular reconstructions. Select case series from high-volume expert centers have demonstrated the feasibility to achieve R0 resection [82–84]. However, these extended resections paired with high morbidity and mortality rates are generally not accepted. Moreover, R0 resections are only achieved in a subset of cases, and even then, over 50% of patients will suffer from a recurrence. In more than 90% of patients, GBC eventually metastasizes to the liver and extra-regional LNs. Other sites of metastatic spread are the lung, bones, and brain [85, 86]. If distant metastases are found at staging for GBC, a resection does not prolong survival [16]. Liver transplantation is not a viable treatment option for GBC due to the high risk of early distant disease, which is not resolved with a new liver [87–89]. No survival benefit by surgery is expected in patients with coeliac or aortocaval LN metastases [16, 43].

In summary, extended resection should only be considered by highly specialized teams in extremely fit patients. Even then, outcomes are poor, and risk of recurrence remains high.

2.5 Surgical Procedures for GBC

The surgeon can encounter GBC in the following two scenarios: incidentally (intra- or postoperatively during/after routine cholecystectomy) or in symptomatic patients with findings suspicious for malignancy on imaging. The majority of patients (60%) is diagnosed incidentally, whereas 40% of patients present with symptomatic disease [90]. According to the situation a different approach by the surgeon is required.

2.5.1 GBC Suspicion During Routine Cholecystectomy

GBC is found at pathological evaluation in about 1% of all laparoscopic cholecystectomies performed for cholelithiasis [91–93]. Gross intra-operative examination and opening the specimen to inspect the mucosa have a detection rate of 92% for iGBC [94]. If neoplasia at laparoscopy is suspected (e.g., due to the presence of a mass), the surgeon should strongly consider to not remove the gallbladder and first perform staging for GBC. Moreover, referral to a specialized hepatobiliary center is needed. The drawback of proceeding with surgery is that the resection may be futile (i.e., in patients with distant metastases). Also, a simple cholecystectomy may result in tumor spill and a R2 resection, while an EC may not be required. If abnormal mucosa is macroscopically noticed after the cholecystectomy, the gallbladder must be sent for frozen-section analysis, and definitive resection (i.e., nonanatomical 2-cm wedge resection of segment 4b and 5 with regional lymphadenectomy) may be undertaken during the same surgical procedure if a hepatobiliary surgeon is available.

In case of concomitant cholecystitis and high suspicion for GBC, it may be recommended to directly perform an EC. In the absence of GBC expertise, it is oncologically safe to abort the procedure and refer the patient to a tertiary center for

further evaluation [17, 95, 96]. EC might not be required, but will decrease the chances of gallbladder perforation and associated risk of tumor spill and peritoneal seeding, as stated in Sect. 2.3.4 "Survival and prognostic factors after resection".

In conclusion, in case GBC is suspected during routine cholecystectomy, it is recommended to refer the patient to a specialized hepatobiliary center and first perform staging. In the presence of concomitant cholecystitis, it is recommended to perform an EC to avoid risk of intra-operative bile spillage.

2.5.1.1 Approach

Historically, a laparoscopic approach for GBC in general has been contraindicated due to concerns about increased risk of port site recurrences, peritoneal metastases due to bile spillage, and nonradical resection [97]. These risks have subsided due to improved recognition of GBC intraoperatively, improvements in laparoscopic skills of hepatobiliary surgeons, and the use of a retrieval bag [98]. Studies found that an initial laparoscopic approach does not influence the course of early-stage GBC if definitive resection during or after LC is performed [99, 100]. A recent meta-analysis by Zhao et al. showed a higher 5-year survival rate in patients who underwent laparoscopic compared to open surgery, though bias may have been present since the laparoscopic approach was more often used in earlier tumor stages [99].

2.5.2 GBC Suspicion Before Surgery

After a complete workup, stage-adjusted resection is scheduled (Table 2.1). Staging laparoscopy is strongly recommended in all patients, particularly in patients with suspected T3/T4 disease or positive resection margin in iGBC [49–51, 101]. If peritoneal or hepatic metastases are found, resection is futile [102]. Both open and minimal-invasive approaches are options for curative-intent resection of GBC. A minimal-invasive approach, however, is only recommended in expert centers [98].

2.5.2.1 Open Approach

In an open approach, adequate exposure can be obtained through a right subcostal incision (Kocher) with or without extension to the left (Chevron) with installation of retractors (e.g., OmniTract, Thompson, or Rochard). The teres ligament is ligated and retracted cranially to expose the undersurface of the liver and the hepatoduodenal ligament. Re-inspection for undetected disseminated disease should be performed because staging laparoscopy may have missed occult metastatic disease. Intraoperative ultrasound can be used to evaluate depth of invasion, location of the primary tumor in relation to vascular structures, and rule-out liver metastases [103].

2.5.2.2 Lymphadenectomy

Assessment of the distant nodal stations is performed to rule out stage IV disease because of extra-regional positive LNs [104]. A Kocher maneuver is executed to assess for aortocaval nodes. Frozen-section analysis of aortocaval nodes prevents a futile resection in 18.6% of GBC patients [105]. Coeliac LNs are also extra-regional

and should be sent for frozen section as well. The LN dissection starts posterior to the head of the pancreas and duodenum, also exposing the vena cava, aorta, and retroportal region. At the cranial border of the pancreas, the common hepatic artery is exposed, and dissection continues toward the celiac arteries. The gastroduodenal branch is preserved, but the right gastric artery is transected to facilitate LN retrieval. The portal vein, common hepatic artery, and common bile duct are freed up from surrounding lymphatic tissue. Regional lymphadenectomy of the hepatoduodenal ligament may be sent for pathological examination as a single specimen, but frequently the lymphadenectomy involves several separately resected LNs. A minimum of six LNs of the hepatoduodenal ligament should be harvested for adequate staging [16]. All lymphatic vessels should be tied to prevent postoperative chyle leak.

2.5.2.3 Assessment of Main Portal Vein and Common Hepatic Artery

Involvement of the main portal vein and common hepatic artery is evaluated. If either structure is affected, or if more than one extrahepatic organ is involved, the tumor is classified as T4 GBC and a resection is futile for almost all patients. Inclusion of the main portal vein and common hepatic artery, however, is unlikely in the absence of jaundice and can be mostly ruled out on preoperative imaging. The cystic artery and duct are divided flush with the right hepatic artery and the common bile duct if no signs of tumor involvement are present, and frozen-section analysis of the cystic duct resection margin is performed.

2.5.2.4 Assessment of the Extrahepatic Biliary Tree

If the cystic duct margin is positive or if the tumor directly invades the common bile duct, extrahepatic bile duct (EBD) resection is required to obtain R0 resection. Involvement of the bile duct is most likely in patients with a tumor in the neck of the gallbladder or in the cystic duct, or when jaundice was present at diagnosis. Jaundice in GBC is a sign of advanced disease with tumor involvement of the EBD. Though, Varma et al. reported 50% R0 resection in jaundiced GBC patients [106]. Tran et al. did observe in 108 patients presenting with jaundice in GBC a higher perioperative morbidity (69% vs. 38%, $p = 0.002$) but no higher 90-day mortality (6.5% vs. 3.6%, $p = 0.35$) compared to nonjaundiced patients who underwent curative-intent surgery. Japanese guidelines recommend preoperative biliary drainage in all jaundiced GBC patients, but there is no consensus regarding the approach and duration for drainage, nor the target bilirubin level. In conclusion, the presence of jaundice reflects T3/4 disease and is a poor prognostic factor [107]. Therefore, resection in GBC patients with jaundice at presentation, should only be considered in selected cases.

Routine EBD resection in nonjaundiced GBC patients to avoid isolated bile duct recurrences is not recommended. In a series of 26 nonjaundiced GBC patients who underwent a radical resection without EBD resection, no isolated recurrences at the EBD were found [108]. Moreover, EBD resection does not result in more harvested LNs [109]. The associated morbidity of an EBD resection has to be taken into account as well. D'Angelica et al. reported that 33% of patients had a complication requiring re-intervention or resulted in permanent disability or death, versus 13% of

patients who had no EBD resection [110]. Thus, only in highly selected patients, the common bile duct is divided as distal as possible posterior to the pancreatic head. The resection margin must be examined by frozen-section analysis. A 70 cm Roux-en-Y jejunal loop with jejuno-jejunostomy is prepared and positioned via a retrocolic route. The hepaticojejunostomy can be performed using running or separate sutures. The mesogap is closed with running or separate 3–0 Vicryl or PDS sutures.

2.5.2.5 Assessment of Right Portal Vein and Right Hepatic Artery

If the right hepatic artery and/or right portal vein are involved, a right hemihepatectomy is required to achieve R0 resection. This should only be performed in highly selected patients and is typically suspected based on preoperative imaging. In order to adequately assess portal vein invasion, the liver is split along the umbilical fissure. R0 resection is possible if the tumor does not invade the left portal vein or left hepatic artery, obviously in a patient with a good performance status and an adequate liver remnant. Aberrant vascular and biliary anatomy should be noted on preoperative imaging. The left hepatic duct is transected and right hepatic artery ligated. Vascular clamps are placed on the main and left portal vein to transect the right portal vein. Transection is executed and depending on the extent of invasion of the right portal vein, either closure of the portal vein stump or a primary end-to-end anastomosis between the main and left portal vein is accomplished with a running, nonabsorbable suture (Prolene™ 5–0). Then, right hemihepatectomy with *en-bloc* resection of the gallbladder is completed preserving the middle hepatic vein. In-flow occlusion might be obtained by applying the Pringle maneuver in an intermittent or continuous fashion, and central venous pressure is kept below 5 mm Hg. The right liver lobe is mobilized by dividing the surrounding ligaments and ligating the short hepatic veins into the cava. Subsequently, the right hepatic vein is identified. The right hepatic vein is then transected, either by vascular stapler or suture ligature. Two traction sutures are placed at the inferior margin of the liver, one at each side of the demarcation line, and transection of the liver parenchyma is initiated. Superficial incision of the parenchyma takes place with diathermy, and further dissection can be performed with Kelly clamping or using an energy device, i.e., Thunderbeat® (Olympus Medical Systems Corp., Tokyo, Japan) or Enseal® (SurgRx Inc., Redwood City, CA, USA). During parenchymal transection, optimal exposure is obtained by either holding the right hemiliver with the left hand or performing a hanging maneuver, i.e., passing a tape between the anterior surface of the inferior vena cava and the liver. Hemostasis and biliostasis is verified with gauzes, and potential leaks should be suture-ligated. In order to prevent rotation of the left hemiliver, the falciform ligament is reattached. Abdominal drainage can be considered if a hepaticojejunostomy was performed or if a percutaneous transhepatic stent has been removed.

2.5.2.6 Nonanatomical or Anatomical Segment 4b and 5 Resection

If the tumor does not invade the porta hepatis or liver parenchyma, the gallbladder is removed with a 2-cm nonanatomical wedge of the adjacent liver parenchyma using an energy device as aforementioned. The primary aim of liver resection in

patients with GBC is to achieve a negative resection margin on the hepatic side. Therefore, liver resection should be performed according to the extent of liver parenchyma invasion and might be more extensive. Intraoperative ultrasound can be useful to delineate the extent of the tumor [111]. The transection line is marked on the liver capsule with electrocautery. Traction sutures can be placed adjacent to the demarcation line at the inferior margin of the liver. Parenchymal transection is performed with Kelly clamping or an energy device, and vessels are ligated or clipped. A vascular stapler can be used to control large intrahepatic vessels. Transection can also be performed along the anatomical border of segment 4b and 5. Then, transection begins medially, encountering the middle vein first and then the segment 5 pedicle. The main anterior pedicle and pedicle adjacent to segment 8 are at risk for inadvertent injury during parenchymal transection. The gallbladder is removed en-bloc. Hemostatic agents can be used according to the surgeons' preference (i.e., TachoSil®, Surgicel®). Abdominal drainage is not needed [112].

2.5.2.7 Laparoscopic and Robotic Approach

In a laparoscopic approach, the same principles as in open surgery are respected. Technical feasibility and safety of laparoscopic wedge resection, anatomical segment 4b and 5 resection, hepatoduodenal lymphadenectomy, and EBD resection have been reported but should only be carried out in expert centers [113–115]. These procedures require an expert advanced laparoscopic surgical team that will still have a long learning curve. For a systematic description of laparoscopic approach in GBC, we refer to a recent review of Vega et al. [116]. Recently, robotic approach for extended resections in GBC has also been described and considered safe and feasible [117, 118]. The surgical technique used in the robotic approach is depicted by Goel et al. [117]. The main advantage of robotic approach is the shorter learning curve.

2.5.3 GBC Diagnosed at Histopathological Analysis After Routine Cholecystectomy

If histopathology results are consistent with GBC, appropriate workup as described in Sect. 2.2.2 "Staging: anatomy and imaging" is warranted. In addition, review of initial imaging results, the operative note, and the histopathology report of the performed cholecystectomy is mandatory. A re-resection is recommended for patients with T1b, T2, or T3 iGBC in the absence of metastatic disease and/or poor performance status [16].

2.5.3.1 Re-resection: Timing and Open Versus Laparoscopic Approach

Re-resection is considered more technically challenging than primary resection as adhesions from the index surgery are expected. Optimal timing for re-resection considering these technical aspects and tumor biology is between 4 and 8 weeks [119]. Data on outcomes of laparoscopic re-resection has only been reported by expert

centers [98]. One retrospective study did not detect survival differences between patients undergoing an open or laparoscopic re-resection [120].

2.5.3.2 Extent of Re-resection

The aim of re-resection is twofold; to remove residual cancer and to perform adequate staging. Re-resection consists of either an open or laparoscopic nonanatomical 2-cm wedge resection of segments 4b and 5 and a lymphadenectomy with a minimum count of six LNs [16]. Rarely, more extensive procedures such as major liver resection, vascular resection, or common bile duct or adjacent organ resection are required to obtain negative resection margins. The same surgical principles apply for re-resection as for primary resection as described in Sect. 2.5.2 "GBC suspicion before surgery" with the exception that the gallbladder already has been removed.

2.5.3.3 Port-Site Resection

Historically, port-sites resection at the time of re-resection for iGBC was recommended because of the high rate of wound recurrences. However, recent evidence shows that excision of port-sites is not correlated with improved overall or recurrence-free survival and causes a 10% rate of incisional herniation [121, 122]. Moreover, port-site resection is a disfiguring operation. If pathological examination of the specimen is positive, the patient has peritoneal metastasis. However, ESMO guidelines recommend resection of port-sites when the gallbladder was perforated at the index cholecystectomy or was not removed using a retrieval bag [123].

2.6 Postoperative Management

Postoperative care should be adjusted to the extent of surgery, with initial surveillance in an intensive care unit after major hepatectomy with bile duct reconstruction. Hemoglobin, coagulation parameters, liver function, and electrolytes should be monitored. Standard care includes adequate pain control, early ambulation, thrombosis prophylaxis, adequate fluid management, and early enteral diet to avoid general surgical complications such as pneumonia, deep vein thrombosis, pulmonary embolism, and pleural effusion.

2.6.1 Complications

Complications specific to liver resection include postoperative hemorrhage, bile leak with biloma formation, and liver failure. Posthepatectomy bleeding occurs in 1–8% of patients and management may be conservative (i.e., blood transfusion) or invasive (i.e., embolization or relaparotomy) depending on severity [124]. Parenchymal bile leaks are mostly self-limiting with percutaneous drainage, although in more severe cases endoscopic sphincterotomy and/or stent placement may be required. Injuries to the right anterior bile duct, segment 8 bile duct, or

extrahepatic bile ducts more likely require endoscopic and/or surgical management. Awareness for the risk of posthepatectomy liver failure in case of major liver resections is important, particularly in jaundiced patients [125].

2.6.2 Postoperative Surveillance

No high-quality studies regarding optimal postoperative surveillance strategies have been conducted. However, the general consensus is that surveillance should consist of physical examination, laboratory testing, and/or CT scan of the thorax and abdomen once every 3 months for the first 2 years, every 6 months for the next 3 years, and annually thereafter [123].

2.6.3 Adjuvant Therapy

At least 50% of patients with resected GBC will suffer from a recurrence [16, 126]. After a potential curative resection the median time to recurrence is only 12 months. 85% develop a distant recurrence without a concomitant loco-regional recurrence, and 15% has a loco-regional recurrence without distant recurrence [127]. In other cancers, adjuvant chemotherapy has shown to increase survival by increasing local control and decreasing distant disease. However, adequately powered trials investigating the value of adjuvant chemotherapy in GBC alone have not been performed. In the past decade, multiple RCTs have investigated the value of adjuvant chemotherapy in all patients with biliary tract cancer (BTC). The BILCAP trial compared adjuvant capecitabine to observation alone in all patients with resected BTC and did not find a significant difference in survival in the primary, intention-to-treat analysis; median overall survival was 51 months in the capecitabine group compared with 36 months in the observation group (HR 0.81, 95% CI 0.63–1.04; $p = 0.097$) [128]. In the per-protocol analysis, a survival benefit of 17 months was found (HR 0.75, 95% CI0.58–0.97, $p = 0.028$). No subgroup analysis, including only GBC patients was conducted.

2.7 Palliative Therapy

The plurality of patients with GBC has noncurable disease due to presentation at an advanced stage of disease or due to recurrence after curative-intent resection. In the palliative setting, obstructive jaundice develops in about half of the patients requiring adequate biliary drainage for symptomatic relief and/or initiation of chemotherapy [129]. Careful patient selection is mandatory for palliative chemotherapy [130, 131]. Endoscopic or percutaneous stenting is preferred to obtain biliary decompression. Saluja et al. performed an RCT comparing palliation of obstructive jaundice by endoscopic versus percutaneous drainage in 44 GBC patients with hilar biliary obstruction [132]. Compared to endoscopic drainage, patients who

underwent percutaneous drainage had a higher rate of relief of obstruction (89% vs. 41%, $p < 0.001$), lower rates of cholangitis (11% vs. 48%, $p = 0.002$), and similar quality of life. However, in both the drainage approaches, procedure-related deaths were reported; 4% in the percutaneous group versus 8% in the endoscopic group. Gastric outlet obstruction might occur due to duodenal compression or infiltration and may be resolved by surgical bypass in selected patients. Nevertheless, endoscopic stenting, decompressive gastrostomy, and endoscopic-guided gastroenterostomy are preferred in most patients [133].

2.8 Conclusion and Future Perspectives

The outcomes of GBC patients across all stages remain poor. Early detection, adherence to guidelines, referral to a hepatobiliary center with GBC expertise, better patient selection for surgery, fine-tuning the extent of surgery, reducing morbidity and mortality of surgery, and more effective systemic treatment options can improve the prognosis of GBC.

Given the rarity and heterogeneity of GBC, development of randomized controlled trials regarding surgical treatment is challenging. Trials investigating the value of (neo-) adjuvant chemotherapy are ongoing and targeted therapy may be the next step to improve treatment. Recent studies show that GBC patients carry several actionable genomic alterations for which targeted therapies are readily available and the first outcomes seem promising [134–136]. In conclusion, a multidisciplinary approach appears vital to further improve prospects of GBC patients.

Key Points

- GBC is the most prevalent biliary tract malignancy and remains highly lethal.
- The surgeon may be confronted with GBC in two scenarios: incidentally (intra- or postoperatively during/after cholecystectomy for cholelithiasis or cholecystitis), or in symptomatic patients with findings suspicious for malignancy on imaging.
- Imaging work-up of patients suspect for GBC includes at minimum local staging and assessment of potential distant metastases by CT. MRI and PET-CT may be considered in a more advanced stage.
- Histopathological confirmation is not required before planning surgery in patients with imaging findings suspect of GBC.
- Staging laparoscopy should be strongly considered in all patients with suspected locally advanced disease (i.e., T3/4 or N1) on preoperative imaging, and in all incidental GBC patients with T3 disease or positive (cystic duct) margins.
- Overall survival is mainly determined by tumor stage, lymph node status, and resection margin. Estimated 5-year overall survival after potentially curative resection is 21% with a recurrence rate of at least 50%.
- The presence of jaundice in GBC patients is a poor prognostic factor. Potential curative-intent surgery should only be considered in selected cases.

- If GBC is suspected during routine cholecystectomy and no expertise in GBC is available, it is recommended to abort the procedure and refer the patient to a specialized center for appropriate staging.
- If GBC is suspected during routine cholecystectomy with concomitant cholecystitis, a cholecystectomy with nonanatomical 2-cm wedge resection of segments 4b and 5 might be recommended to avoid the risk of intra-operative bile spillage.
- The principal aim of surgical resection is attainment of negative margins.
- A simple cholecystectomy suffices for the treatment of T1a GBC.
- Resection for GBC with invasion in or beyond the muscular layer includes a cholecystectomy with nonanatomical 2-cm wedge resection of segments 4b and 5 and lymphadenectomy of the hepatoduodenal ligament (minimum of 6 LNs). If more extended resections are necessary to achieve R0 resection, shared decision-making should weigh surgical morbidity and mortality versus expected survival benefit.
- Re-resection for iGBC is recommended for patients with pT1b, pT2, or pT3 disease without metastatic disease and/or poor performance status.
- Extrahepatic bile duct resection should not be performed routinely and is only recommended for selected patients with a positive cystic duct margin or direct tumoral involvement of the hepatic duct.
- Resection of laparoscopic port-sites in patients with GBC is not recommended because it is not associated with better survival.
- A multidisciplinary approach appears vital to further improve prospects of GBC patients.

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Conventional Therapy in Gallbladder Cancer

3

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3.1 Introduction

Although it is considered a rare disease, gallbladder cancer (GC) is responsible for approximately 180,000 deaths worldwide each year. In the same way, it is the most common malignant tumor of the biliary tract [1]. The outcome of the disease is poor, with overall 5-year survival of less than 5%. However, the early stages of the disease show survival up to 75%. The disease is more commonly observed in areas such as South America, Northern India, Eastern Europe, and some countries of Asia [2]. Chile has the highest incidence in the world; Data originated from Valdivia in Chile shows an incidence rate of 12.3/100,000 for males and 27.3/100,000 for women. This geographical distribution is probably due to different genetic susceptibility [2, 3].

Although the pathogenesis is likely multifactorial, some risk factors can be identified. Of the risk factors, gallstones are considered the most important. Besides gallstones, GC has been associated with factors such as infection, polyps, obesity, pancreaticobiliary maljunction anomalies, and gender [4].

Association with gallstones varies depending on the geographical location. While in Japan and other Asian countries, this association is approximately observed in 70%, in countries such as Chile, the association is observed in more than 95%. On the other hand, GC is found in 0.2–3% of cholecystectomies depending on the place where this exam is obtained [5].

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Although the association between gallstones and malignancy is not fully established, stones would produce chronic inflammation of the mucosa, and this phenomenon would be the initial step in the malignant transformation. The relation between the cholecystectomy rate and GC has been highlighted. The increase in the cholecystectomy rate has been associated with a decline in the GC incidence rate. Because of the above, prophylactic cholecystectomy has been proposed as a recommendation in countries such as Chile where the incidence of GC is extremely high. In this country, patients aged between 35 and 49 years are advised to undergo cholecystectomy irrespective of the gallstones symptoms [5, 6].

Chronic bacterial infection is also considered a risk factor. *Salmonella typhi*, *Helicobacter* sp., *E. coli*, *Enterobacter* sp., and other bacteria have been identified in the bile of patients harboring a GC [7, 8].

The employment of abdominal ultrasonography during the evaluation of abdominal symptoms has brought the detection of polyps in the gallbladder. This diagnosis is a common cause of medical visits and worry. At least in Chile, cholesterol deposit is the most common etiology, and no special treatment is needed [9]. Predictors of possible malignancy are: size greater than 10 mm, solitary polyp, age more than 50 years, sessile shape, and faster growth. When we deal with this type of lesion, it is important to reassure the patient and perform a complete evaluation of the polyp characteristics. In most cases, no complementary treatment is necessary [10]. Adenoma to adenocarcinoma is the more commonly observed sequence in tumors arising in gallbladder polyps. Adenomatous polyps occur in only 1% of cholecystectomy specimens, while up to 7% of lesions have concomitant malignancy. These types of tumors originated from an adenoma are more commonly observed in Asia [10].

Pancreaticobiliary maljunction is an abnormal union of the biliary and pancreatic duct outside of the sphincter. This anomaly allows the reflux of pancreatic juice into the biliary duct and gallbladder producing chronic mucosal inflammation [10]. Concerning gender, females are three times more commonly affected than males. The higher prevalence of gallstones among women would be the main reason for this association [11].

Finally, a genetic factor explains the high incidence in the southern part of Chile, where the Mapuche ethnicity possesses the higher incidence of both gallstones and tumors. The above fact is also observed from studies performed in Sweden, which showed a higher incidence in Chilean and Indian immigrants when compared with immigrants from other areas [10].

3.2 Diagnosis

Unfortunately, and because the tumor is located in a complex area near vascular and biliary structures, early diagnosis is a difficult task. When a patient develops symptoms, the main cause is the compromise of some neighboring organs [12]. Because of the proximity with the bile duct, compromise of this structure and the development of jaundice are commonly observed. The rest of the symptomatology is not specific and common to different tumors [12].

During the last two decades, an increase in the detection of incidental tumors has been observed. An incidental tumor is defined as a tumor not suspected before the cholecystectomy and detected only after the exam of the cholecystectomy specimen [12]. Incidental tumor is detected in approximately 0.2–2% of all laparoscopic cholecystectomies. The increase in the detection of incidental tumors is probably due to the spread of the employment laparoscopic cholecystectomy [13]. The study of the cholecystectomy specimen is important to know the exact depth of invasion and the presence of factors associated with the prognosis such as perineural and perivascular infiltration, lymphatic invasion, and Rokitansky Aschoff sinus invasion.

The cholecystectomy specimen must be examined to detect lesions not already observed during the surgery. After a careful macroscopic exam, the specimen must be histologically examined [14]. Three different areas from the gallbladder mucosa are examined, and the whole mucosa should be evaluated in case of detection of dysplasia or cancer. The random sampling method to examine the gallbladder, which is employed in many western centers, could explain the lower survival rates reported by some groups in commonly good prognosis tumors. Only the complete exam of the specimen will allow knowing the depth of tumor invasion. An example of the above is the difference in the survival between centers that employ a well-defined protocol versus the poor prognosis observed when only a portion of the gallbladder is studied [14].

3.2.1 Ultrasonography

The ultrasound examination is the basic way of studying a patient with biliary pathology. This examination can give the first orientation in the management of a patient in whom GC is suspected (Fig. 3.1). Unfortunately, in cases of early tumors,

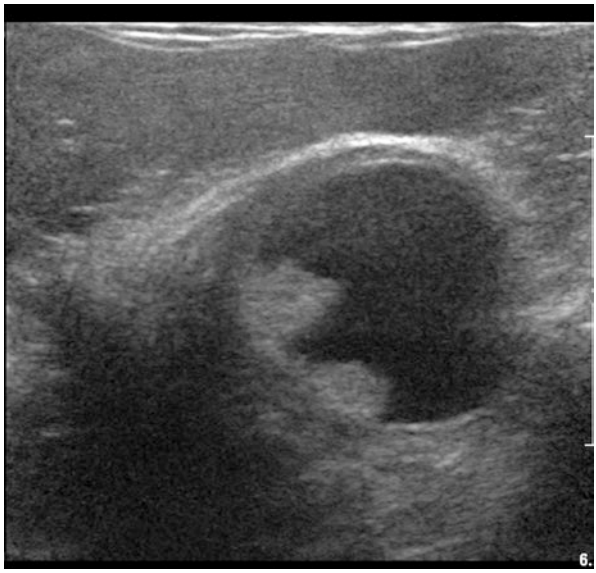

Fig. 3.1 Ultrasonogram showing a mass inside the gallbladder, suspicious of gallbladder cancer

the majority of them correspond to flat lesions which are not well defined by the ultrasonogram. Further, the presence of gallstones and mucosal inflammation interferes with the clear observation of the gallbladder wall [15]. On the other hand, in cases of incidental tumors, postoperative changes make the value of ultrasound limited.

Findings such as biliary duct dilatation, intrahepatic masses, intraluminal gallbladder growth, and changes in the gallbladder wall thickening can be the first sign in the diagnosis of a GC [16].

3.2.2 CT Scan

This study method is the cornerstone in the evaluation of a patient suspected to have gallbladder cancer. CT allows a clear visualization of the gallbladder and surrounded structures allowing a complete staging of the disease [15]. Findings such as diffuse infiltration of the liver and peritoneal carcinomatosis are clear signs of inoperability. Furthermore, CT scan detects portal and biliary tract invasion, invasion of distant lymphadenopathy and neighbor organs (Fig. 3.2).

3.2.3 PET/CT

This diagnostic method combines metabolic and anatomical findings. Its main employment is in the preoperative evaluation of patients suspected to be

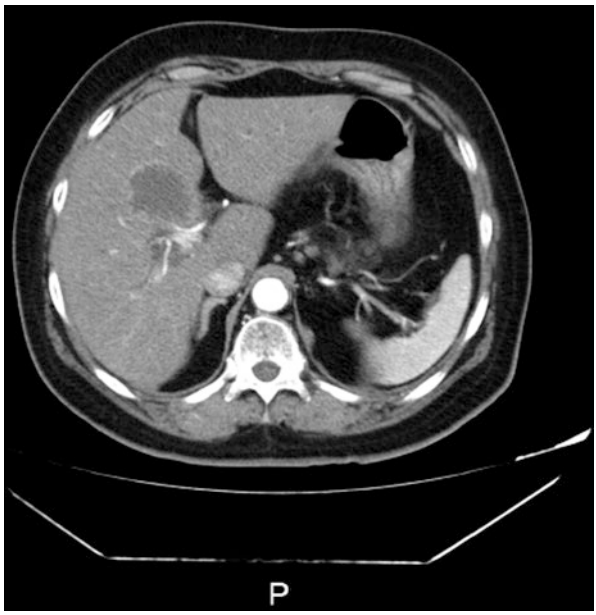

Fig. 3.2 Locally invasive, gallbladder cancer

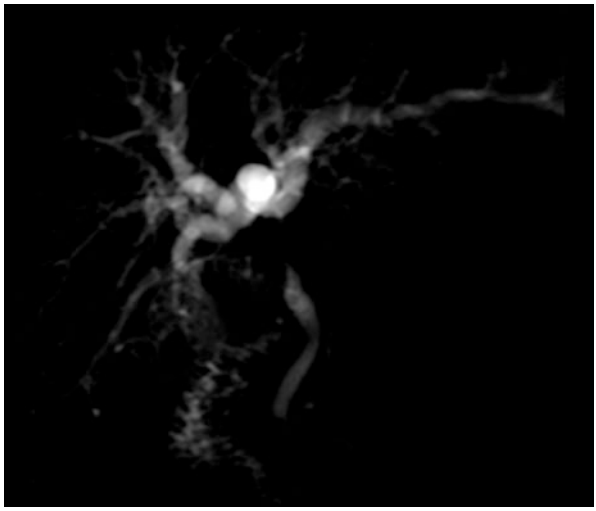

Fig. 3.3 Typical cholangiographic image of common bile duct compromise from a gallbladder cancer

non-resectable or during the evaluation of patients harboring an incidental tumor [17]. In patients harboring an incidental tumor, this method shows a 78% sensitivity and 80% specificity to detect residual disease. The main problem of this method is the possibility of false positives due to inflammation. This fact is mainly observed in patients with the diagnosis of incidental tumors. These patients can show positive areas in the place of ports and in the gallbladder bed [17].

3.2.4 MRI, MRA, and MRCP

The combination of the above exam methods is useful in the detection of vascular and biliary invasion. MRCP gives valuable information at the moment of evaluating a patient who developed jaundice by delineating the exact anatomy of the biliary tract and the level of the biliary compromise (Fig. 3.3) [18].

3.3 Surgical Treatment

Surgery is considered the cornerstone in the management of GC. An R0 resection is imperative to obtain a curative result. Complete extirpation of the tumor is often challenging because of the complex area where the tumor is located. To approach the management of GC, we prefer to divide the patients according to the moment when the diagnosis is performed [12]. Diagnosis can be done or suspected before the surgery, during a procedure performed for a benign condition, or after the result of the biopsy of cholecystectomy specimen [15].

3.3.1 Preoperative Diagnosis

By the employment of image exams, GC can be suspected during the evaluation of a patient with biliary symptomatology. Before proceeding with the surgery, it is necessary to rule out inoperability.

Of the above, peritoneal invasion, bilobar liver compromise, distant lymph node compromise, and distant invasion are typical findings that contraindicate the surgery [17].

A common problem faced is the difficulty to obtain a histologic diagnosis before performing the surgery. The risk of track dissemination in a potentially resectable patient precludes the employment of fine-needle aspiration biopsy [18]. After discarding inoperability, an exploratory laparoscopy must be considered as part of the evaluation and performed before the respective procedure. The laparoscopy will be useful to explore potentially compromised areas that can make to change the indication of resection [19].

3.3.2 Intraoperative Diagnosis

This category represents a big challenge for surgeons who deal with biliary pathology. The classical setting is a surgeon operating a presumed benign pathology that during the surgery has the suspicion of malignancy. Factors to take into account before proceeding with the decision are the following: Is there histologic confirmation of the neoplasia? Did the patient give informed consent for a surgery different from the initially planned? Does the patient have an adequate staging protocol? Does the surgeon have enough experience to accomplish the resection? After the analysis of the above, the surgeon has to decide whether to perform only an exploratory laparotomy with biopsy of critical zones or to accomplish an oncologic procedure [13]. Perhaps the worst scenario is to face a suspicious tumor without getting the diagnosis of malignancy.

3.3.3 Postoperative Diagnosis

This entity identified as incidental cancer is a common way of presentation and responsible for the majority of the curative results. The incidence of these tumors is different depending on the center and the area where the cholecystectomy is performed [19, 20]. Even at present, the debate about the routine pathological exam of cholecystectomy specimens exists. In our center routine, rather than a selective exam is employed. The incidence of GC has increased parallel with the increase in the cholecystectomy rates. The quality of the specimen information is important to adopt the therapeutic strategy. Prognostic factors such as differentiation grade, Rokitansky Aschoff sinus invasion, and lymphovascular and perineural invasion should be informed together with classical pT and lymph node invasion [20].

The management of the patients will mainly depend on the level of invasion of the tumor in the gallbladder wall. Residual tumor at the moment of re-resection has been highlighted as an important poor prognosis factor. To delineate the management, the patients will be divided into different groups according to the level of wall invasion. The main advantage to employ the level of wall invasion is that it is known in all patients who underwent cholecystectomy [19].

Concerning the timing for reoperation, it is well known that delaying the re-resection could improve the patient selection, avoiding operating patients with aggressive tumor biologic behavior. In our center, among the patients who underwent re-resection, we did not observe a relation between the time when the re-resection was done and the final prognosis [17, 19]. The biology of the tumor is probably the most important factor for the final prognosis of the patients.

T1a: This group includes patients with invasion restricted to the mucosa or lamina propria, and they should be considered already treated after the cholecystectomy. General results from different centers show 5-year survival ranging between 90% and 99%. These patients require a complete and detailed examination of the gallbladder specimen to be certain of the level of invasion [17]. Sometimes the examination of areas adjacent to the apparent main tumor can show areas of the tumor with deeper invasion than the area already studied. In the same way, the presence of Rokitansky Aschoff sinus invasion must be excluded [2, 17].

T1b: The management of patients with invasion of the muscular layer remains controversial. Strategies vary according to the center, and 5-year survival ranges between 80% and 37%. In this group, the analysis of cholecystectomy specimen is extremely important. The depth of wall infiltration can change according to the study methodology. Patients with muscular infiltration can change to deeper infiltration when the complete specimen is studied. The above can explain the lower survival reported for T1b patients by some authors [17, 19, 20].

Management of these patients ranges between those who recommend the resection of the gallbladder bed and a lymph node dissection to those who state that a simple cholecystectomy is enough as therapy. Unfortunately, taking into account the present level of knowledge, it is difficult to adopt a valid conclusion about the best treatment for this group of patients [19]. In our center, we have employed both strategies without reaching a consensus.

T2: This group of patients includes those with a tumor invading the peri muscular connective tissue. These patients have an intermediate prognosis between those with mucosal and serosal layer invasion. According to the last classification proposed by the AJCC, subserosal tumors should be divided according to the place where the tumor is located [19, 20]. Tumors located in the hepatic plane would have a worse prognosis compared with those in which the tumor is located on the peritoneal side.

Two factors could explain the existence of a worse prognosis. The first factor is the existence of venous and lymphatic channels in the plane between the liver and the gallbladder [12, 20]. Furthermore, this plane is the space where the resection

of the gallbladder is performed. Then, when the cholecystectomy is performed, this plane is disrupted.

Although there are no prospective randomized studies supporting the value of radical surgery in these patients, the majority of the surgical community advocates the performance of lymphadenectomy and the resection of the gallbladder bed to treat these patients [21]. The optimal extent of the resection is not well defined. The resection of the gallbladder bed with a 3 cm margin and the lymphadenectomy of the hepatic pedicle would be the minimum recommended procedure to accomplish the oncological goals.

The magnitude of the liver resection is variable, while some centers advocate a formal segment IVb and V resection; others show similar results with more limited resections. Among the patients who underwent re-resection, residual disease varies between 30% and 60% [19, 21].

T3: This group of patients includes those who have a direct invasion of liver, duodenum, stomach, or colon. Although the surgery is indicated to get an R0 resection, this could be associated with lower survival rates and high morbidity. Better results could be obtained in cases when the tumor only invades the liver, thus allow performing a curative resection without compromising other organs [10]. Because T3 corresponds to an advanced tumor, the association with extensive lymph node compromise is commonly observed and in part responsible for the lower survival [19].

T4: These types of tumors have a compromise of the portal vein or hepatic artery. Because of the above, jaundice is a typical symptom of the disease and resectability is extremely low. However, infrequent focal portal compromise could be amenable to undergo resection. In general terms, morbidity and mortality associated with the procedure outweigh any survival benefit [13].

3.4 Special Topics

In the following sections, some procedures and events related to the management of GC will be discussed:

3.4.1 Lymphadenectomy

Lymphadenectomy is a common procedure in the management of patients with gallbladder cancer. Lymphatic spread goes from the gallbladder through the cystic lymph node to the nodes located in the hepatic pedicle. From the above lymph nodes, tumor cells go to the nodes located around the hepatic artery toward the celiac axis, and also from the hepatic pedicle to the nodes located in the pancreas head [10]. From the nodes located in the pancreas head, invasion goes to the periaortic lymph nodes [17].

When we treat a patient with gallbladder cancer, we need to decide the limits of the lymphatic dissection. Frozen section biopsy of the nodes located in the

dissemination route should be employed to know the status of the spread. The presence of positive lymph nodes around the hepatic pedicle is a common finding, and it supports the execution of the lymphadenectomy. On the other hand, when the invasion affects the nodes around the hepatic artery or the nodes located in the pancreas head, survival is poor, and the performance of a lymphadenectomy will depend on the institution protocol [19, 20]. The nodes located in the choledochal duodenal angle are very important in the process decision. According to some authors, the invasion of this complex lymphatic structure could be an indication to stop the procedure [20]. In our institution, lymphadenectomy of the nodes around the hepatic artery is performed independent of the level of invasion; however, the compromise of the lymph nodes located in the pancreas head is an indication of no resection. On the other hand, a complete resection of the above lymph nodes would be only possible by performing a pancreaticoduodenal resection [21].

3.4.2 Pancreaticoduodenectomy as Treatment of Gallbladder Cancer

Pancreaticoduodenectomy could be an indication for the treatment of gallbladder cancer in selected cases. Patients with invasion of the nodes located in the head of the pancreas or those with direct invasion of pancreas by the tumor could be candidates for this type of therapy [21]. The results obtained by the employment of this therapy are in general poor. Morbidity and mortality associated with this procedure in GC is high and the survival is low.

Concerning morbidity and mortality, some pancreas characteristics such as the soft consistency and the lack of dilatation of the duct could be in part responsible for the associated complications [10]. In spite of the above, pancreaticoduodenectomy should be taken into account at the moment of deciding what therapy to offer. The analysis should be done case by case considering the extension of the disease and the clinical characteristics of the patient [10].

3.4.3 Gallbladder Wall Perforation

During a cholecystectomy for gallstones, the perforation of the gallbladder wall is a common event that ranges between 13% and 40% of the cholecystectomies. A thinner wall, gallbladder distension, and the absence of a well-defined plane between the liver and gallbladder make possible the wall rupture during the cholecystectomy. Since the beginning of laparoscopic cholecystectomy, recurrences at the peritoneum and port scars were described [12, 14]. Because of this, laparoscopy thought to be a contraindication when GC was suspected. Horkof was the first author to analyze the relation between gallbladder perforation and prognosis in incidental GBC [17]. In a series of 82 patients in whom GC was diagnosed from the cholecystectomy specimen, 13 of 14 patients with peritoneal recurrence had the antecedent of bile spillage during the previous cholecystectomy [18]. This result highlights the importance of

bile spread in the development of tumor recurrence in patients with incidental gallbladder cancer. In our center, when we studied this relationship, the bile leak magnitude was correlated with the prognosis. Then, only in the group affected by massive bile spillage, survival was affected. Massive spillage was defined as the spread of not only bile but also stones. Because of the importance of bile spillage as a risk factor in gallbladder cancer, we must highlight the role of the cholecystectomy surgical technique [18]. Surgeons operating cholecystectomies must know the value of a meticulous technique to avoid the chance of perforation [12]. The above is crucial in areas where GC is common.

In the future and considering bile spillage as a prognostic factor, we should analyze the employment of a complementary therapy in patients suffering from perforation in which an incidental tumor is detected.

3.4.4 Management of Advanced Tumors

A high proportion of gallbladder tumors are detected when they have already spread to distant areas or are locally invasive. Unfortunately, the possibility of performing a curative resection in these patients is extremely low [21]. The invasion of vascular structures such as portal vein and hepatic artery are classical difficulties to carry out a curative resection. Besides the above, the peritoneum is another commonplace of spreading resulting in unresectability. The peritoneal invasion is commonly detected by the employment of CT scan or during the laparoscopic exploration before performing the laparotomy for resecting the tumor [21, 22].

3.4.5 Jaundice

In some patients, jaundice is the only symptom of the disease. Because of not only the involvement of the intrahepatic and extrahepatic bile ducts but also the associated compromise of neighbor and vascular structures, jaundice is commonly considered a cause of no resectability [22, 23]. In spite of the above, a complete evaluation of the patient is warranted. The presence of jaundice may be secondary to the following mechanisms: (a) involvement of lymph nodes around the common bile duct, (b) direct infiltration by the tumor, (c) growing of the tumor inside the common bile duct by dissemination from the gallbladder. Obstructive jaundice can also have systemic consequences such as impairment of cellular immunity, changes in the gut barrier, and reduction in the sinusoidal flow [23].

The resectability rate is generally low, and median survival ranges between 11 and 26 months. Some authors have reported higher survival rates, but their series includes patients with invasion of the biliary tract irrespective of the existence of jaundice. Although the survival of these patients is poor, surgery is the only method to get survival benefit. Because of the above, we should not discard the possibility of resection, and patients must be analyzed case by case before getting a final decision. The employment of neoadjuvant therapy after the biliary decompression can be considered an alternative strategy in the management of this group of patients.

Japanese surgeons showed a 65% response rate with 40% R0 resection rates with Gem-Cis/Gem-Ox neoadjuvant chemotherapy [16, 23, 24]. The above study included all patients with locally advanced tumors and not only those with jaundice.

3.4.6 Resection of Portal Sites

It was considered an essential part of the reoperation in patients harboring an incidental tumor. Recent studies have shown no survival benefit when patients who underwent port site resection were compared with those who did not undergo resection after adjusting for T and N stages. At present, routine port excision is no longer advised in the management of incidental tumors [5, 20].

3.4.7 Laparoscopy: When and Where

The introduction of laparoscopy in the management of gallstones brought uncertainty about the possible detrimental effect that this method could have on the survival in those patients in whom an incidental tumor was detected [5]. Several studies showed that the perforation of the gallbladder wall instead the laparoscopy itself would be the explanation for the observed worse prognosis.

Concerning reoperations, laparoscopy is a good method to perform the resection. Laparoscopy allows a complete exploration of the abdominal cavity before proceeding with the resection [19]. In the same way, numerous authors have highlighted that the laparoscopic method has the same oncological standards as the open method. This conclusion is based on the number of dissected lymph nodes and also in the quality of liver resection [19]. Vega published a report comparing both the open and the laparoscopic methods to perform the resection in patients with incidental tumors. The study did not show differences in the survival rate between both the methods of resection [19].

3.4.8 Palliative Management

Patients without the option to receive curative management are candidates for palliative management. Jaundice, outlet obstruction, and pain are the main symptoms to palliate [21]. Jaundice and hitching are important complaints. Biliary tract drainage is the way to alleviate the obstruction and treating the symptoms. The main difficulty to drain the biliary duct is the fact that obstruction from gallbladder cancer involves the upper portion of the bile duct. Because of the proximity between the neck of the gallbladder and the upper portion of the bile duct, hepatic hilum and segmental branches are commonly obstructed by direct infiltration [23, 24]. Internal drainage by employing an endoscopic cholangiography is the preferred method. Before proceeding with the procedure, cholangiography by magnetic resonance is necessary. The existence of multiple compromises of segmental branches makes the procedure much more difficult to perform and also carries the risk of contamination of liver segments with the subsequent infection of obstructed areas [23, 24].

Percutaneous drainage can be an alternative to drain the obstruction. The main problem associated with this type of drainage is the necessity of performing more than one drainage due to the obstruction affects segmental branches independently. No differences have been shown for one approach versus the other [24]. Gastric outlet obstruction is also a potential complication observed in up to 30% of patients. These patients may benefit from surgical gastrojejunostomy or an endoscopic approach [25].

3.5 Chemotherapy and Radiotherapy

3.5.1 Chemotherapy in Advanced Cases

The introduction of gemcitabine made possible the employment of this drug in the management of advanced tumors. The ABC-02 (2010) trial compared gemcitabine/cisplatin with gemcitabine alone in locally advanced or metastatic cholangiocarcinoma and GC. The results showed clear superiority of the combination regimen, with significant improvements in overall survival (11.7 vs. 8.1 months) and progression disease-free survival (8 vs. 5 months) [26]. Tumor control was achieved in 81.4% of patients who received cisplatin plus gemcitabine compared with 71.8% of patients who received gemcitabine alone ($p = 0.049$). Adverse events were similar between the two arms aside from liver function, which was significantly worse in the gemcitabine-only group (27.1%) than in the cisplatin-gemcitabine group (16.7%) [22, 23].

Other combination regimens have been studied including gemcitabine plus capecitabine, gemcitabine plus oxaliplatin (GEMOX), and gemcitabine plus S1. These combinations may have similar effects that gemcitabine plus cisplatin in the first line setting [24–26].

Recently, a phase II trial of gemcitabine, cisplatin, and nab-paclitaxel for biliary tract cancers patients, including gallbladder cancer was reported [23, 25]. The median follow-up was 12.2 months, and median progression-free survival was 11.8 months (95% CI: 6.0–15.6). Median overall survival was 19.2 months (95% CI: 13.2 to not estimable).

In the European Union, 5-fluorouracil, irinotecan, and oxaliplatin (FOLFIRINOX) regimen is being investigated versus gemcitabine and cisplatin for advanced biliary tract cancers, including gallbladder cancer. Finally, the ABC-06 trial, randomized patients to FOLFOX versus supportive care; 21% had gallbladder cancer. Median overall survival was 6.2 months for the FOLFOX arm vs. 5.3 months for supportive care only arm [25]. The results of these studies may redefine the current first-line therapy options for gallbladder cancer.

3.5.2 Neoadjuvant Chemotherapy

The employment of neoadjuvant chemotherapy should be considered in the management of patients with gallbladder cancer [25–27]. This approach could be useful

not only before the respective surgery when the diagnosis is performed in the preoperative setting but also after the diagnosis of an incidental tumor before the reoperation [28]. Unfortunately, information about this method of management is limited to get definitive conclusions.

Neoadjuvant therapy should be considered in cases of locoregionally advanced disease, such as a mass invading the liver and or nodal involvement [27]. Although the evidence is limited to define a standard regimen, options include gemcitabine/capecitabine, capecitabine/cisplatin, capecitabine/oxaliplatin, 5-fluorouracil/oxaliplatin, and 5-fluorouracil/cisplatin [28].

3.5.3 Adjuvant Chemotherapy

Considering the higher relapse rate of GC, adjuvant chemotherapy seems to be a good option in the management of patients after resection. Adjuvant strategies have been explored, in the form of chemotherapy, radiotherapy, and chemoradiotherapy [29, 30]. The effect of these regimens has been limited, and often the studies have included other biliary malignancies. The following studies have been performed in GC:

The PRODIGE-12/ACCORD-18 compared adjuvant gemcitabine and oxaliplatin (GEMOX) versus surveillance, in patients harboring either cholangiocarcinoma or gallbladder adenocarcinoma [25]. The trial was designed to detect a difference in median relapse-free survival. The results showed 18 months of relapse-free survival in the surveillance arm compared with 30 months in the GEMOX arm, but difference did not get statistical differences. There was also no difference in the overall survival between both study arms. Subgroup analyses by lymph node status, margin status, and primary disease site did not suggest any subgroup that would benefit from adjuvant GEMOX [26].

The BILCAP (2017) compared adjuvant capecitabine with only observation after macroscopic complete resection in patients with cholangiocarcinoma or gallbladder cancer. This study remains the first and only trial to demonstrate a benefit in adjuvant therapy for biliary tract malignancies [21]. In the group of GC patients, it should be highlighted that the study was performed in patients harboring a GC with muscular invasion. It is well known that this type of tumor has a good prognosis irrespective of the type of therapy. The trial showed an improvement in the overall survival of 20–32%, corresponding to an HR of 0.71. Approximately 54% of patients had microscopic positive margins and 38% of patients had lymph node-positive disease [31]. Results failed to show a significant difference in unadjusted intention-to-treat overall survival. However, there was a significant difference in overall survival in a prespecified intention-to-treat analysis adjusted for nodal status, disease grade, and sex. Adjuvant capecitabine is currently recommended for all patients who undergo resection of GC [31].

SWOGo809, this trial was designed to evaluate the role of postoperative therapy in extrahepatic and gallbladder carcinoma. Eligibility criteria included patients after radical resection, stage Pt2-4 or N+, or positive resection margin. Patients received

gemcitabine and capecitabine associated with radiotherapy. Results showed that the combination had promising efficacy. From the analysis of the above studies, it is highlighted that patients with resected biliary tract cancer including GC should receive adjuvant capecitabine while those with R1margins could receive chemoradiotherapy [30].

3.5.4 Chemoradiotherapy (CRT)

CRT has been expected to show a synergetic activity associated with chemotherapy. Due to the lack of randomized clinical trials including CRT, this form of therapy has not been established as standard of care in GC. In a retrospective cohort study, the effect of CRT (5- FU + 45–54 Gy) was assessed following surgery with curative intent. The overall survival at 3 and 5 years was 57% and 51%, respectively [32].

In another study including patients with T1-3 N0-1 treated with surgery and adjuvant CRT. It was confirmed that CRT was associated with an overall survival advantage at 3 years (HR: 0.47 (0.39, 0.58). In the same way, a series of patients undergoing surgical resection (R0, R1, or R2), followed by adjuvant RT (some patients also received 5-FU as a radio-sensitizer), showed a 5-year overall survival of 53%, 20%, and 0%, respectively ($p = 0.0038$). Adjuvant CRT employing chemotherapy (fluoropyrimidines) and radiotherapy (total abdominal radiation, 20–100 cGy/day) was applied in a series of patients with R0, T1b-2-3 N0–1 M0 disease. In these patients, a 41% of 5-year overall survival was observed [33].

3.5.5 Radiotherapy (RT)

Before the introduction of effective chemotherapy treatment, radiation therapy was employed as the only method for adjuvant therapy in GC. A systematic review determined that adjuvant RT reduced the risk of death and recurrence vs. surgery alone. In another series of patients, including patients with stage III-IV, those treated with resection combined with adjuvant RT had an overall survival at 1, 3, and 5 years of 56%, 24%, and 18%, respectively, while in those who underwent only surgery, overall survival was 43%, 23%, and 17%, respectively ($p < 0.05$) [34].

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Adjuvant Therapy of Gallbladder Cancer

4

Zachary J. Brown, Daniel B. Hewitt, and Timothy M. Pawlik

4.1 Introduction

Gallbladder cancer (GBC) is a rare gastrointestinal malignancy that carries a poor prognosis largely due to its late presentation, early invasion, and lack of effective systemic therapy [1]. Complete resection of GBC is the most effective therapy and the only chance for cure. However, patients with GBC are plagued by high recurrence rates after resection where up to 70% of patients develop recurrent disease at a median time of 11.5 months. Isolated locoregional recurrence as the site of failure has been found to occur in 15% of patients while recurrence involving distant sites with or without locoregional recurrence occurs in 80–85% of patients [2]. Thus, effective adjuvant therapies are needed to decrease disease recurrence and improve survival.

As GBC is rare, it has been difficult to study and find effective therapies. Clinical trials of adjuvant chemotherapy and radiation are limited. Additionally, in order to achieve sufficient statistical power, GBC studies often include patients with other biliary malignancies such as intra- or extra-hepatic cholangiocarcinoma [3]. In addition to heterogeneous study populations, early studies of adjuvant therapy for GBC were small, nonrandomized, and retrospective [4]. In turn, data on the use of adjuvant therapy for GBC have often been inconclusive, as well as largely underpowered [5]. Recent clinical trials such as ABC-02, PRODIGE-12/ACCORD-18, and BILCAP have attempted to address the topic of adjuvant therapy for biliary cancers. We herein review data on adjuvant treatment of GBC as well as highlight strategies for treating patients in the adjuvant setting.

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4.2 Rationale for Adjuvant Therapy and Risk Factors for Recurrence

Surgical extirpation with negative margins remains the only chance for cure in patients with gallbladder cancer [6]. Simple cholecystectomy with negative margins is adequate treatment for patients with T1a disease, with long-term survival approaching 100% [7]. For patients with T1b disease or greater, operative management consists of cholecystectomy, partial liver resection of segments IVB and V, as well as a portal lymphadenectomy [8]. In some instances, extended liver resections may be required along with bile duct excision in order to obtain negative margins. However, routine use of extended hepatic resection increases morbidity without improvement in survival [8, 9]. Lymphadenectomy generally includes portal/hepatoduodenal ligament and pericholedochal/hilar area. Extended lymphadenectomy including celiac, peripancreatic, periduodenal, and superior mesenteric nodes is not required [10]. Lymphadenectomy not only provides important staging information but may decrease local recurrence rates [11].

Despite the best attempts at curative resection, a majority of patients with GBC will recur. Recurrence following surgery is a major barrier to providing long-term survival in patients with GBC. In fact, depending on stage of presentation, up to 30–60% of patients can develop recurrent disease within 1–2 years after resection. Jarnagin et al. noted that the majority of patients (72%) developed distant metastasis as the initial site of GBC recurrence including the peritoneum (72%) or the lung (12%); in contrast, a minority of patients (15%) had locoregional recurrence only [2]. Risk factors for recurrence include R1 resection, >T2 disease, lymph node metastasis, and presence of perineural invasion [12]. The high incidence, as well as the frequent systemic nature of recurrence, highlights the rationale for the use of adjuvant systemic therapy [2]. While some patients who undergo an R0 resection may simply be observed following surgery, current National Comprehensive Cancer Network (NCCN) guidelines suggest adjuvant systemic therapy, preferably in a clinical trial, for all patients with T1b disease or higher T category. Controversy surrounding the role of adjuvant remains, however, due to the traditional lack of robust data from randomized clinical trial [13–15].

Several retrospective studies provided conflicting data regarding the role of adjuvant therapy in the treatment of GBC [2, 16, 17]. Glazer et al. reported that neoadjuvant or adjuvant therapy prolonged overall survival (OS) [16]. In a separate study of the National Cancer Database (NCDB), Tran et al. reported on patients with T1-T3 N1 M0 GBC as well as intrahepatic cholangiocarcinoma who underwent non-operative treatment, surgery, or surgery plus adjuvant therapy. These authors noted an improvement in OS among patients who underwent resection plus adjuvant therapy. Among surgery patients, the addition of adjuvant chemotherapy was associated with a survival advantage regardless of margin status [17]. These studies, like many involving biliary tract cancers, included patients with GBC and cholangiocarcinoma, limiting inferences for patients with GBC. A meta-analysis by Ma et al. did, however, investigate studies published between 1967 and 2014 that only included patients with GBC to evaluate adjuvant therapy versus curative-intent

surgery alone [18]. Ten retrospective studies that included 3191 patients demonstrated that adjuvant chemotherapy was associated with an improvement in OS compared with surgery alone (HR, 0.42; 95% CI, 0.22–0.80). Patients who had the greatest benefit from adjuvant therapy were those with R1 disease (HR, 0.33; 95% CI, 0.19–0.59) and metastatic lymph node disease (HR, 0.71; 95% CI, 0.63–0.81) [18].

Additional retrospective studies have reported that adjuvant chemotherapy may improve OS in selected patients with T2 or T3 tumors and lymph node metastasis. For example, Cho et al. noted that patients with GBC and no lymph node metastasis did not have a survival advantage with the addition of adjuvant chemoradiation therapy (5-FU or gem concurrently with radiation 45Gy in 25 fractions for 25 days). In contrast, patients with lymph node metastatic disease benefitted from adjuvant chemotherapy relative to disease-free survival (DFS), with adjuvant chemotherapy being an independent prognostic factor for improved survival [19]. In a separate multi-institutional national database study of 291 patients with GBC who underwent curative resection, adjuvant chemotherapy was utilized in 36% of patients. Patients with high-risk features, such as T3 or T4 tumors, lymph node metastasis, or R1 resection, had improved OS and DFS with adjuvant therapy [20]. In a different study, Horgan et al. reported a meta-analysis of studies published between 1960 and November 2010 in which the authors examined the impact of adjuvant chemotherapy, radiotherapy, or both compared with curative-intent surgery alone for resected biliary tract cancer [21]. The authors noted a nonsignificant improvement in OS with the use of any adjuvant therapy compared with surgery alone ($p = 0.06$). Additionally, there was no difference in outcomes between gallbladder and other bile duct cancers ($p = 0.68$). Patients who received chemotherapy or chemoradiotherapy had a greater benefit than individuals who received radiation alone, and the greatest benefit was among patients who had an R1 resection, as well as patients with lymph node metastasis [21].

The use of radiation therapy in the adjuvant setting also remains controversial. In one study using the national cancer database (NCDB), neoadjuvant therapy was utilized in 13.5% of patients, while 28.8% received adjuvant therapy [22]. The patients having T3 or N1 disease, and patients who had an R1 resection were more likely to receive adjuvant therapy. Use of adjuvant therapy was associated with a modest early survival advantage, which appeared to dissipate at 5 years of follow-up [22]. In another study, adjuvant chemoradiation was associated with improved survival especially among patients with metastatic nodal disease; however, adjuvant chemotherapy was not associated with improved survival [23]. In a meta-analysis of 50 studies, Ghidini et al. reported that the use of adjuvant therapy increased survival by 4.3 months compared with surgery alone [24]. In a different multi-center US study of the 112 patients, patients who received adjuvant radiation were more likely to have had a higher T-category (57% vs. 16%, $p < 0.01$), lymph node disease (63% vs. 18%, $p < 0.01$), as well as R1 surgical margins (37% vs. 9%, $p < 0.01$) versus patients treated with surgery alone [25]. Adjuvant radiation was associated with decreased isolated local failure, but did not improve overall survival; 71% of recurrences included a distant site of failure [25].

The use of adjuvant therapy has been relatively low. For example, a study of the NCDB noted that only 22.1% of patients received adjuvant chemotherapy [26].

Patients receiving adjuvant therapy were younger, had less comorbidity, more often had nodal disease, and were more likely to have had an R1 resection. Despite the low use, adjuvant chemotherapy was associated with improved OS among patients with metastatic nodal disease, as well as in patients who had inadequate nodal staging [26]. Interestingly, the use of adjuvant treatments has remained largely unchanged from 2005 through 2013 among patients diagnosed with T1-3N0-GBC [27]. In fact, use of adjuvant radiation decreased from 4.2% to 1.7% ($p < 0.001$), while adjuvant chemotherapy increased from 8.3% to 13.8% ($p < 0.001$) [27]. Among patients who did receive adjuvant therapy, it was associated with improved 3-year OS, with an adjusted hazard ratio of 0.47 (95% CI = 0.39–0.58) for chemoradiation, 0.77 (95% CI = 0.61–0.97) for chemotherapy, and 0.63 (95% CI = 0.44–0.92) for radiation therapy [27].

4.3 Randomized Clinical Trials

Over the past several decades, several randomized clinical trials have been performed to investigate the use of adjuvant therapies in biliary tract malignancies (Table 4.1). In 2002, Takada et al. investigated the use of intravenous mitomycin C (6 mg/m^2) on the day of surgery and intravenous 5-FU (310 mg/m^2) in two treatments for 5 days during postoperative weeks 1 and 3, followed by oral 5-FU (100 mg/m^2) daily from postoperative week 5 until recurrence [28]. In total, 436 patients were randomized; 158 patients with pancreatic adenocarcinoma, 118 with bile duct cancer, 112 with GBC, and 48 with cancer of the ampulla of Vater. Interestingly, 5-year OS was better among patients with GBC who received adjuvant mitomycin C and 5-FU compared with the control group (26.0% vs. 14.4%, respectively; $p = 0.0367$); 5-year DFS was also improved (20.3% vs. 11.6%, respectively; $p = 0.0210$) [28]. There was no difference in OS or DFS noted in patients with pancreatic, bile duct, or ampullary cancer.

4.3.1 ABC-02

The Advanced Biliary Cancer (ABC-02) phase III randomized controlled trial compared cisplatin plus gemcitabine versus gemcitabine alone among patients with locally advanced or metastatic cholangiocarcinoma, GBC, or ampullary cancer [32]. In this trial, 410 patients were randomized to receive cisplatin (25 mg/m^2) plus gemcitabine (1000 mg/m^2) every 3 weeks for 8 cycles, or gemcitabine (1000 mg/m^2) alone every 4 weeks for 6 cycles for up to 24 weeks. The primary end point was OS, and secondary endpoints were of progression-free survival (PFS), tumor response, and adverse events. All patients had an Eastern Cooperative Oncology Group (ECOG) performance status of 0, 1, or 2 and a life expectancy of at least 3 months. Of the 410 patients randomized, 149 patients had GBC, 241 had cholangiocarcinoma, and 20 had ampullary cancer; 204 received cisplatin plus gemcitabine and 206 received gemcitabine alone.

Table 4.1 Randomized clinical trials of chemotherapy for biliary tract cancer

	Takada et al. [28]	PRODIGE12/ ACCORD18 [29]	BILCAP [30]	BCAT [31]
Study arms	5FU + mitomycin vs. observation	GEMOX vs. observation	Capecitabine vs. observation	Gemcitabine vs. observation
Recruitment period	April 1986–June 1992	July 2009–February 2014	March 2006–December 2017	September 2007–January 2011
Total sample size	436	196	447	225
Disease distribution	GBC 112 (26%) CCA 118 (27%) PDAC 158 (36%) Ampulla 48 (11%)	GBC 38 (20%) ICC 86 (44%) Hilar CCA 15 (8%) Distal ECC 55 (28%)	GBC 79 (18%) ICC 84 (19%) Hilar CCA 128 (28%) Distal ECC 156 (35%)	GBC 0 Hilar CCA: 101 Distal ECC: 124
Primary endpoints	OS	RFS and time to definitive deterioration of HRQOL	OS	OS
Secondary endpoints	DFS, ECOG PS, improvement in body weight, adverse events	OS, toxicity, and exploratory translational end point	Per-protocol analysis of OS/RFS, RFS, toxicity, health economics, and quality of life	RFS and toxicity
Completion of therapy	80% completion	Median of 10 cycles of 10 for gemcitabine and oxaliplatin	55% completed chemotherapy, 10 patients (4%) had 0 cycles, 32% discontinued therapy due to toxicity	52.1% completed chemotherapy. 18 patients stopped gem due to need for dose reduction
Results	5-year OS improved in patients with GBC who received adjuvant therapy (26.0% vs. 14.4%, $p = 0.0367$) and 5-year DFS (20.3% vs. 11.6%, $p = 0.0210$). No difference in OS or DFS in patients with PDAC, CCA, or ampullary cancer	No difference in RFS or deterioration of HRQOL. Patients with GBC who received GEMOX had a significantly worse RFS and OS	No significant difference in OS in intention-to-treat population. Significant improvement with capecitabine in OS and RFS in prespecified per-protocol analysis	Gemcitabine provided no difference in OS or RFS
Relapse rate	79.9% adjuvant therapy 88.4% observation	62.1% adjuvant therapy 67.7% observation	60% adjuvant therapy 65% observation	53.8% adjuvant therapy 56.5% observation

GBC gallbladder cancer, *CCA* cholangiocarcinoma, *PDAC* pancreatic ductal adenocarcinoma, *ECC* extrahepatic cholangiocarcinoma, *ICC* intrahepatic cholangiocarcinoma, *5FU* 5-fluorouracil, *OS* overall survival, *DFS* disease-free survival, *RFS* recurrence-free survival, *ECOG PS* Eastern Cooperative Oncology Group Performance Status, HRQOL Health-Related Quality of Life

At a median follow-up of 8.2 months, median OS was higher among patients who received cisplatin-gemcitabine than the gemcitabine alone (11.7 months versus 8.1 months; hazard ratio, 0.64; 95% confidence interval 0.52–0.80; $P < 0.001$). Additionally, patients who received cisplatin-gemcitabine had improved PFS (8.0 versus 5.0 months, $P < 0.001$) and a higher tumor control rate (81.4% versus 71.8%, $P = 0.049$). Adverse events were relatively similar in both the groups except for neutropenia which was found to be greater in patients who received cisplatin-gemcitabine; liver function was worse in the gemcitabine-only group. On prespecified subgroup analysis, there was no difference in the hazard ratio for death according to primary tumor site. These data provided evidence that cisplatin plus gemcitabine was an effective treatment for locally advanced or metastatic biliary tract cancer including gallbladder cancer [32]. Additionally, these results established gemcitabine with cisplatin in the adjuvant setting and set the stage for the following clinical trials [3].

4.3.2 PRODIGE-12/ACCORD-18

The PRODIGE-12/ACCORD-18 trial was a phase III multi-institutional study that compared gemcitabine and oxaliplatin (GEMOX) to surveillance alone among patients who received an R0 or R1 resection of localized biliary tract cancer [29]. In a previous phase II study, GEMOX had been demonstrated to be well tolerated among patients with advanced biliary tract cancers with an objective response rate of 20.5% (9/44) among patients with intrahepatic or extrahepatic cholangiocarcinoma, but only 4.3% (1/23) for GBC [33]. The PRODIGE-12/ACCORD-18 study aimed to determine if adjuvant GEMOX would improve outcomes versus surgery alone. In total, 196 patients were randomized to receive either GEMOX (gemcitabine 1000 mg/m^2 on day 1 and oxaliplatin 85 mg/m^2 on day 2 of a 2 week cycle) for 12 cycles or surveillance only. The primary endpoints were relapse-free survival (RFS) and time to definitive deterioration of health-related quality of life. Secondary endpoints included OS, toxicity, and exploratory translational end points. Among the 196 patients included, 38 patients had GBC while 86 patients had intrahepatic cholangiocarcinoma, 15 perihilar cholangiocarcinoma, and 55 distal cholangiocarcinoma.

At a median follow-up of 46.5 months, there was no difference in RFS among patients who received GEMOX versus individuals who had surgery alone (30.4 months vs. 18.5 months; hazard ratio 0.88; 95% CI, 0.62–1.25; $P = 0.48$). In addition, there was no difference in time to deterioration of HRQOL. Overall survival was not different between the study groups (75.8 months versus 50.8 months; HR, 1.08; 95% CI, 0.70–1.66; $p = 0.74$). Patients who received GEMOX experienced more grade 3 and grade 4 adverse events. Furthermore, on pre-planned subgroup analysis, disease site, lymph node status, or margin status was not associated with a differential improvement in subgroups relative to the benefit of GEMOX. Interestingly, patients with GBC who received GEMOX ($n = 17$) had

worse RFS and OS versus surveillance alone (n = 21) (P = 0.034 for RFS and P = 0.017 for OS) [29]. This study has been criticized as being underpowered to detect an effect size of HR 0.6, as well as including a low proportion of high-risk patients who may have benefited the most from adjuvant therapy (13% had R1 resection and 37% had metastatic lymph node disease) [3].

4.3.3 BILCAP

The BILCAP study was a phase III multi-institutional study that compared adjuvant capecitabine versus observation in patients with cholangiocarcinoma or muscle-invasive GBC who underwent a macroscopically complete resection with curative intent [30]. Patients who had not completely recovered from surgery were excluded. Capecitabine is an oral fluoropyrimidine that has been shown to have efficacy in treating biliary tract cancers [34]. In this trial, 447 patients were randomized to receive oral capecitabine (1250 mg/m^2) twice daily on days 1–14 of a 21-day cycle for 8 cycles versus observation only. The primary endpoint was OS and secondary endpoints included a per-protocol analysis of outcomes, RFS, toxicity, health economics, and quality of life. Of the 447 patients randomized, 84 patients had intrahepatic cholangiocarcinoma, 128 hilar cholangiocarcinoma, 156 lower common bile duct cholangiocarcinoma, and 79 muscle-invasive gallbladder cancer.

At a median follow-up of 60 months, in the intention-to-treat analysis, the median OS was 51.1 months in the capecitabine group versus 36.4 months in the observation group (adjusted hazard ratio 0.81, 95% confidence interval 0.63–1.04; p = 0.097); RFS was 24.4 months in the capecitabine group versus 17.5 months in the observation group (p = 0.033). A prespecified per-protocol analysis demonstrated a median OS of 53 months in the capecitabine group versus 36 months in the observation group (adjusted hazard ratio 0.75, 95% confidence interval 0.58–0.97; p = 0.028), as well as a median RFS of 25.9 months in the capecitabine group and 17.4 months in the observation group (p = 0.0093) [30]. However, there was no evidence of difference in RFS beyond 24 months, indicating capecitabine may have just delayed recurrence [3, 30].

4.3.4 BCAT

The BCAT trial was a randomized, controlled, multi-institutional phase III Japanese trial that investigated adjuvant gemcitabine versus observation among patients with resected bile duct cancer. Similar to the ABC-02 trial, gemcitabine (1000 mg/m^2) was administered on days 1, 8, and 15 every 4 weeks for 6 cycles [31, 32]. The primary endpoint was OS. Secondary endpoints included RFS and toxicity. Overall, 225 patients were included: 45% had hilar cholangiocarcinoma and 55% had distal cholangiocarcinoma; no patients with GBC were included. On analysis, gemcitabine provided no difference in OS or RFS.

4.3.5 TOSBIC01 (Tokyo Study Group for Biliary Cancer)

Recently, Itano and colleagues reported results using S-1 in patients with resected biliary malignancies. Their regimen consisted of S-1 (80 mg/m^2/day orally twice daily on days 1–28 of each cycle) given within 10 weeks postsurgery and was continued up to 1 year postsurgery. Forty-six patients met the inclusion criteria of whom 19 had extrahepatic cholangiocarcinoma, 10 had gallbladder carcinoma, 9 had ampullary carcinoma, and 8 had intrahepatic cholangiocarcinoma. The investigators observed that 54.3% of patients completed adjuvant therapy; among patients who did not complete adjuvant therapy, the recurrence-free survival was 62.5% at 1 year. Among the 7 out of 10 patients with GBC who completed therapy, OS and DFS at 1 year were 91.2% and 80.0%, respectively [35].

4.3.6 Chemoradiotherapy: SWOG0809

SWOG08909 trial was a clinical trial investigating chemoradiotherapy for GBC [36]. In this study, 25 of 79 patients had GBC while the remainder had extrahepatic cholangiocarcinoma. Two-year OS was 68% (95% CI, 54%–79%) among patients with bile duct cancer and 56% (95% CI, 35%–73%) among patients with GBC ($p = 0.87$). Two-year DFS was 54% (95% CI, 39%–66%) for extrahepatic cholangiocarcinoma and 48% (95% CI, 28%–66%) for GBC ($p = 0.71$) [36].

4.4 Future Directions

Despite the use of capecitabine, relapse rates still remain high. In the BILCAP study, 65% of patients in the observation group and 60% of patients treated with capecitabine reported disease recurrence [30]. As such, novel therapies are needed to improve survival and decrease disease recurrence among patients with GBC (Fig. 4.1). The use of next-generation sequencing has facilitated more personalized medicine by identifying unique mutations that may prove to be therapeutic targets. While GBC is considered with other biliary tract cancers in clinical trials, improved genetic analysis may yield a better understanding of the shared and distinct somatic genomic landscapes of cholangiocarcinoma versus GBC [37]. For example, mutations in isocitrate dehydrogenase 1 (IDH1) and IDH2 are much more predominant in intrahepatic cholangiocarcinomas, while these specific mutations are not as prevalent in extrahepatic cholangiocarcinomas or GBC. In contrast, KRAS and TP53 were more common in extrahepatic cholangiocarcinoma, and PIK3CA was more common in gallbladder cancer [38]. Weinberg et al. reported on the genetic profile of 1502 biliary tract cancers using next-generation sequencing. Intrahepatic cholangiocarcinoma had higher rates of IDH1, BAP1, PBRM1 mutations and FGFR2 fusions, and extrahepatic cholangiocarcinomas has higher rates of KRAS, CDKN2A, and BRCA1 mutations, while GBC had higher rates of homologous recombination repair deficiency and Her2/neu overexpression [39]. These genetic differences

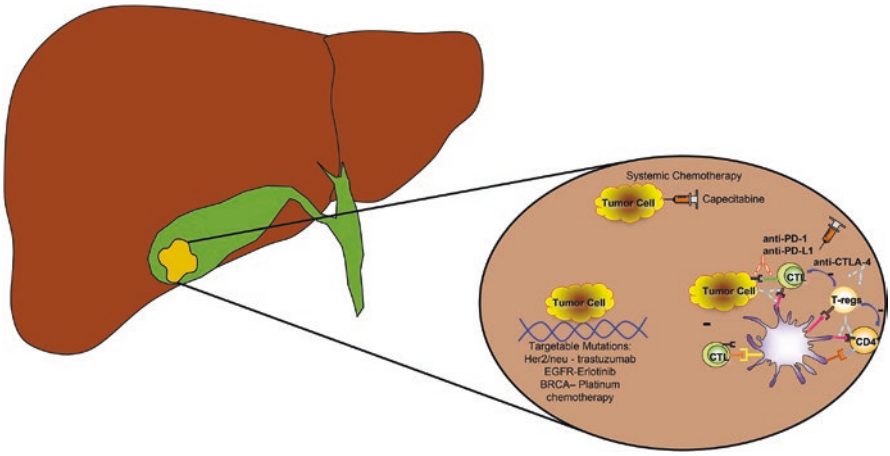

Fig. 4.1 Adjuvant strategies for gallbladder cancer. *T-regs* regulatory T cells, *PD-L1* programmed death ligand-1, *PD-1* programmed death-1, *CLTA-4* cytotoxic T-lymphocyte-associated protein-4, *CTL* cytotoxic T cell

among intrahepatic cholangiocarcinoma, extrahepatic cholangiocarcinoma, and GBC could perhaps account for differences in outcomes noted in randomized clinical trials such as BILCAP, PRODIGE-12/ACCORD-18, and BCAT [40].

The ability to successfully apply the information obtained from next-generation sequencing in still under investigation. Next-generation sequencing has been used to evaluate circulating-tumor DNA (ctDNA) and/or tumor-based DNA among patients with biliary tract cancers. The most common alterations occurred in TP53, KRAS, and PIK3CA for ctDNA versus TP53, CDKN2A, and KRAS for tissue-DNA. Among patients included in this study, 80 patients had systemic therapy initiated after molecular profiling of their tumor; 43% of patients were administered at least one drug matched to their profiling. The matched therapies included targeted therapies for genomic alterations, immunotherapies for PD-L1 immunohistochemistry status, or mismatch repair deficiency, as well as a combination of targeted therapy with immunotherapy based on tumor mutational burden. Interestingly, partial response rate was higher among patients who received the matched regimen versus the unmatched patients (24% versus 4.7%, $p = 0.02$); the progressive rate of disease was lower in the matched patients versus the unmatched patients (39% versus 65%, $p = 0.04$) [41].

Biliary tract malignancies including GBC may have several targetable mutations (Table 4.2). Studies utilizing targeted therapies in patients with GBC are currently under investigation. Similar to ovarian and breast cancers, biliary tract cancers with BRCA mutations are more sensitive to platinum-based therapies [42–44]. Erlotinib, an epidermal growth factor receptor (EGFR) inhibitor, has been shown to have clinical efficacy as monotherapy in patients with advanced biliary cancer; in fact, up to 60–80% of patients with GBC may have HER1/EGFR expression by immunohistochemistry [45]. Erlotinib combined with bevacizumab, a vascular endothelial

Table 4.2 Targetable genetic mutations in biliary tract cancers

Specific target	Prevalence	Therapy
Gallbladder cancer		
Her2/neu	10–16%	Trastuzumab, pertuzumab, lapatinib, T-DM1
CDKN2A/B	6–19%	Palbociclib
AR1D1a	15%	mTOR inhibitor, anti-PD-1 for MSI tumors
KRAS	4–13%	Trametinib, selumetinib
EGFR	4–13%	Afatinib, erlotinib, cetuximab
PIK3CA	6–14%	mTOR inhibitor, AKI inhibitor, PI3K inhibitor
ERBB3	0–12%	Seribantumab, pertuzumab, trastuzumab, T-DM1
PTEN	0–4%	mTOR inhibitor, AKI inhibitor, PI3K inhibitor
Intrahepatic cholangiocarcinoma		
IDH1/2	22%–28%	AG-120, AG-881
BAP1	15%–25%	Vorinostat, panobinostat
FGFR2 fusions	10%–20%	BGJ398, ponatinib, FGFR antibodies
Extrahepatic cholangiocarcinoma		
Her2/neu	11%–22%	Trastuzumab, pertuzumab, lapatinib, T-DM1
PRKACA/B	9%	Protein kinase A inhibitor
ARID1A	5%–12%	Vorinostat, panobinostat

growth factor (VEGF) inhibitor, has shown promising results among patients with advanced biliary tract cancers [46]. However, a phase III study that combined erlotinib with GEMOX did not improve OS or PFS, although it was associated with tumor response [47]. The combination of sorafenib with erlotinib similarly has not produced promising results in patients with cholangiocarcinoma and GBC with a median progression-free survival of only 2 months and median overall survival of 6 months [48]. Amplification of HER2/neu has also been identified in GBC with a prevalence of 4–13% [49]. A retrospective study investigated patients with GBC and cholangiocarcinoma treated with HER2/neu directed therapy. In eight patients with GBC who had HER2/neu amplification, HER2/neu directed therapy produced one complete response, four partial responses, and three patients had stable disease [50].

Over the last several years, the use of immunotherapy including immune checkpoint inhibitors has gained increased interest in the treatment of patients with advanced disease, as well as in the adjuvant setting. Mismatch repair deficiency can predict clinical benefit from immune checkpoint blockade among patients with solid gastrointestinal malignancies, including GBC [51, 52]. Unfortunately, in a series of 321 patients with biliary tract cancers, DNA repair mutations were only identified in 6% of patients with GBC [53]. The use of immunotherapy including immune checkpoint inhibitors is still largely unknown in patients with GBC. In 2017, pembrolizumab, an immune checkpoint inhibitor that targets PD-1, received FDA approval for the treatment of solid tumors, including GBC, with mismatch repair deficiencies [51]. Preliminary data had demonstrated objective radiographic response rates (53%), including complete responses (21%), in patients with advanced solid tumors [51]. While PD-1/PD-L1 expression can

only be found in 18–23% of patients with GBC [54, 55], agents targeting these immune checkpoint proteins can provide acceptable response rates with durable antitumor activity, both in monotherapy and in combination regimens, when administrated to patients with advanced GBC in early phase clinical trials [56–59]. In turn, the use of immunotherapy, especially immune checkpoint inhibitors, has emerged as a promising strategy for patients with GBC, but the absolute benefit is largely unknown and requires further investigation. As such, the use of next-generation sequencing to directed therapy is still under investigation for GBC. In the future, advances in the understanding of molecular characteristics and carcinogenesis of GBC will hopefully be used to develop prognostic biomarkers to guide therapy and risk stratify patients [60]. In particular, the use of microRNAs, tyrosine kinase receptors, or neutrophil-to-lymphocyte ratios may be used in the future as potential biomarkers [60].

4.5 Conclusion

Even after curative-intent resection, patients with GBC still have a high chance of recurrence. Retrospective data on adjuvant therapy have been equivocal, while data from more recent randomized clinical trials have provided level one evidence to inform the use of adjuvant therapy for biliary malignancies. Next-generation sequencing and a better understanding of the underlying genetics of GBC may help to risk stratify patients to improve patient selection and direct decisions around adjuvant therapy. In particular, patients with the highest chance of recurrence are likely to benefit the most from adjuvant treatment strategies. Future work will need to determine molecular or genetic characteristics to assist in proper patient selection for adjuvant therapy, as well as identify the next generation of more effective agents to treat GBC.

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5 Incidental Gallbladder Cancer: The Role of Routine Versus Selective Histopathological Examination of Gallbladder Specimens After Cholecystectomy

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5.1 Introduction

Two thirds of gallbladder cancers are discovered by chance on histopathological examination after cholecystectomy [1, 2]. This condition is referred to as incidental gallbladder cancer (IGC) and occurs in 0.2–3.3% of the specimens [3]. Given its low incidence, there is an argument as to whether histopathological evaluation of the gallbladder should be performed routinely or on a selective basis, after cholecystectomy.

5.2 Routine Versus Selective Histopathological Assessment

Histopathological examination of gallbladder specimens is performed either on a regular basis or according to a more selective approach. The latter entails a critical review of preoperative imaging, intraoperative findings, and inspection of the specimen at the end of the surgery: if the gallbladder is thick-walled, there are dense local adhesions or the mucosa feels abnormal to touch, the specimen is sent to the pathology laboratory [4].

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Regular histopathological assessment of the specimens eliminates virtually the risk of potential oversight of IGC cases [5, 6]. A Swedish study, based on the national registry of gallstone surgery and endoscopic retrograde cholangio-pancreatography [7] found that hospitals who examined systematically all the gallbladders diagnosed a higher proportion of IGC than hospitals where a selective approach was applied. Moreover, once cancer is diagnosed, staging the disease allows the patients to receive the most appropriate treatment; those who are eligible to undergo secondary resectional surgery have improved survival rates [8]. Also, diagnosis of IGC grants the enrolment into follow-up programs. Finally, routine histopathological examination may have medico-legal relevance in case of disputes or diagnostic uncertainty [9].

An argument in favor of a selective approach is that IGC is unlikely to be found in a normal-looking gallbladder [10]. IGC is associated with abnormal preoperative imaging in less than 50% of cases [11], while an abnormal looking gallbladder is detected almost invariably during surgery [12]. The routine practice of submitting all the gallbladders constitutes a significant workload for the pathology departments. Therefore, reducing this practice would save time and costs for unnecessary examinations [13]. Also, since early-stage disease is the most common finding of IGC, cholecystectomy alone would be curative, and no further treatment would be needed [4, 14].

5.3 Histopathology

The majority of gallbladder cancers develop in the fundus (60%), less commonly in the body (30%) and the neck (10%) of the organ [15]. Macroscopically, most early lesions are subtle and appear flat with mucosal granularity and are seen within an area of thickened wall or as a discrete polypoid mass. It is often difficult to distinguish carcinoma from chronic cholecystitis preoperatively or in the operating room or even at the pathology cut-up bench. It has been reported that even thorough macroscopic examination can miss as many as 30% of muscle-confined cases [15, 16]. More frank tumors may show grossly thickened and indurated gallbladder walls with exophytic or polypoid friable mucosal lesions.

The current pathology guidelines advise that if the gallbladder appears macroscopically normal, at least one section of the gallbladder wall (fundus) and one of the cystic duct margins are submitted along with any sampled lymph nodes. If the gallbladder wall is thickened or there are lesions, then more generous sectioning is advised [17]. During histopathological examination, if the pathologist sees dysplasia on any of the sections, this should prompt a return to the specimen to put through the entire gallbladder wall, to exclude the presence of more severe dysplasia or invasive carcinoma [18].

The most common type of gallbladder cancer found is adenocarcinoma (90%). Most gallbladder adenocarcinomas are of biliary type. Other less common types include intestinal type, gastric foveolar type, adenosquamous carcinoma, carcinosarcoma, cribriform carcinoma, clear cell adenocarcinoma, mucinous, signet ring cell, squamous cell, and undifferentiated carcinomas [18]. Tumors are classified based on their differentiation: well, moderate, and poorly differentiated. Staging

depends on the depth of invasion through the gallbladder wall and involvement of the peritoneal surface, adjacent liver, other organs, or major vessels. The important distinction between stage pT1a and pT1b is determined by invasion of the muscle layer of the gallbladder wall. pT2 tumors extend beyond the outer limit of the smooth muscle without involving any of the following structures: peritoneal surface, adjacent liver, extra-hepatic organ. Involvement of the latter constitutes pT3 [18]. Patients with incidental gallbladder cancer should be referred to the hepatobiliary cancer center multidisciplinary team meeting for review and to see if further intervention is warranted. The evidence for selecting patients for further surgery is currently inconclusive and cases should be evaluated on an individual basis; pT1 and superficial/limited pT2 carcinomas are considered to have been successfully treated if the surgical margins are clear.

Inflammation appears to be the main event in gallbladder carcinogenesis, and coexisting pathologies are frequently seen in conjunction with GBC [19, 20], such as acute and chronic cholecystitis, xanthomatous cholecystitis, porcelain gallbladder (hyalinization and calcification of the wall), and dysplasia (intraepithelial neoplasia). Each of these conditions can result in thickening of the gallbladder wall and the appearance of mucosal lesions in their own right and explains how IGC can be easily overlooked and dismissed as an inflammatory process.

5.4 Personal Experience

We reviewed the routine histological examinations of 5779 gallbladder specimens of a population based in the United Kingdom [21]. IGC occurred in six patients (0.1%), there were five women and patients were older than those with benign disease (mean age was 73.7 vs 55.8). Preoperative imaging showed a thick-walled gallbladder in three cases, while in all six the organ looked abnormal upon surgery. On histopathological examination, three patients had locally advanced disease (AJCC stages II, IIIA, IIIB) and three had metastatic disease (stage IVA). In all the six cases, other conditions were found together with cancer: chronic cholecystitis [3], dysplasia [1], chronic cholecystitis and dysplasia [2]. Our results from a low incidence geographical area corroborated data on known risk factors of gallbladder cancer– female gender, advanced age, chronic cholecystitis, and dysplasia.

5.5 Conclusions

IGC occurs more commonly in elderly women with history of symptomatic gallstones.

Views on routine and selective examination of gallbladder specimens are still under debate. While local policies depend on epidemiological, clinical, and financial factors, the presence of a macroscopically abnormal gallbladder on preoperative imaging and/or intraoperative assessment demands histopathological examination of the specimen.

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Minimally Invasive Surgery for Management of Gallbladder Cancer

6

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6.1 Introduction

Gallbladder cancer (GBC) is a disease process that has a diverse variation worldwide. It has a very high incidence in parts of northern India; Karachi, Pakistan; and Quito, Ecuador. It has a notable incidence in South Asia and some central and eastern European countries. Females have a higher incidence, with factors such as cholelithiasis, obesity, and infections related to *Salmonella paratyphi* and *typhi* showing a higher relative risk [1]. A subset of patients with cholelithiasis develops porcelain gallbladder, which is a consequence of a chronically inflamed wall. However, not every patient with a porcelain gallbladder will develop GBC, with the risk more in the range of 10–20% [2]. However, despite these possible etiological factors, the exact pathway behind GBC remains insidious and likely multifactorial [3, 4].

As an anatomic structure it lies below segments IVb and V with close proximity to the portal structures. As it has only a single muscle layer, the tumor has easier access to the serosa, with its close proximity to the structures of the hepatoduodenal ligament often making surgical resection difficult or impossible. The first echelon nodes of drainage are the cystic and peri-choledochal nodes, with further

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connection to the portal and common hepatic artery nodes making their dissection a critical part of any surgical resection [5]. The disease process can be frustrating with delays in diagnosis, resulting in presentation at an advanced stage and resultant incurability. For those who present at an earlier stage, surgery remains the only chance of cure with the addition of adjuvant chemotherapy and radiation providing potential benefit.

In the era of laparoscopic cholecystectomy, the identification of incidental GBC now represents the majority of presentations for this disease process [2]. Gallbladder cancer is a relative rare disease with the incidence rate in the US estimated at approximately 1.2 cases per 100,000 per year. It is associated with a poor prognosis with survival for GBC in recent years being reported at 5–10% for 5 years with a median survival of 3–6 months from time of diagnosis. This has however been improving with groups reporting median survival of 50 months for those amenable to surgical resection. Since George Pack first suggested a radical liver resection for GBC in 1955, treatment paradigms have ranged from partial hepatectomy to wedge resection and even to formal hepatectomy. The advent of minimally invasive surgical techniques, laparoscopy and robotics, to the field of hepatobiliary surgery has further added to the surgical options and bears discussion.

6.2 Operative Indications

Resectability in GBC relies on preoperative staging, considerations of findings at an initial laparoscopy for cholecystectomy or as part of a staging workup. Early tumor stages, Tis, T1, and T2 are resectable in most cases. A routine cholecystectomy is adequate for T1a stages where tumors are confined to lamina propria. When the muscular layer is involved in T1b or greater tumors, a more radical approach is needed because of the higher risk of nodal invasion. Cholecystectomy thus needs to be extended to adjacent liver segments IVb and V. Laparoscopic radical cholecystectomy survival rate at 10 years approaches 90% for T1 disease [6–9].

Stage IV is characterized by vessel invasion, specifically the portal vein, hepatic artery, or more than two extrahepatic organs. The AJCC has established their eighth edition, which now includes a prognostic dichotomy in perimuscular connective tissue invasion. Tumors on the hepatic side, now classified as T2b, have a poorer prognosis and a higher risk of vascular, nodal, and neural invasion. Conversely, tumors which have developed toward the peritoneum are classified T2a. T2 disease, if resectable, must be treated by an extended or radical cholecystectomy [10]. The presence of metastases to periaortic, pericaval, superior mesenteric artery or coeliac nodes or distant metastases further categorizes Stage IVB disease (AJCC/UICC TNM eighth edition) and contraindicates any surgical treatment.

The extent of liver resection needs strong consideration, with differing opinions on nonanatomic resections versus complete resection of segments IVB and V. Either is feasible with the achievement of R0 the governance behind these decisions. A major hepatectomy may be a consideration in those instances when an R0 resection is to be achieved with groups suggesting no difference between minor or major hepatic resections. Nodal disease stretching down the portal chain to lie posterior to the pancreas or duodenum may present a challenge. For those that are not invading

vascular structures, resection is often achieved satisfactorily; however, larger nodes may not be removed successfully without the addition of a pancreatoduodenectomy. The decision to pursue this course of action must be considered with caution due to the significant morbidity and mortality associated with this procedure when compared to radical cholecystectomy alone. Pooled analysis suggests that involvement of the common bile duct is associated with advanced T stage and is an independent prognostic factor in survival. Positive margins at the cystic duct mandate further resection of the common bile duct to achieve R0 resection, which while a poor prognosticator has a better survival outcome than R1 resection [11].

6.2.1 Lymphatic Nodes Resection

Hepatoduodenal ligament lymphadenectomy must be done for staging and to reduce the risk of local recurrence. Aorto-caval node removal has no benefit on survival; however, its frozen sampling at the beginning of surgery may have an impact on the procedure because a positive result may preclude surgical resection with patients then being treated with chemotherapy alone [12, 13].

6.2.2 Preoperative Evaluation

Most patients are referred for resection with a diagnosis of GBC of either a resected specimen for routine gallbladder surgery or after fortuitous diagnosis on imaging. While distant metastatic disease is a formal contraindication for resection, pertinent imaging investigations must be engaged to assess liver and nodal involvement [13].

6.2.3 Imaging

Ultrasound scan (USS) is often the first imaging technique in those undergoing cholecystectomy for unsuspected GBC, due to its greater sensibility and sensitivity in diagnosing cholelithiasis. High-resolution imaging is required to assess resectability, while USS is limited in the diagnosis of early lesions and as such unreliable for staging. An abdominal computerized tomography (CT) scan is thus needed to assess nodal status, local invasion involving adjacent organs, vessels, and peritoneum. An abdominal MRI is also recommended to better analyze hepatic parenchymal and biliary ductal involvement, with chest CT utilized to rule out thoracic lymph nodes and/or metastases [13].

In some 20–30% of cases, GBC may present as an asymmetric wall thickening, which has an expanded differential diagnosis ranging from cholecystitis, adenomyomatosis, acute hepatitis, portal hypertension to congestive heart failure. In cases where a mass occupying lesion is noted, as is the case in some 40% of patients, USS with a heterogenous and hypoechoic tumor is classic [9]. Asymmetric wall thickening with persistent arterial enhancement or isodensity during hepatic venous phase should, however, heighten suspicion. Furthermore, GBC arising on a background of chronic inflammation certainly makes radiological interpretation more difficult [13].

The role of 18F-fluorode-oxyglucose positron emission tomography (FDG-PET) in GBC is still in flux. With the observation that GBC is often highly PET avid, studies have suggested that it may change operative decisions in some 25% of cases. In cases of incidentally found GBC, up to 50% of cases were noted to have metastatic spread by FDG-PET, for instance on laparoscopy incisions. While these are highly suggestive, false-positive results may be noted in areas of inflammation from recent laparoscopic cholecystectomy, with recent data also noting FDG-PET to have negative predicted value of 65%, suggesting a greater extent of residual disease might be missed. It may be that the role of FDG-PET is best served to rule out distant spread while residual disease is best assessed at re-exploration [14, 15].

6.2.4 Other Complementary Investigations

Preoperative routine work-up including chest X-ray, EKG, serum complete blood count, chemistries, liver function studies, and serum tumor markers consists of CEA and CA 19-9 levels. A DPD gene sequencing is also needed if a 5-fluorouracil chemotherapy treatment is considered. A nutritional support needs to be engaged for every cancerous disease, hence the need to assess a baseline in vitamin serum levels, coagulation factors, albumin, and prealbumin. Indocyanine Green (ICG test) is a routine test in Asia because of the high rate of hepatitis-related cirrhosis in the population that may influence the extent of surgery. All planned hepatectomies should be assessed for residual liver volume, Child classification, and pathologic examination for underlying liver cirrhosis grade especially if the liver is not chemotherapy naive, thus more vulnerable [16].

6.2.5 Preoperative Laparoscopy

GBC has a rate of peritoneal metastases that ranges from 25% to 70% with T stage being a strong correlate. It is still unfortunate that despite high-quality preoperative imaging up to 20–50% of laparoscopies have a diagnostic yield. As a preoperative tool, it is unlikely to be warranted if a recent LC detecting GBC has been performed, and if adequate thought has been given to peritoneal and hepatic evaluation [17]. Such exploration is thus considered for only stage pT2a or higher, to be done with a laparoscopic ultrasound if available so that the hepatic parenchyma to the surface can be evaluated [17].

6.3 Laparoscopic Radical Cholecystectomy

6.3.1 Patient Positioning

The patients should ideally be placed in a modified lithotomy position with both arms out for the anesthesiologists. The patient is on a bean bag with a lower strap across the pelvis to assist when extended lateral positioning is needed. For formal right hepatectomies, a bump to elevate the retroperitoneal aspect can be

used. Two monitors are placed at the head of the patient for the surgeon and assistant [18] (Gumbs and Hoffman 2010).

6.3.2 Positioning and Placement of Trocars

Entry into the abdomen is achieved with either a Veress needle or Hassan open technique. The Veress needle is positioned under either subcostal region. Entry is confirmed by initial aspiration and then verification of intra-abdominal pressure. After establishing pneumoperitoneum, a 12 mm trocar is placed a hands breath below the subcostal cage in the medial midclavicular line (Fig. 6.1) [18–20]. This is achieved through a modified open technique with dissection to the anterior fascia and using needle aspiration to confirm lack of adhesions. The remaining tracers are placed under direct visualization. The trocars are generally placed in a curved line extending across the midline with a 10/12 mm port placed 4 finger breath lateral to the optic port and a further 5 mm port on the opposite side. A final 5 mm port is placed on the medial aspect, subxiphoid, along the curvilinear line. Generally, four trocars are necessary and can be placed so that they can be utilized for conversion if necessary (Fig. 6.1).

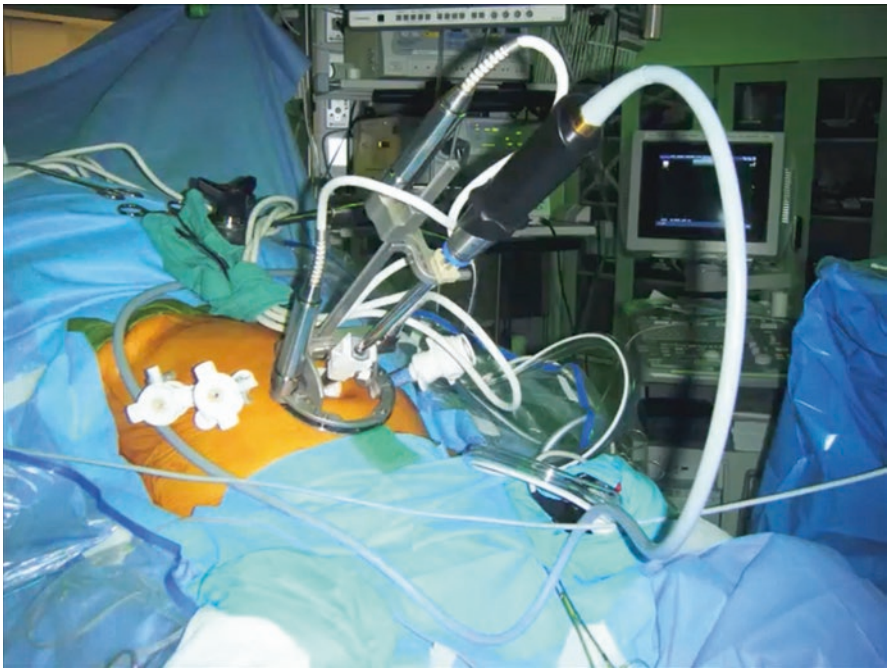

Fig. 6.1 Trocar placement. Patient is in the French-position with the surgeon operating in-between the legs. A robotically controlled laparoscope holder is being used (ViKY, Videoendoskopy, Endocontrol, Grenoble, France)

6.3.3 Exposure

The abdomen is initially inspected for evidence of peritoneal carcinomatosis. Any suspicious lesions are biopsied and sent for frozen section. Positive biopsy results preclude resection. The operation proceeds if there is no evidence of carcinomatosis. Adhesions to the gallbladder fossa are preserved while other adhesions are lysed using ultrasonic shears. Hepatic ultrasound is performed to evaluate the liver for metastasis and to locate the extent of parenchymal disease to assure a margin free transaction [18]. The patient is placed into 45° reverse Trendelenburg position, and the liver is retracted superiorly via the assistant's medial port to expose the tumor (Fig. 6.2).

6.3.4 Dissection

Using the ultrasound dissector in one hand and a laparoscopic bipolar device (Medtronic, Jacksonville, FL, USA) in the other hand, the pars lucida of the lesser omentum is incised to expose the common hepatic artery. Lymphadenectomy

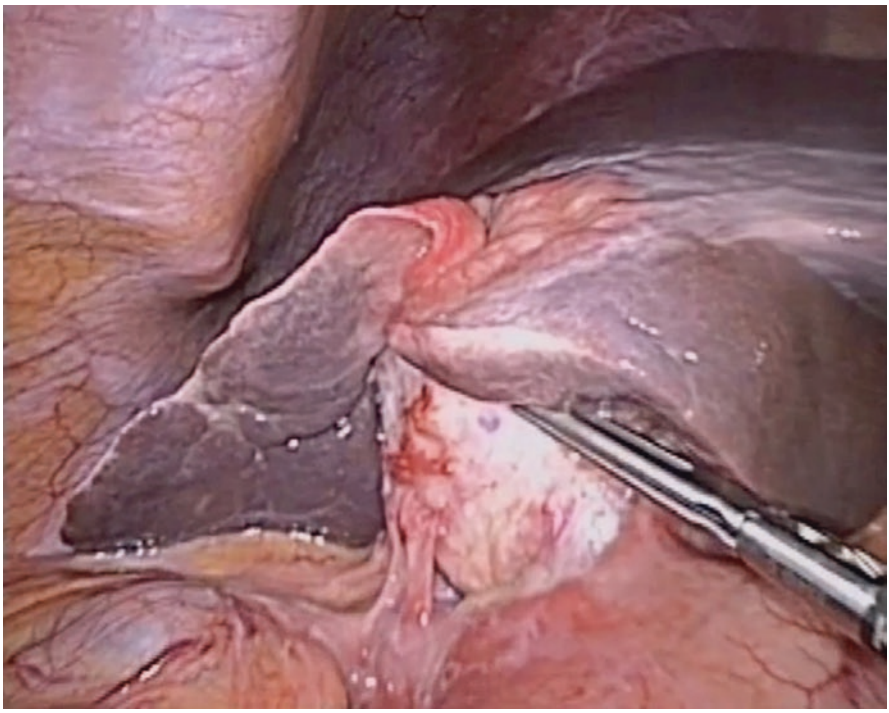

Fig. 6.2 A 5 cm T3 gallbladder cancer diagnosed preoperatively. The patient ultimately required laparoscopic radical cholecystectomy, common bile duct excision, and Roux-en-Y hepaticojejunostomy

begins at the common hepatic artery lymph node and proceeds toward the *porta hepatis*. All lymphatic containing tissue is dissected, from the superior portion of the duodenum to the liver hilum, exposing the gastroduodenal artery, proper hepatic artery, and bifurcation of the right and left hepatic arteries [19]. During this dissection, the portal structures are assessed for invasion by tumor and the common bile duct (CBD), hepatic arteries, and portal vein are skeletonized (Fig. 6.3). Up to 10% of patients can have anatomical variations such as a replaced right hepatic artery arising from the superior mesenteric artery (Fig. 6.4). If the patient had a cholecystectomy previously and the cystic duct margin was assessed and negative for malignancy, the liver parenchymal dissection is initiated. If the cystic duct margin was not assessed in the previous operation, it is imperative that the residual cystic duct be identified, resected, and sent to pathology for frozen section. A positive cystic duct margin warrants resection of the common bile duct with reconstruction to achieve negative margins, which can be done laparoscopically [20].

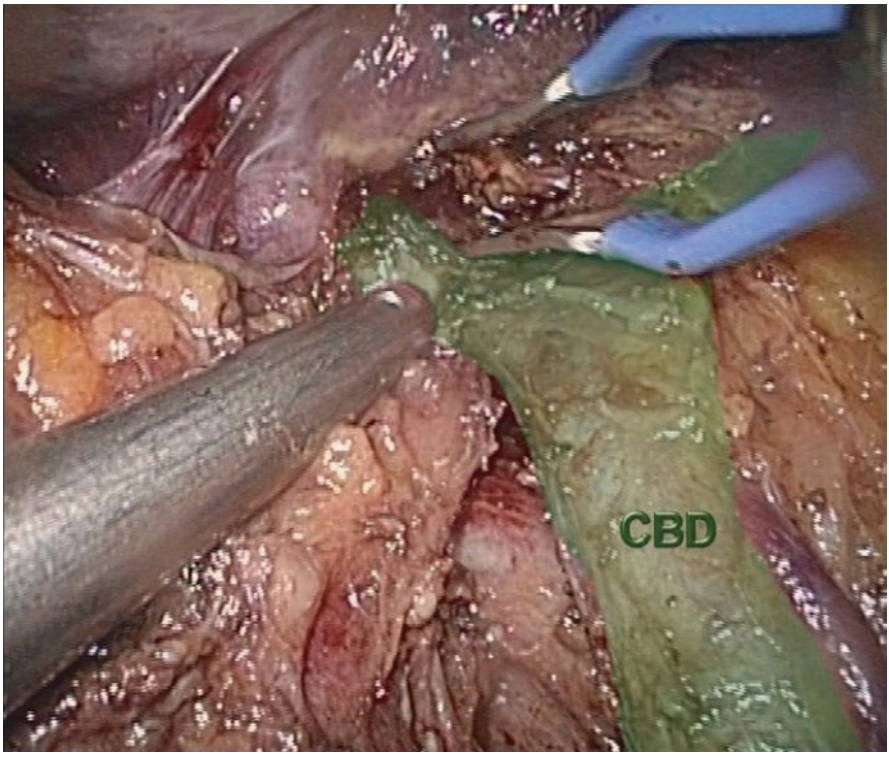

Fig. 6.3 After the hepatoduodenal ligament is incised, the lymph nodes in the portal triad are removed, so that the common bile duct (CBD) is skeletonized. Between the laparoscopic bipolar forceps and the aspiration device is seen the right hepatic duct

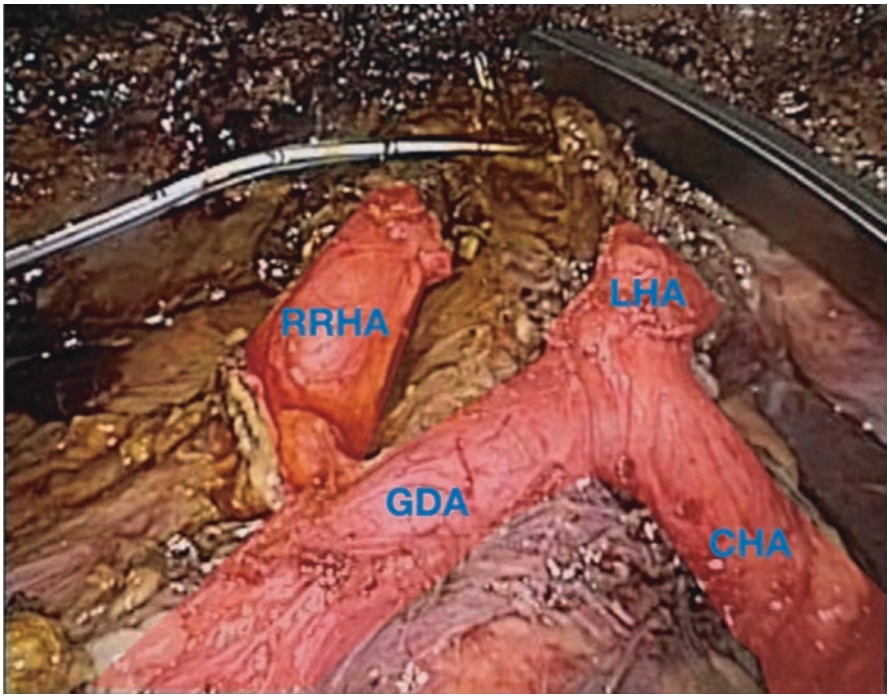

Fig. 6.4 Patient with invasion into the common bile duct (CBD) necessitating a CBD excision. This patient also had a replaced right hepatic artery (RRHA) coming off the superior mesenteric artery. The common hepatic artery (CHA), gastroduodenal artery (GDA), and left hepatic artery (LHA) are all labeled

6.3.5 Resection

Wedge resection of the gallbladder bed is begun by confirming the extent of resection using the laparoscopic ultrasound. If the gallbladder is still present, it is left attached to the gallbladder fossa for *en bloc* resection. Hepatic parenchymal resection is performed with the ultrasonic shears in one hand and the laparoscopic bipolar device (Medtronic, Jacksonville, FL, USA) in the other (Fig. 6.5). The laparoscopic ultrasound can be used to identify the middle hepatic vein in the superior aspect of the gallbladder bed prior to transection with clips or ultrasonic shears. The assistant's role during parenchymal transection involves retraction and suction with the laparoscopic aspirator. The laparoscopic bipolar device helps to achieve hemostasis during parenchymal transaction [19, 20].

The specimen is placed in a specimen retrieval bag and removed from the abdomen. It is then sent to pathology for frozen section analysis of the cystic duct margin and hepatic parenchymal margins [18]. Additional parenchymal margins are taken if pathology reveals a positive margin. If the cystic duct margin is positive for malignancy, a resection of the common duct can be performed laparoscopically or after

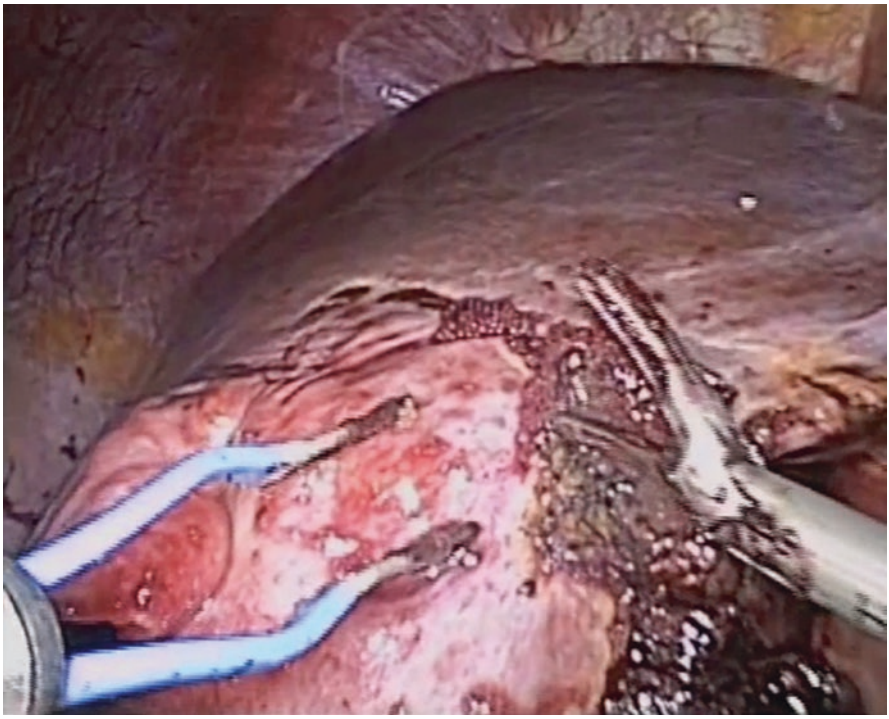

Fig. 6.5 The hepatic parenchymal dissection is done with the ultrasonic shears in one hand, and the laparoscopic bipolar device is used to obtain hemostasis simultaneously

conversion to an open approach. The common bile duct is dissected circumferentially and transected below the confluence of the right and left hepatic ducts. The free end of the common bile duct is grasped, retracted anteriorly and inferiorly, and transected at the most distal extrapancreatic portion of the duct using a vascular stapler [19–21].

6.3.6 Choledochojejunostomy

If frozen section analysis of the common bile duct reveals negative margins, reconstruction can be done laparoscopically via choledochojejunostomy. The ligament of Treitz is identified, and the small bowel is transected approximately 45 cm distal using a GIA stapler. The Roux limb is positioned adjacent to the common bile duct to assess whether the anastomosis will have any tension. The jejunum can be further mobilized by dividing a small portion of the mesentery using the ultrasonic shears. An enterotomy is made in the Roux limb using the ultrasonic shears [21, 22]. A 5-Fr. pediatric feeding tube is placed across the biliary anastomosis to prevent inadvertent closure of the lumen during construction of the anastomosis (Fig. 6.6). A single layer, running anastomosis is created using a 4–0 absorbable suture (Fig. 6.7).

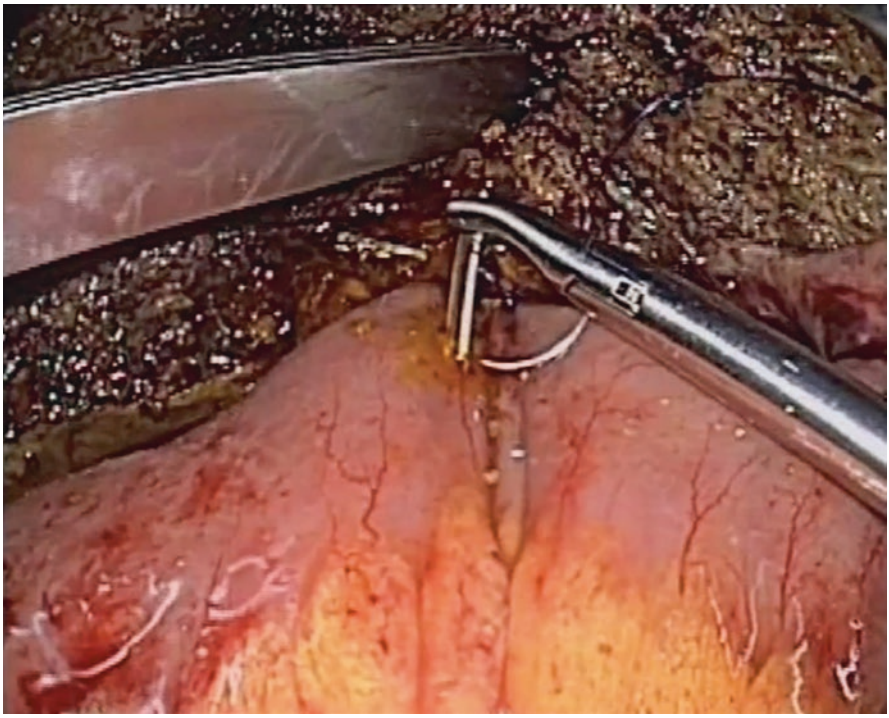

Fig. 6.6 Laparoscopic Roux-en-Y hepaticojejunostomy after common bile duct (CBD) excision. The Roux limb is seen at the bottom of the image. A 5 Fr. pediatric feeding tube is being placed into the small intestine as an internal biliary stent to prevent inadvertent closure of the bile duct

The jejunojejunostomy is created using a GIA stapler in a side-to-side fashion. The remaining enteroenterostomy is closed with running 3-0 silk suture. This anastomosis could also be created extracorporally via the extraction site. The abdomen is irrigated and suctioned free of excess fluid. It is not our routine to place closed suction drains. All fascial incisions larger than 5 mm are closed [22].

6.3.7 Postoperative Care

Low molecular weight heparin is given preoperatively and continued postoperatively unless there are signs of bleeding or evidence of coagulopathy. If a significant amount of liver was resected, coagulation factor levels and prothrombin time are checked for the first three postoperative days and low molecular weight heparin continued if these remain within normal limits. Liver function tests can be followed for signs of regeneration or failure. Nasogastric tubes are not left in after surgery unless patients have a history of gastroperesis. Early ambulation is encouraged as is incentive spirometry. Diet is advanced as tolerated to a low fat one if the gallbladder has been removed [23].

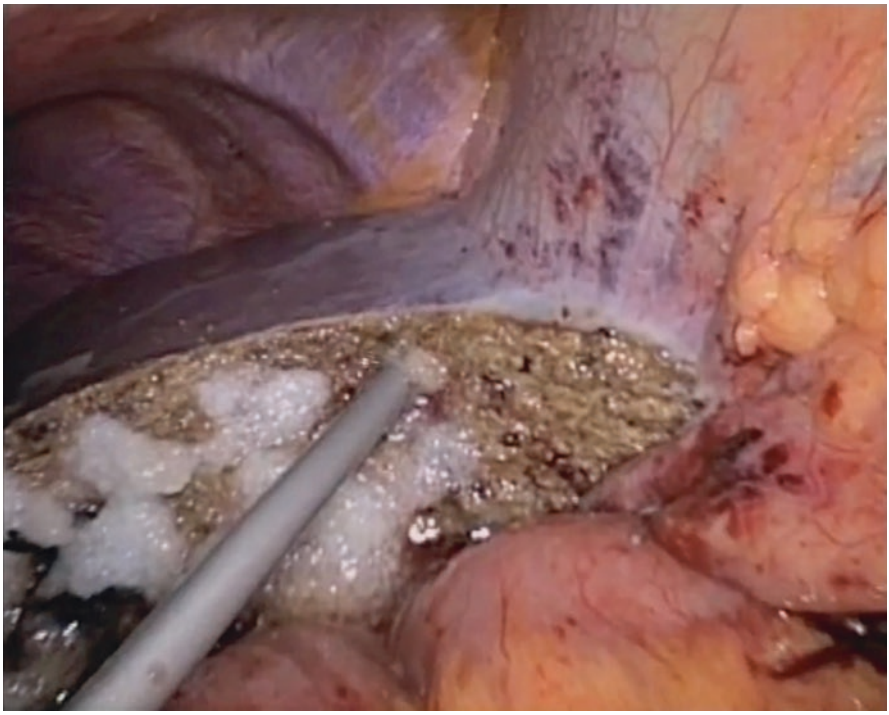

Fig. 6.7 Laparoscopic radical cholecystectomy and Roux-en-Y hepaticojejunostomy. The Roux limb is seen at the bottom of the image. In this patient, complete resection of hepatic segment IVb was necessary due to the extent of the hepatic parenchymal invasion

6.4 Complications

6.4.1 Bleeding

The major source of bleeding during this procedure arises from injury to the middle hepatic vein during parenchymal transection of segments IVb and V. This can be avoided through the use of intraoperative ultrasonography. If bleeding persists from the middle hepatic vein after transection, control is obtained with gentle pressure and then clip placement. Segmental vessels encountered during parenchymal resection can be clipped. Hepatic parenchymal bleeding is controlled with a combination of techniques including use of the laparoscopic bipolar device mentioned previously, maintaining low central venous pressures during parenchymal transection and meticulous dissection. The liver is inspected for bleeding after decreasing the amount of pneumoperitoneum to assure that small amounts of bleeding are not being controlled by the positive pressure of insufflation prior to abdominal closure [24, 25].

6.4.2 Bile Leak

At the conclusion of hepatic resection, the transected liver surface should be inspected carefully for signs of bile drainage. Any areas of bile leak can be over sewn or clipped. Prevention of bile leak is through meticulous dissection and understanding of liver anatomy. Postoperative biliary fluid collections are treated with percutaneous drainage. These fluid collections may evolve into a biliary fistula if drainage continues. As mentioned, drain placement is not routine, except if a biliary anastomosis is created [24].

6.5 Discussion

Minimally invasive surgical resection of gallbladder cancer was reported in 2008 and consisted of an extended right hepatectomy for a T2 gallbladder cancer [26]. Since then, multiple international centers have reported case series on laparoscopic radical cholecystectomies for gallbladder with comparisons with open procedures [27]. Over the last decade or so, studies comparing laparoscopic and open surgery for gallbladder cancer have reported similar lymph node retrieval with an average of eight vs. nine lymph nodes retrieved whether or not laparoscopy or open resections are done, respectively (Table 6.1).

Operating room times have also been similar with a mean of 222 min for laparoscopy compared to 226 min for open resections. This is possibly due to the likelihood that larger more difficult to resect tumors are being resected minimally invasively. This confounding factor could explain the tendency for decreased estimated blood loss (EBL), complication rate, and length of stay (LOS) when surgery for gallbladder cancer is done laparoscopically compared to via laparotomy, 265 mL, 9.6%, and 5.5 days vs. 308 mL, 14.8%, and 10 days, respectively [28]. As a result, comment on overall survival rates between these 2 surgical approaches is currently not possible. This is highlighted by the high degree of variability in TNM stage for resected gallbladder cancer among the reported literature. As robotic surgery becomes more prevalent, a less clear pattern is seen whether or not resections are done robotically or via laparotomy [29]. Although lymph node retrieval is similar between robotic and open cases, operating room times are longer when hepatic resection are done robotically with the mean averaging 236 min compared to 200 min, respectively (Table 6.2). Furthermore, there does not seem to be an advantage to LOS with hospitalizations averaging 5.1 days after robotic resections compared to six for open ones. That said, EBL and complication rates certainly appear improved when hepatectomy for gallbladder cancer is done robotically with EBL averaging 220 mL vs. 535 mL, robotic and open, respectively. Lastly, the complication rate seems to be lower after robotic resections with a mean complication rate of 7.7% vs. 19.2%, when resection is done robotically compared to via laparotomy.

Table 6.1 Literature review of laparoscopic vs. open hepatectomy and hepatoduodenal lymph node dissection for gallbladder cancer T1b-T3 (*LNN* lymph nodes, *OR* operating room, *EBL* estimated blood loss, *Conv.* conversion, *OS* overall survival, *LOS* length of hospital stay)

	(n)	LNN	OR Time	EBL	Conv.	Complication (%)	OS (%) 5 year	LOS
Gumbs et al. (2013)								
Laparoscopic	15	4 (1–11)	220 (120–480)	160 (0–400)	6.7	0		4 (2–8)
Agarwal et al. (2015)								
Laparoscopic	24	10 (4–31)	270 (180–240)	200 (100–850)		4.2		
Open	46	11 (5–26)	240 (180–360)	275 (100–800)	NA	8.7		
Feng et al. (2019)								
Laparoscopic	41	5 (3)	137	358 (390)		7	T2 = 48.1 T3 = 12.5	5 (3)
Open	61	5 (3)	168	386 (391)	NA	9.8	T2 = 64.7 T3 = 16.0	11 (5)
Jang et al. (2019)								
Laparoscopic	55	7.6 (3.4)	231	225 (328)		12.7	73.1	5.8 (5,3)
Open	44	9.9 (6,8)	252	310 (260)	NA	13.6	65.7	9.5 (4,8)
Nag et al. (2021)								
Laparoscopic	30	11.9 (5.2)	286	158 (85)	23.3	16.6	79	6.4 (3.1)
Open	38	12.7 (4.2)	274	219 (87)		31.5	62	9 (8.0)
ALL								
Laparoscopic	165	7.8 (1–31)	222	265	17.8	9.6		5.5
Open	189	9.1 (3–26)	226	308	NA	14.8		10.0

Table 6.2 Literature review of robotic vs. open hepatectomy and hepatoduodenal lymph node dissection for gallbladder cancer T1b-T3 (*LNN* lymph nodes, *OR* operating room, *EBL* estimated blood loss, *Conv.* conversion, *LOS* length of hospital stay)

	(n)	LNN	OR time min.	EBL mL	Conv.	Complication % (n)	LOS
Goel et al. (2019)							
Robotic	23	10 (2–10)	295 (200–710)	200 (20–700)	14.8 (n = 4/27)	4.3 (1)	4 (2–12)
Open	70	9 (2–25)	200 (85–400)	600 (50–3000)	NA	21.4 (15)	5 (3–15)
Ahmad (2020)							
Robotic	10	5 (2–8)	173 (95–240)	88 (30–200)	0	10 (1)	3.5 (2–6)
Yoonhyeong et al. (2020)							
Robotic	16	7.2 (3.3)	198.3	295 (200–635)	0	6.3 (1)	7 (6–8)
Open	34	7.4 (4.5)	200	400 (240–850)	NA	14.7 (5)	8 (8–10)
Araujo et al. (2020)							
Robotic	3	4 (3–6)	392	186	0	0	2
Byun et al. (2020)							
Robotic	13	7.2 (3.1)	188	271	NA	15.4 (2)	6.6
ALL							
Robotic	65	7.7	235.5	219.7	5.8	7.7	5.1
Open	104	8.5	200.0	534.6	NA	19.2	6.0

The large variation in degree of hepatic resection for gallbladder cancer makes any comparisons between approaches with the current literature available extremely difficult. Also, the large heterogeneity in pathological staging among the published literature make meta-analyses difficult. Gallbladder cancer is different from other hepatobiliary malignancies in that the degree of resection varies dramatically based on the degree of tumor invasion with dramatically different hepatic parenchymal resection requirements and overall survival depending on T-stage. There is also a large degree of debate as to how many lymph nodes need to be retrieved and some centers still insist on systematic CBD resection regardless of tumor involvement.

6.6 Conclusions

Although laparoscopic cholecystectomy is an accepted standard for the treating of biliary colic and cholecystitis, laparoscopic management of gallbladder cancer staged T1b or higher is less discussed. As the skill of laparoscopy further emerges allied with technological advancement in parenchymal transection and tumor mapping, the role of laparoscopic radical cholecystectomy is likely to come to the fore. Carcinologic quality concerns should not be held against laparoscopic gallbladder cancer surgery anymore, even if GBC is discovered during routine surgery for another disease [6, 30]. Lymph node yield and excision are comparable between minimally invasive and open approaches [2]. Bile leaking during procedure explains dissemination and recurrence rates in old laparoscopic series of cases, and at the same time, GBCs are being diagnosed pre-operatively more frequently. Thus, any procedure should seek to avoid leakage of bile from the gallbladder. There is indeed no evidence that laparoscopy worsens overall survival as long as definite resection is complete [12]. Minimally invasive approaches may not be suitable for extended liver invasions in nonexpert centers and for acute cholecystitis on nonoperated GBC [17, 28].

Conventional (open) and minimally invasive approaches (laparoscopic and robotic) for gallbladder cancer surgery have been reviewed with comparison on lymph yield, operating time, blood loss, conversion rate, overall survival, complication rate, and hospital stay. Most of all cases had negative margins reported by authors. Most tumors were T2–T3 adenocarcinoma. Robotic surgery offers multiple theoretical advantages such as stabilization of hand tremor, functional imagery with indocyanine green [16] for vessel dissection, and a three-dimensional imaging. Although some studies found that laparoscopic approaches for gallbladder cancer surgeries may yield less lymph nodes than the open approach, this was not found when multiple studies were reviewed, and furthermore, no significative difference in survival was found [25]. Operative time, blood losses, and complication rates often appear to be in favor of minimally invasive surgery [27, 31, 32]. Moreover, such procedures seem to yield shorter hospital stays [33, 34].

As a result, it appears that a randomized-controlled trial (RCT) will be necessary to truly see if there are any benefits to the minimally invasive approach for these tumors. That said, due to the degree of societal acceptance of minimally invasive techniques, an RCT comparing laparoscopy with robotic resections might be even more interesting.

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Thick-Walled Gallbladder: Differential Diagnosis and Surgical Approach to a Thickened Gallbladder

7

Ashish Gupta

7.1 Introduction

Gallbladder forms an integral part of the human biliary system. This acts as a reservoir for bile and helps to concentrate the biliary secretions. The gallbladder contracts and releases the biliary fluid into the alimentary canal in response to the intake of fatty food by the individual [1]. Hormones like cholecystokinin and secretin are primarily involved in this physiological activity. The stasis of bile and concentrating capacity of the GB predisposes this organ to various benign and malignant pathological process. The stasis of the bile causes nucleation of the bile salts, and this along with hypokinesia leads to the formation of gall stones. The repeated inflammation of the gallbladder and blockade of the cystic duct due to the stones cause cholecystitis and subsequent thickening of the GB wall [2].

The GB is very thin walled, and this may be attributed to lack of submucosa in the GB wall [3]. The absence of submucosa causes early migration of inflammatory and malignant cells to the serosa and subserosal layer. The gallbladder wall usually measures less than 3 mm on ultrasonogram. The thickness of more than 4 mm is considered as thick-walled GB [4]. There are multiple pathologies local and systemic and benign as well as malignant that can have thickened GB (Table 7.1).

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Table 7.1 Various pathologies causing thickened gallbladder

Benign	Malignant
Acute calculus/Acalculous cholecystitis	Carcinoma gallbladder
Chronic cholecystitis	Neuro endocrine carcinoma gallbladder
Xanthogranulomatous cholecystitis	Mesenchymal tumors like rhabdosarcoma, angiosarcoma
Pyocele/mucocele	Gastrointestinal stromal tumor
Gallbladder polyp	Malignant tumors of liver, duodenum, colon infiltrating the GB wall
IGG4 related cholecystitis	
Vascular lesions like hemangioma/ arteriovenous malformation	
Hydatid cyst and biliary worm infestation	
Tuberculosis of the gallbladder	

7.2 Pathogenesis of the Thickened Wall

The common entity that precipitates the thickened wall is the GB calculus (Fig. 7.1). The formation of the calculus can lead its impaction in the neck and the Hartman pouch of the GB. This impaction causes the increased backpressure changes which along with repeated attacks of inflammation lead to thick-wall GB. The increased backpressure changes might lead to rupture of Rokitansky-Aschoff sinuses leading to seepage of bile and biliary pigments in the muscular layer. This seepage leads to further inflammation and thickening of the wall. Chronic impaction and inflammation might precipitate metaplasia and carcinoma sequence that leads to the malignancy of GB wall. The formation of GB calculi also causes injury to lining epithelium of the GB predisposing this to granulomatous inflammations like schistosomiasis and tuberculosis.

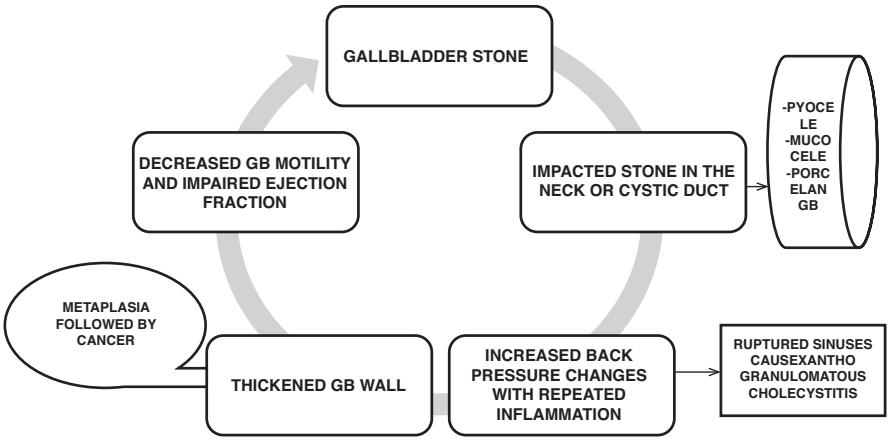

Fig. 7.1 Pathogenesis of the gallbladder thickening

7.3 Differential Diagnosis

7.3.1 Xanthogranulomatous Cholecystitis

Xanthogranulomatous cholecystitis (XC) is a rare form of chronic inflammation of the gallbladder wall. It is characterized by the formation of xanthogranuloma, foam cells, and fibrosis. XC camouflages the malignancy and usually proves a nightmare for the surgeon. As already discussed, the rupture of sinuses causes bile spillage in the muscular layer which causes the above changes [5, 6]. The peri cholecystic inflammation leads to adhesions between the GB, liver, and adjacent viscera. Chronic inflammation and infection can cause pericholedochal, periportal lymphadenopathy. Many a time past history of TB along with XC can be clinically treated as carcinoma [7].

Radiology shows a typically thickened GB wall with irregular thickening and presence of stones. Multiple calcifications could also be visualized in the GB wall [8]. The spillage and inflammation of the GB wall lead to the formation of collection/chronic inflammation typically seen as infiltration into the liver bed. Contrast-enhanced CT scan is usually required as these cases are managed on the lines of malignancy [7] and undergo further management as per the protocol followed in malignancy.

7.3.2 Chronic Cholecystitis

Chronic cholecystitis occurs secondary to gall stone disease. Repeated impaction of gallstone in the neck leads to colicky pains and mild inflammation of the GB wall which subsequently leads to fibrosis and thickening of the wall [1]. This along with impacted stone in the neck leads to the formation of mucocele and infection which

is called as pyocele. These conditions are commonly encountered during clinical practice. Patients with these conditions present with pain abdomen radiating to the right shoulder and fever. History of multiple episodes of such pain may be elicited. The ultrasonogram shows a thick-walled GB along with multiple radioopaque structures with distal acoustic shadowing [9]. These intraluminal lesions change the position along with the position of the patient. This is used as one of the differentiating tests between a GB polyp and calculus. The treatment of patients with normal wall thickness and symptomatic stones is cholecystectomy.

7.3.3 Gallbladder Polyps

Polyps in gallbladder affect around 5% of the population [10]. These lesions are defined as protrusion of the mucosa in the lumen of the organ. The polyps of the gallbladder can be true polyps or the pseudo polyps (Fig. 7.2). True polyps can be malignant or benign. Single, sessile, and polyps >10 mm with wall thickness more than 4 mm can be malignant, whereas the smaller ones usually turn out to be benign ones [11]. Multiple polyps are usually benign and contain cholesterol crystals or inflammation. Patients having polyps should be evaluated for symptoms and other gallstone pathologies like cholelithiasis. The gallbladder shows smooth pedunculated or sessile soft tissue mass protruding into the lumen. These masses lack the

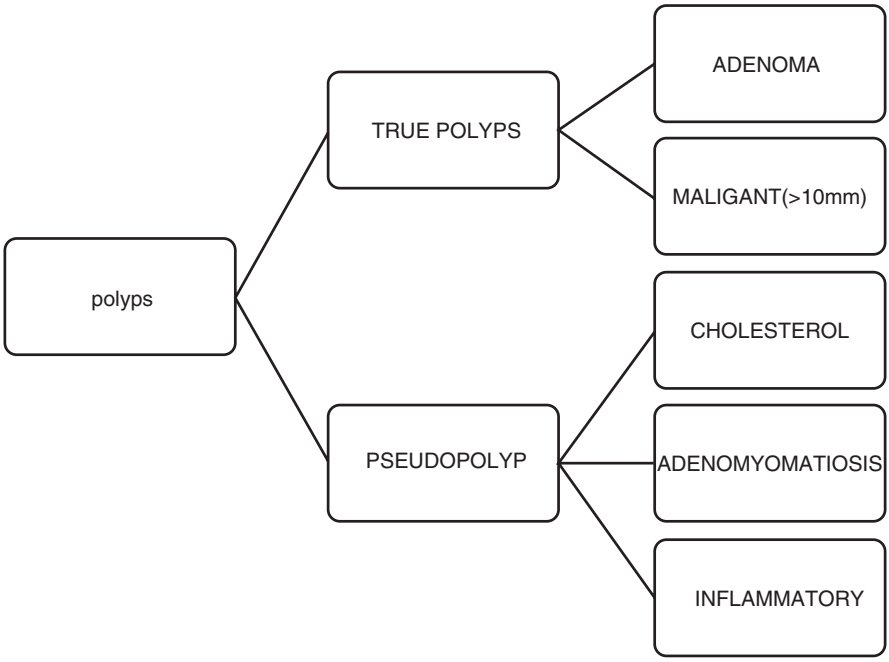

Fig. 7.2 Classification of the gallbladder polyps

capability to change the position and posterior acoustic shadowing which differentiates this from calculus. Patients having symptoms attributable to the gallbladder and a definitive pathology should be assessed and prepared for cholecystectomy. Patients who are having gallbladder polyps and deemed unfit for surgery should be kept on regular follow-up. Patients with single polyp less than 10 mm should be kept on regular follow-up [11].

7.3.4 Gallbladder Tuberculosis

The gallbladder inherently possesses the resistance to tubercular infection. The alkaline nature of the bile and continuous mucosal lining prevent these infections [12]. However, calculus in the gallbladder cause injury to the mucosal lining and predisposes this organ for granulomatous inflammation. The dysmotility and stasis of the biliary fluid also have synergistic effect on the infections. Gallbladder is usually involved as a part of the miliary process, direct infiltration of the gallbladder along with the adjacent viscera, and hematogenous spread from the focus elsewhere in the body. The TB cause thickened gall wall can cause mass formation and can have associated lymphadenopathy [13]. Tuberculosis of the GB is a typical mimicker of the malignant disease. The patients in endemic zone of the cancer are usually treated on the lines of malignancy, and they undergo staging and surgery on the malignant lines. There are subtle signs that can lead the physician toward its diagnosis; however, there is a very thin line of distinction between the two.

Biochemical and hematological investigations can help to establish granulomatous infection, but these are nonspecific and lack definitive role in the assessment of these individuals [14]. Ultrasound and cross-sectional imaging can aid in the distinction between the two. Discrete nodules and peritoneal deposits signify metastatic disease, and this favors malignancy; however, presence of thickened omental and the mesenteric fat along with caking and matting of the bowel loops with inflammatory stranding favors benign infective etiology [12] (Fig. 7.3).

Presence of ascites and peritoneal nodules can be misleading as these can be a presentation in both the diseases. The ascetic fluid cytology and laparoscopic-assisted peritoneal biopsy are definitive aids to establish the diagnosis. Preoperative histopathology although gold standard is possible only in surgically unresectable and advanced disease.

7.3.5 IGG4-Related Cholecystitis

IGG4-related cholecystitis is a part of the systemic disorder which leads to autoimmune involvement of various glands and organ systems of the body [15]. The systemic involvement usually causes fibrosis and destruction of these organs.

IGG4 cholecystitis is an uncommon entity seen as a part of the systemic sclerosing cholangitis. There are two morphological forms of the disease. One form is the

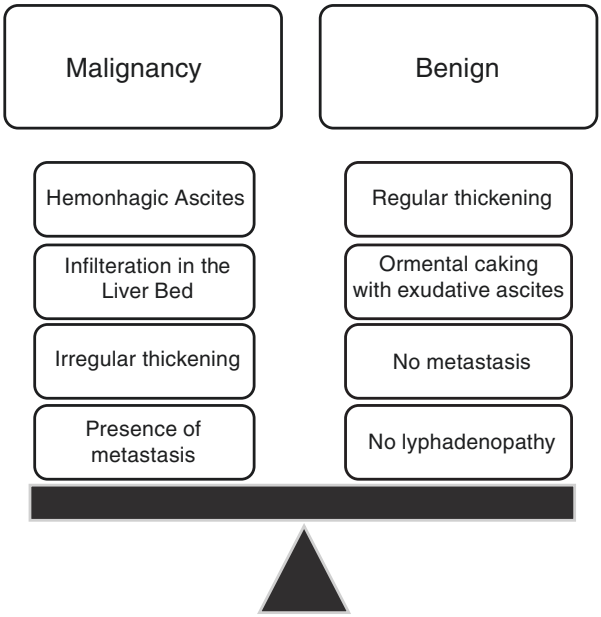

Fig. 7.3 Differentiation between gallbladder tuberculosis and cancer

mass forming type, and other one is the diffuse thickening of the gallbladder wall. Only few cases are reported in English in literature. Till 2018, only 13 cases of IGG4-related cholecystitis have been reported. The age of these patients varied between 18 and 82 years. Fundus of the gallbladder was commonly involved and neck was involved only in few cases. Most of these patients underwent surgery, and nonoperative management was possible only in few cases [16].

Histopathology of the resected specimen offers the only chances of definitive diagnosis. The diagnosis is confirmed by clinical and histological involvement of various endocrine and exocrine glands. These glands typically show infiltration by the plasma cells, storiform fibrosis, and destruction of the native glands. The immune histochemistry using IGG4 stain confirms the diagnosis [17]. These patients are treated on the lines of other autoimmune disorders. These usually respond to a course of systemic corticosteroids.

7.3.6 Vascular Lesions of the Gallbladder

Hemangioma is the venous malformation of the submucosal veins of the gallbladder. These can be cavernous, capillary hemangiomas, or the arteriovenous malformations. Unlike hepatic hemangiomas, the gallbladder hemangiomas are rare and are usually a diagnostic surprise [18]. Like hepatic hemangiomas, cavernous hemangiomas are the commonest vascular lesion in the gallbladder wall [19].

Two hypotheses have been proposed for the origin of the hemangioma in this organ. One hypothesis is the hamartomatous proliferation of the vascular tissue in the subepithelial layer. Secondly these may also arise from the proliferation of the

primitive mesodermal cells sequestrated in the sub-epithelial layer [18, 20]. These patients usually have a thickened GB wall, but these may be differentiated from other gallbladder tumors depending on the site of origin of these lesions. The gallbladder is commonly affected by the malignant and benign pathologies originating from the mucosa, whereas lesions like mesenchymal tumors and GIST arise from the submuosal layers and have smooth mucosal linings. These lesions are challenging to diagnose on a routine ultrasound. Cross-sectional imaging like computerized tomogram shows peripheral nodular enhancement with a centripetal flow the data on the description of these lesions by MRI is lacking [21]. Elective cholecystectomy is the treatment of choice, whereas emergent cholecystectomy is required in perforated GB with ruptured hemangioma.

7.3.7 Hydatid Cyst of the Gallbladder

Echinococcosis or the hydatid cyst is a common parasitic infection involving the human body. Man is a dead-end host for the infection and is accidently involved by ingesting food contaminated with fecal matter of dogs and sheep. These patients usually present with a right hypochondrial mass and pain abdomen [22]. Rupture of the cyst into the lumen of the gallbladder can present with Charcot's triad and subsequently Reynolds Pentad. These patients are misdiagnosed as calculus disease of the gallbladder on clinical examination, but ultrasound can help establish the definitive diagnosis. The ultrasound of the abdomen can classify these lesions based on the characteristics as visualized on the scan [23]. The lesions can vary from active cyst to a dead calcified one. CT scan of the abdomen will show the origin of the cyst, its extension, and presence of any daughter cysts with or without any compression symptoms. These lesions are first managed with a course of antiparasitic treatment using Albendazole 15–20 mg per kg body weight followed by cholecystectomy.

7.3.8 Gallbladder Cancer

Gallbladder cancer (GBC) is the most common malignant etiology involving the GB. The gallbladder carcinoma although rare in the western world, it is quite common in the Indian subcontinent along the Indo-Gangetic belt [24]. The cancer presents as mass, thick-walled gallbladder or polypoidal lesions. The thickening in the wall is usually irregular and causes mucosal destruction with infiltration into the adjoining segment IVB and V of the liver. These lesions may also have liver deposits, mesenteric and peritoneal nodules with ascites signifying the metastatic disease. These findings on ultrasound and cross section help the clinician toward establishing a malignant pathology rather than a benign one. Adenocarcinoma is the commonest pathology. These lesions present in an advanced stage and are usually not amenable to surgical excision. Chemotherapy and palliative care are only modalities of management in advanced cases [25]. The average life expectancy in advanced cases is 6–9 months.

7.3.9 Gallbladder Gist

Gastrointestinal stromal tumors (GIST) are rare tumors of the GB. These lesions originate from the interstitial cells of Cajal (ICC). These cells are commonly found in the gastric tissue and are the pacemakers of the intestine. So as per the distribution of these cells, tumors are common in the stomach followed by small intestine. The origin of the GIST can be explained by the metaplasia of the cells due to chronic irritation by the gallstones; these cells may also originate from the progenitor stem cells sequestrated during the embryonal development [3].

These tumors are mass forming or cause thickening of the wall. These may be benign or malignant depending upon the size and mitotic figures [26, 27]. These lesions are subepithelial and have smooth mucosa. These lesions may present with cholangitis, pain abdomen, and jaundice depending upon the nature and size of the tumor.

Surgery is the cornerstone of the treatment. The negative margins are the major determinant of postoperative life expectancy. The extent of surgery and the lymphadenectomy are not defined for gallbladder due to rarity of cases in available literature.

The lesions show whorled shaped smooth muscle cells which may be well to poor differentiated cells with mitotic figures. The immunohistochemistry shows CD34 and CD117 cells positivity although CD117-negative cases have also been reported [28]. These cases respond to imatinib therapy and require regular follow up after resection.

7.3.10 Neuroendocrine Tumors of Gallbladder

Neuroendocrine tumors of the gallbladder arise from the Kulchitsky cells [29]. Small intestine is the commonest site of the tumor followed by stomach. Gallbladder comprises 0.5% of the total cases of neuroendocrine tumors [30]. Well differentiated tumors are rare in gallbladder. Most of the carcinoid tumors arising from the gallbladder are neuroendocrine carcinomas and carry poor prognosis. These lesions may present as mass, thickened wall, or polypoidal lesions. These lesions may have associated features of carcinoid syndrome in less than 1% cases. The neuroendocrine carcinomas of gallbladder are large, subepithelial without mucosal destruction, show rim enhancement on MRI, and have hepatic metastasis more commonly than an adenocarcinoma [31]. These lesions show positivity to synaptophysin and chromogranin on IHC. Somatostatin receptor scintigraphy plays an important role in staging of the disease. Somatostatin analog can be used as therapeutic chemotherapy as adjunct to surgery for unresectable lesions [32].

7.3.11 Mesenchymal Tumors of the Gallbladder

These arise from the mesenchymal cells present in the connective tissue of the gallbladder. The lesions can be rhabdomyosarcoma, hemangiosarcoma, lymphangiosarcoma, and other soft connective tissue lesions. These tumors usually present at an advanced stage and carry a poor prognosis [33, 34]. If resectable extended cholecystectomy is the treatment of choice. Palliative care is option for unresectable diseases.

7.4 Approaches to a Thick-Walled GB

Any patient with a right hypochondrial pain must undergo an ultrasound. The gallbladder should be assessed for smoothness and thickness of the GB wall. The wall more than 4 mm thick should be considered pathological, and these patients must undergo further evaluation. CECT abdomen will be the first modality of investigation. Thickened GB wall, irregular wall, mass in the GB wall along with the infiltration of the liver, and presence of metastatic deposits are soft markers toward cancer; however, early cancerous lesion may just have thickened wall with or without lymphadenopathy.

Endoscopic ultrasound (EUS) is helpful to characterize the lesion. It can also help to establish the origin of the pathology, and the lesion can be aspirated for cytological examination if it is deemed unresctable.

The resectable patients on CECT should straight away be planned for a diagnostic laparoscopy followed by a surgery. The thick-walled GB must be resected along with wedge of liver. The specimen must be subjected to a frozen section, and if the malignancy is reported, the surgeon must complete lymphadenectomy before closing the abdomen.

The surgery is deemed complete if the specimen is reported as benign. The unresectable patients must be subjected to fine needle aspiration which may be ultrasound, CECT, or EUS guided. Palliation is the only treatment for unresectable malignancy; however, benign lesions can undergo medical/surgical excision as per the treatment protocol.

7.5 Conclusion

Thick-walled gallbladder is commonly encountered in north India. Though a diagnostic and therapeutic challenge, the pathology must be staged and investigated preoperatively before contemplating a morbid procedure. These patients must be prepared for a major surgery although the disease may turn out to be benign on table. Thorough evaluation, surgical planning, and treatment will help prevent any misadventure.

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8 Management of Incidentally Detected Gallbladder Cancer After Cholecystectomy

K. Søreide

8.1 Introduction

Gallbladder cancer (GBC) belongs to the biliary tract cancers [1], is overall rare compared to other solid organ cancers but has a variable presentation and disease frequency worldwide (Fig. 8.1). While GBC has a very dismal prognosis overall [2–4], patients who present with early-stage disease have a much more favorable outcome with up to 80–80% survival at 5 years. Early GBCs are most often discovered incidentally at histopathological examination of the surgical specimen after cholecystectomy has been performed for presumed benign indications. Due to the rarity of such incidental findings, the routine histopathological examination of cholecystectomy is controversial and continues to be debated [5–11]. However, as laparoscopic cholecystectomy has become one of the most frequently performed procedures in general surgery [12, 13], almost half of all GBCs are currently detected incidentally. In a recent series from Western countries, 0.25–0.89% of specimens demonstrated GBC as an incidental, unexpected finding [14–18], with lower prevalence in low-incidence regions compared to high-incidence regions [7]. The frequency of GBC is even higher (up to 2%) and age of presentation much younger (around 40 years) in endemic regions, such as Chile and India [1, 19, 20]. Notably, although GBC usually presents in older people (age > 60 years) and has a low incidence in most Western countries, the majority (>50%) of GBCs are now detected as incidental findings after cholecystectomy [21, 22].

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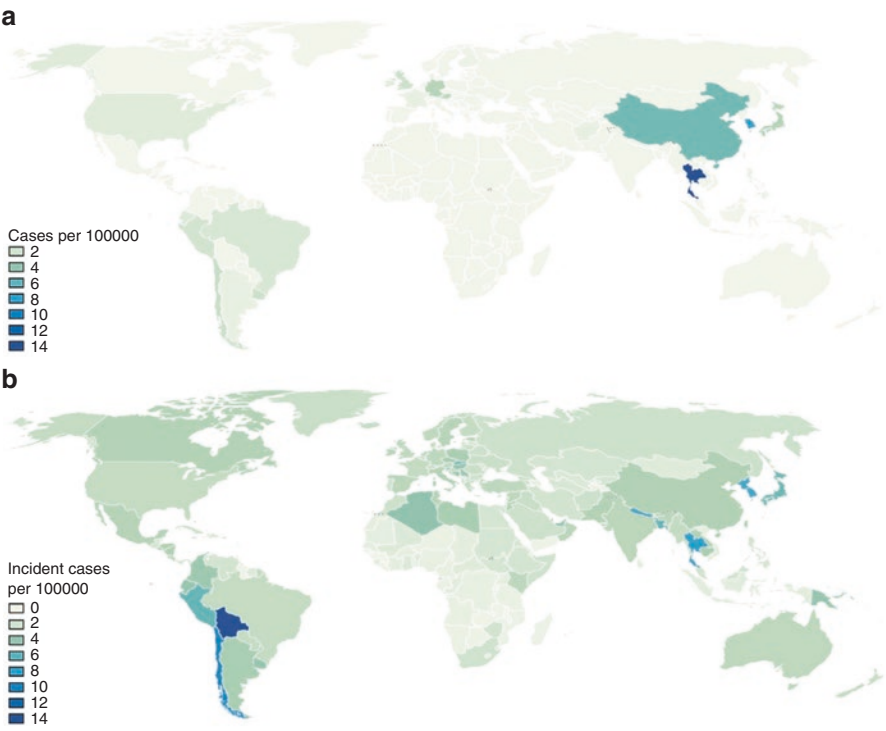

Fig. 8.1 Global incidence of cholangiocarcinoma (**a**) and gallbladder cancer (**b**). (Reproduced from Valle et al. [1] with permission from Elsevier © 2021)

8.2 Defining the Dilemma When Diagnosing an Incidental GBC

The incidental and unsuspected finding of GBC may pose several dilemmas for further management [23–25]. Incidental GBCs have a more favorable prognosis than cancers presenting with symptoms. However, the role, timing, and extent of further surgery, and the impact on outcome, remain controversial. The current available guidelines propose best evidence for management [26, 27]. However, it is recognized that this group of patients are undertreated, with several complex factors contributing to this observation [28–30]. In the following, a set of recommendations (Fig. 8.2) will be given based on previous systematic overview [24] and updated data from the contemporary literature.

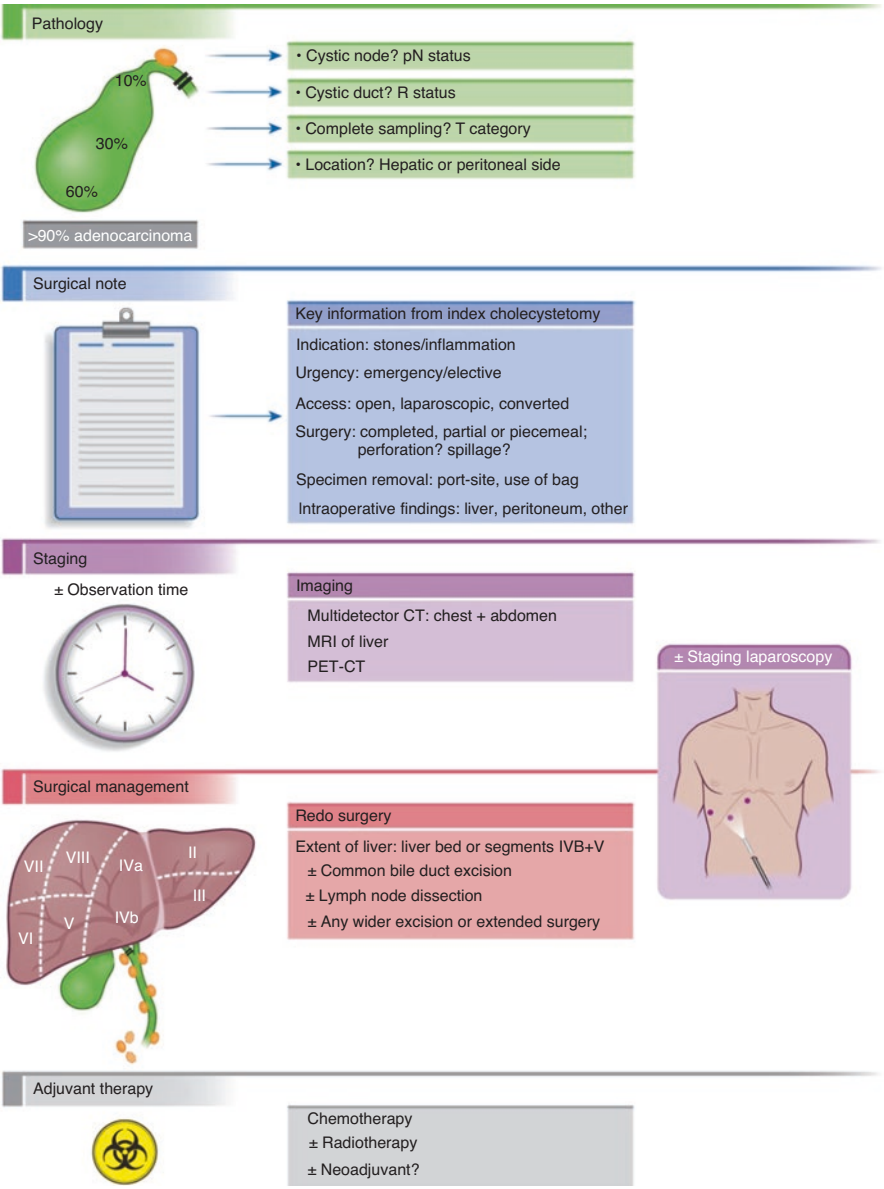

Fig. 8.2 Factors of clinical importance in incidental gallbladder cancer. (Reproduced from Søreide et al. [24] with permission from Oxford University Press © 2019. Please see main text for details)

8.3 Pathology Notes for Incidental GBC

Pathological examination is important for appropriate staging and further management in incidental GBC and should me prompted as soon as a cancer diagnosis has been made, preferably in the institution planning or discussing the case for re-resection. When a diagnosis of gallbladder neoplasia is confirmed, it is essential to establish the correct pathological stage (Fig. 8.3) for planning of further management [31, 32]. Neoplasia contained within the mucosa (Tis or pT1a) is considered to have a very low risk of recurrence and essentially to be cured by cholecystectomy alone, whereas invasion into the gallbladder muscle wall (pT1b) is considered to require further surgery.

8.3.1 The Role of pT-Stage Category at Presentation

In a systematic review of over 2000 incidental GBCs [14], the most frequent stage at presentation was pT2, followed by pT3 and pT1. Although higher rates of T4 disease are seen in symptomatic and unresectable GBCs, the distribution of T2

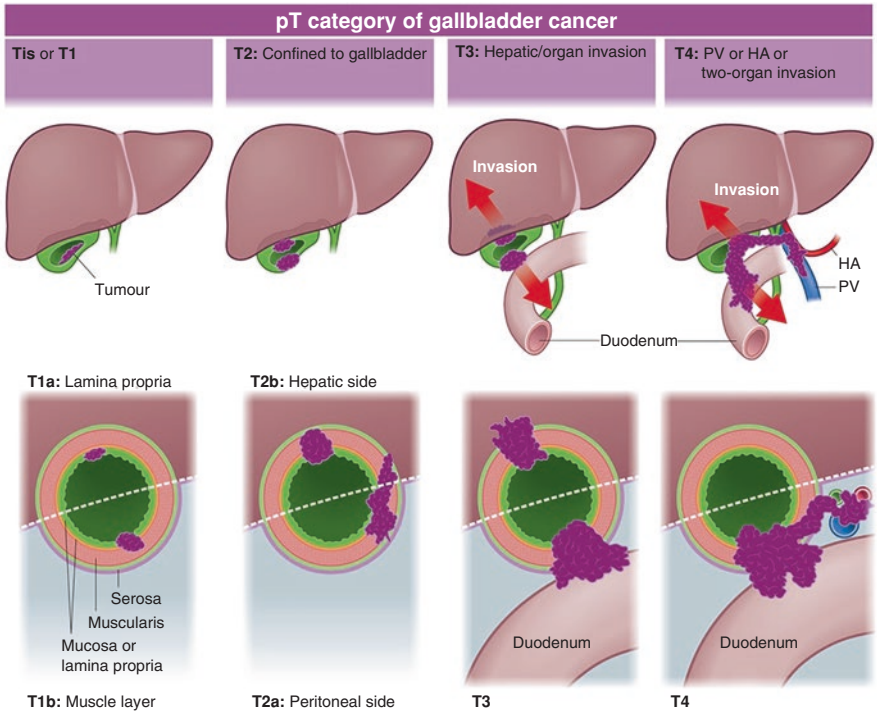

Fig. 8.3 Illustration of pT categories of the TNM system for gallbladder cancer. Based on the AJCC eighth edition. *PV* portal vein, *HA* hepatic artery. (Reproduced from Søreide et al. [24] with permission from Oxford University Press ©2019)

(about half) and T1 (about one-third) status corroborates well with findings from other studies [18, 22, 33, 34]. When exclusively looking at incidental GBCs found on histopathological examination alone, the rate of T1 cancers make up about 66%.

Determination of node status has also been viewed as essential in GBC [35–37], as the presence of nodal metastasis (pN+) is considered an adverse prognostic factor with poor overall survival. Finally, the cystic duct margin should be reported as part of the resection margin, as involvement would suggest need for reresection.

Studies on the quality of pathology reporting in incidental GBC are lacking. One small multicenter study from France [33] found that pathology reports frequently had missing data for key prognostic factors, including tumor stage, size, grade, and resection margins. Several histopathological factors beyond pT and pN category have been reported [22, 38] to be of prognostic relevance, such as grade, lymphovascular, and perineural invasion, and should be obtained together with pT and node status. Despite the controversies, the TNM stage is important for prognostication, as the prognosis drops considerably even with the presence of node-positive disease, with very dismal prognosis for metastatic disease (Table 8.1).

8.3.2 Is Sidedness Important in GBC?

For pT2 cancers, the location in the gallbladder is important, as cancers located on the liver side (as opposed to the peritoneal side) have a worse prognosis as reported in some studies [39, 40]. Tumor location is important for further treatment decision-making in T2 GBC and is incorporated in the current (eighth edition) staging system. However, more recent data suggest that this may not be the case [41–43], and controversy concerns the sidedness of T2 cancers [42]. Furthermore, another meta-analysis found that hepatectomy in T2 GBC may not even improve outcomes, and hence, it may not be necessary [41]. Obviously, this topic continues to be debated.

Table 8.1 TNM stage according to the AJCC eighth edition

Stage	Tumor category	Node category[a]	Metastasis category	Estimated 5-year survival (%)
0	Tis	N0	M0	80–100
I	T1a (lamina propria)	N0	M0	80–100
	T1b (muscularis)			
IIA	T2a (peritoneal side)	N0	M0	40–75
IIB	T2b (hepatic side)	N0	M0	28–50
IIIA	T3	N0	M0	8–28
IIIB	T1–3	N1	M0	8
IVA	T4	N0–1	M0	7
IVB	Any T	N2	M0	4
	Any T	Any N	M1	0–2

[a] *N1* one to three metastatic nodes, *N2* more than three metastatic nodes

8.3.3 Recording of Intraoperative Events at Primary Surgery

For the surgeon, it is of importance to obtain knowledge of any intraoperative event that may influence further management. Based on data from the German registry [44], intraoperative perforation of the gallbladder was associated with a higher risk of local recurrence. Perforation or bile spillage may be associated with an almost universal risk of peritoneal carcinomatosis and a poor prognosis [45–47] and may preclude surgical cure. If perforation occurred, this should be taken into consideration for the next strategy steps, and discussion in MDT should include this in the considerations.

8.3.4 Timing of Reresection: Does It Matter if Early or Late?

When an unexpected diagnosis of cancer is obtained, early contact with a hepatobiliary center should be made [48]. In one US study, increasing travel distance to a treating center was associated with poorer outcome, suggesting barriers to care [49]. However, the time interval from index operation to reresection (or evaluation at a hepatobiliary center) is not straightforward, with contradictory results reported between studies in relation to the importance of the time interval.

Overall, the time interval from index cholecystectomy to reresection is reported with considerable variation across studies, with a median usually at 2–3 months and range between 1 and 11 months [34, 50–52]. The unexpected finding of a cancer comes with a sense of urgency for both treating surgeon and patient, and, even from the side of the receiving center of the referral, urgent or even emergency transfers may occur after the diagnosis is confirmed. However, there are few data to support a need for an emergency referral and immediate redo surgery if an incidental cancer is detected, although the timing of surgery remains debated [53–56].

In several studies, the unresectability rate at restaging (before redo surgery) is as high as 50% for incidental cancers [48, 54, 56, 57], despite early referral. Indeed, in one study [54] early referral was a strong predictor of unresectability. Together, this may suggest that biology, rather than time, is the most essential factor for progression of disease. In particular, if perforation occurred during first operation, a period of observation may be allowed, as perforation may be associated with higher risk of disease dissemination and poor survival.

Early surgery is not associated with an improved outcome if the cancer has spread beyond the resection planes of the initial surgery [54]. In a large multicenter study [55], the investigators found inferior outcomes for patients treated within the first 4 weeks of the primary operation, and also for those treated more than 8 weeks after surgery; a 4–8-week window had the best outcome. However, there is potential bias in the retrospective observational design of these data accrued from several centers, and the plausibility of a 4–8-week window for resection has been questioned [53]. Indeed, others [50, 51, 54, 57] have shown that it is primarily the pT category at first operation, rather than time interval that determines risk of residual disease and operability at second operation. Residual disease was found in half of 22 patients with

T1b/T2 cancers after redo surgery, with very poor prognosis in those with residual disease [57]. Consequently, surgery may simply act as a staging procedure rather than change the natural history of the disease, and an argument could be made for a time window for observation and reimaging for optimal clinical restaging before commencement of surgery based on this. "Urgent" reresection (at less than 4 weeks) may be associated with ongoing or not yet resolved inflammation from index surgery, and complicate further resection. The "test of time" interval before further redo surgery should take into account the operative report at index surgery and the pathological assessment of the resected specimen [24]. Proper staging, underlying tumor characteristics, previous gallbladder perforation with risk of tumor spillage, and the risk of residual disease determine the long-term prognosis [24, 25].

8.4 Preoperative Cross-Sectional Imaging for Restaging before Resection

After establishing a diagnosis of incidental GBC, cross-sectional imaging should be performed to exclude disseminated disease or obvious early recurrence. The findings on cross-sectional imaging may depend largely on the time since primary resection and pathological stage, but also on events during index surgery (e.g., severe cholecystitis? perforation? spillage of content?). Chest and abdominal CT should be a minimum requirement for restaging, but other imaging modalities may be considered for higher sensitivity and specificity. PET-CT may have an increasing role in the setting of staging before an eventual reresection of incidentally detected GBC, but diagnostic accuracy may be influenced by such factors as time since index surgery or inflammation that may make interpretation somewhat difficult. However, as technology evolves, the sensitivity of PET scan may be proven to be superior and add to conventional imaging [58, 59]. Sensitivity for disseminated disease is considered high [60–66]. Also, GBC is a rather PET-avid malignancy and thus may be suitable for preoperative staging by PET scan [58, 60, 62, 63, 65].

8.4.1 Role of PET Scan?

Although data are currently based on few series of incidental GBC [64–66], these suggest that PET–CT has a role before reresection in any T1b cancer and above for detection of disseminated disease [65] and for ruling out local residual disease in T1b cancers [64]. One study [64] advised against undertaking redo surgery in patients with T1b cancers if PET–CT findings were negative, as the likelihood of finding residual disease was very low. While the data for GBC is overall low and evidence scarce, a recent metanalysis [58] found pooled sensitivity and specificity of 18F-FDG PET/CT for the detection of local disease estimates of 96% (95% confidence interval (CI), 90–99%) and 91% (95% CI, 77–98%), respectively. Pooled sensitivity and specificity for the detection of metastatic disease are 95% (95% CI, 88–98%) and 97% (95% CI, 90–100%), respectively. For nodal disease, these

values are 75% (95% CI, 53–90%) and 91% (95% CI, 77–98%), respectively. Others have also highlighted that GBC is typically FDG avid, and when anatomic imaging is equivocal, PET can be used to assess metastatic involvement with high specificity and inform subsequent management [59].

8.4.2 Role of Staging Laparoscopy and Laparoscopic Reresection

Previous studies have investigated laparoscopic staging in incidental GBC given the high rate of associated peritoneal metastasis. Staging laparoscopy may avoid a non-therapeutic laparotomy in about half of patients with disseminated disease but has the lowest yield in early stages [67]. Overall, the diagnostic yield is low, but may be considered in poorly differentiated and higher T categories (e.g., T3) with a greater risk of disseminated disease [67, 68].

Increasingly, investigators also report on the use of laparoscopic (or robotic) resection for GBC, stating at least similar outcomes for early-stage GBC [69, 70]. In experienced hands, a minimal invasive surgical access has been reported to be feasible also for reresection of incidentally detected GBC [71, 72], but usually to very selected patients only [73]. In an expert consensus, it was stated that while laparoscopic reresection may be used with similar results as for open surgery, it is still considered to be early days for general recommendation [74].

8.5 Type and Extent of Surgical Reresection

Considerable debate still exists over re-excision, aggressiveness of surgery, and its influence on outcome in incidental GBC [75, 76]. There is consensus that R0 resection represents the strongest prognostic factor for long-term outcome and chance for cure. The timing, type and extent of reoperation, and patient selection are still widely debated [24, 77–82].

In stage T1a GBCs, the 5-year survival rate approaches 100%, with a less than 2% risk of pN+ disease on reresection; thus, simple cholecystectomy is considered curative for these patients [83, 84]. This has reached consensus in most guidelines [85–88]. Current guidelines suggest that T1b cancers should undergo extended resection with lymphadenectomy, as about 10% of these tumors will have pN+ status [83, 85]. However, although there is a difference in survival between T1a and T1b cancers, this does not appear to be influenced by simple cholecystectomy or extended lymphadenectomy [85]. In a systematic review [83] covering 29 studies and including 1266 patients with T1 incidental GBC, 1.1% of patients with T1a disease died from cancer, compared with 9.3% of those with T1b disease. The authors concluded that there is no firm evidence that extended surgery confers a survival benefit in T1b cancers [83].

In stage T2, consensus suggested to perform an extended lymphadenectomy at reoperation [86, 89, 90]. However, with the recently introduced subdivision of T2

into pT2a and pT2b, there appears to be new debate over the need for reoperation and extended surgery in all peritoneal-side T2 cancers [40, 91]. Although T2 sidedness is already included in the TNM system, it is based on a rather limited number of patients [39, 40] and has more recently been challenged for its actual prognostic role [42, 92]. The variation in outcome related to T2 cancers seems to be more complex than previously thought and may include genetic differences in the cancer biology as well as treatment-related factors [93] (Fig. 8.4).

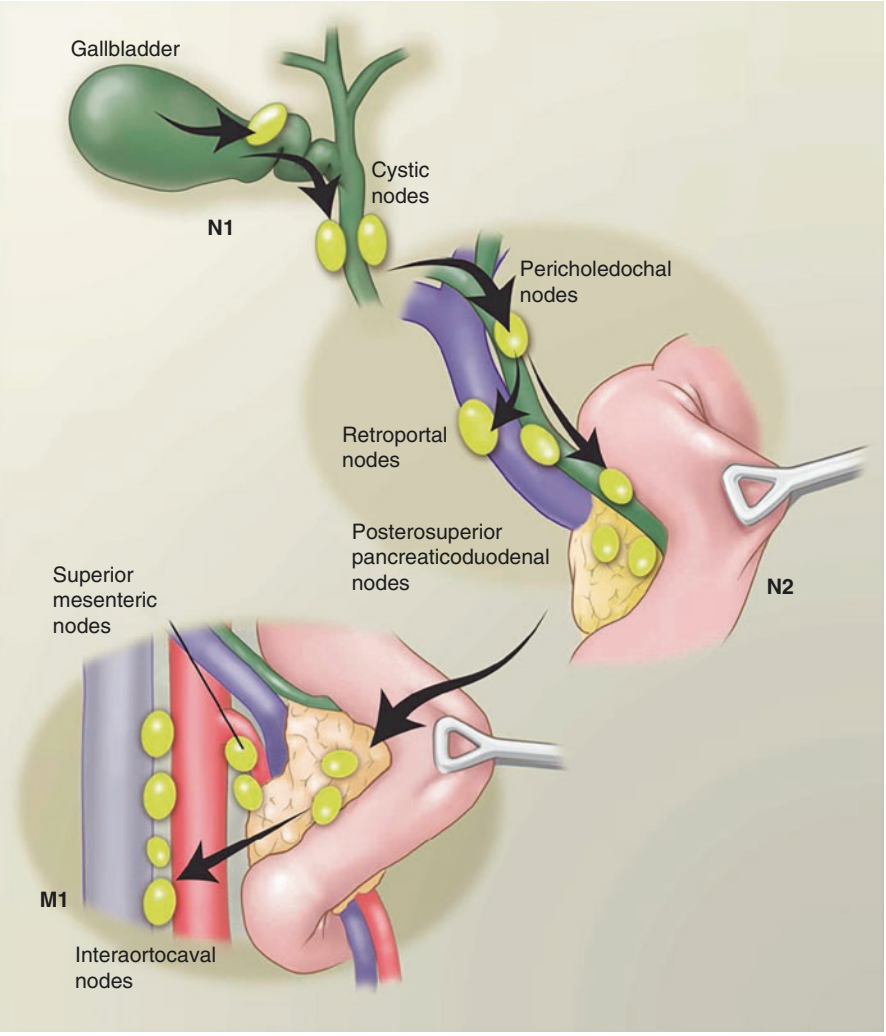

Fig. 8.4 Lymphadenectomy and lymph node drainage basins in GBC. (Reproduced from Qadan & Kingham [77] with permission from Elsevier)

8.5.1 Biology Trumphs Surgery

Biological features of the cancer, rather than extent of surgical reresection, dictate the outcome of patients with early-stage incidental GBC. These findings may be controversial in relation to current recommendations and previous findings [94], but on closer inspection of studies reporting on outcome in T2 cancers in the past and benefit of extended surgery, it is usually the presence of liver involvement or node metastasis that is related to poor survival [94, 95]. Indeed, previous extensive surgery reported from tertiary-center series have not yielded an effect of improved survival after either excision of common bile ducts (CBDs) [96] or multiple organ resections [97].

8.5.2 Should Laparoscopic Port Sites be Resected?

GBC is prone to the development of peritoneal metastasis [98], and early reports after laparoscopic cholecystectomy reported high rates of port-site metastasis. However, a recent systematic review [99] found that since the 1990s, compared with the 2000s era, the incidence of port-site metastasis in incidental GBC has decreased significantly from 18.6% before 2000 to 10.3%. The extraction site is at significantly higher risk than nonextraction sites, and the risk of port-site metastasis is associated with increased T category and the presence of poor histopathological features. Several studies [100–102], including a multicenter consortium study from the USA and a French registry study, reported no survival benefit from routine port-site excision, and this practice is largely obsolete in modern practice. The European Society for Medical Oncology guidelines [88] suggest port-site excision if there is documented intraoperative perforation of the specimen, but this is not supported by available data.

8.5.3 Should the Common Bile Duct be Resected?

Resection of the CBD is a further controversial area. In patients without involvement of the CBD (for instance, based on cystic duct evaluation), there was no benefit in terms of overall survival, lymph node yield, and similar recurrence rates with extrahepatic bile duct resection to a "radical cholecystectomy," but the associated morbidity was higher [34, 96]. Recent studies [34, 103–105] further showed no improvement in lymph node retrieval with resection of the CBD, and overall survival was worse. When adjusting for disease stage, survival was similar in patients undergoing CBD resection and those having no resection [104], suggesting that it is disease stage that drives the biology and thus the outcome, rather than extent of surgery. Similar findings have been reported from Japan [106].

8.6 Outcome Prediction and Prognostic Score

Among the most important factors associated with outcome in incidental GBC is the ability to achieve an R0 resection, whereas both a higher T category and the presence of lymph node metastasis are strong predictors of poor survival [107–110]. Several attempts at refining prognostication have been entertained, with a GBC Predictive Risk (GBPR) score developed from a multicenter series of incidental gallbladders [38]. The GBPR score consists of four pathology-derived risk factors associated with either locoregional or disseminated disease according to risk groups. In the original study, of the 262 patients with incidental GBCs, only 88 (33.6%) had data on all four factors to allow for predictive use of the score. Even though the score helps to redistribute T1b cancers with higher risk based on additional poor prognostic factors, the validity of the score remains uncertain based on the low proportion of patients available for constructing the score. A single-center study from Japan [111] of 56 patients has validated the prognostic role of the GBPR score as being an independent factor for overall and recurrence-free survival. A valid and robust risk score may be useful in selecting patients for both redo surgery and adjuvant therapy.

8.7 Adjuvant Chemotherapy

The role of adjuvant chemotherapy in GBC is poorly documented, with data from series or trials grouping all types of biliary tract cancer together, based on large retrospective comparisons in registries or occasional multicenter experiences [112–119], as compiled in four meta-analyses [112, 114, 115, 117]. Since 2010, four randomized phase III clinical trials including ABC-02, PRODIGE-12/ACCORD-18, BILCAP, and BCAT and a single-arm phase II trial (SWOG0809) have been reported on the use of adjuvant strategies for biliary malignancies. These trials have led to the recommendation that patients with resected biliary tract cancer should be offered adjuvant capecitabine chemotherapy and those with R1 margins could be considered for chemoradiotherapy [1, 26, 120].

Overall, adjuvant chemotherapy seems to be associated with better survival for all biliary tract cancers [112, 115], as well as in series of incidental GBC [22], with gemcitabine being the drug of choice in the investigated studies. Since 2010, cisplatin and gemcitabine have been the preferred combination, based on results obtained in advanced biliary tract cancer [121]. However, overall application is low (<30%) and the overall treatment effect small [122, 123]. Application of adjuvant therapy has remained low in the population at risk, despite data suggesting a survival benefit [124]. This may reflect a largely elderly and comorbid population, but also reluctance to subject any patient with early-stage cancer (such as T1b or T2 with no node metastases) to chemotherapy when the effect is documented to be marginal. Thus, it will be paramount to define the appropriate risk groups who should be good candidates with the greatest benefits for receiving adjuvant therapy.

In a registry study, with patients matched for characteristics, median survival was longer for extended cholecystectomy with adjuvant therapy (23.3 months) than for simple cholecystectomy with adjuvant therapy (16.4 months) and was significantly longer than either simple (12.4 months) or extended (10.7 months) cholecystectomy alone [113]. Notably, in the registry only about one-third of patients ever received chemotherapy, and almost 90% of resections were simple cholecystectomies [113]. The authors proposed that adjuvant chemotherapy could be considered rather than extended resection in some patients. However, there is a bias toward chemotherapy in younger, more fit patients with higher likelihood of both T3 and node-positive cancers and having extended resections [113].

Of note, adjuvant chemoradiotherapy was not associated with improved survival in a collective review [112], but had higher toxicity. A registry series [125] proposed better survival for all patients with reresected cancers who received adjuvant radiotherapy, but not for patients who had chemotherapy alone. Another study [124] could not confirm a benefit for radiotherapy. Radiotherapy is controversial as it is performed (and, thus, considered) only in some centers. There are no randomized trials for radiotherapy as adjuvant therapy. A systematic review [114] reported favorable survival for patients who had radiotherapy after radical surgery, whereas receiving radiotherapy was a negative prognostic factor in another register study [126].

The heterogeneity in the data should be noted: the mix of symptomatic and incidental cancers; gallbladder and other extrahepatic cholangiocarcinomas, studies also including intrahepatic cholangiocarcinomas; the type and duration of chemotherapy used in various time intervals and a selection bias for both surgery and adjuvant therapy in most of the reports. As the concept of neoadjuvant therapy [127, 128] is not possible, by definition, in incidental GBCs, and symptomatic cancers may have a different inherent biology, findings from the present data would need to be extrapolated. Consequently, guidelines and consensus statements are vague [86, 88], but recommend adjuvant chemotherapy for most patients after resection, in particular those with any T2 disease and above with N1 disease, given the high risk of recurrence and nodal dissemination. A French multicenter study [129] found no difference in recurrence-free survival between GEMOX (gemcitabine–oxaliplatin) and observation alone in biliary tract cancers, based on data presented in abstract form only. The randomized BILCAP trial demonstrated better survival for capecitabine after radical surgery of biliary tract cancer [130]. GBCs made up but a subset of the BILCAP population with about one-third of all biliary tract cancers. Another ongoing European trial, the ACTICCA-1 trial [131], which compares cisplatin–gemcitabine combination with observation alone after radical surgery, may possibly change the trial protocol to test that combination of cisplatin–gemcitabine versus capecitabine, rather than a simple "observational" arm. The optimal regimen or selection of subgroups for adjuvant chemotherapy is currently not known based on available data. It is hoped that better targeted therapy, immune therapy and drugs based on genetic aberrations may improve both patient selection and treatment effect in the future (Fig. 8.5). Further trials to guide best treatment, such as the OPT-IN trial, are underway (Fig. 8.6). This trial is specifically designed for T2 and T3 incidental GBC [132].

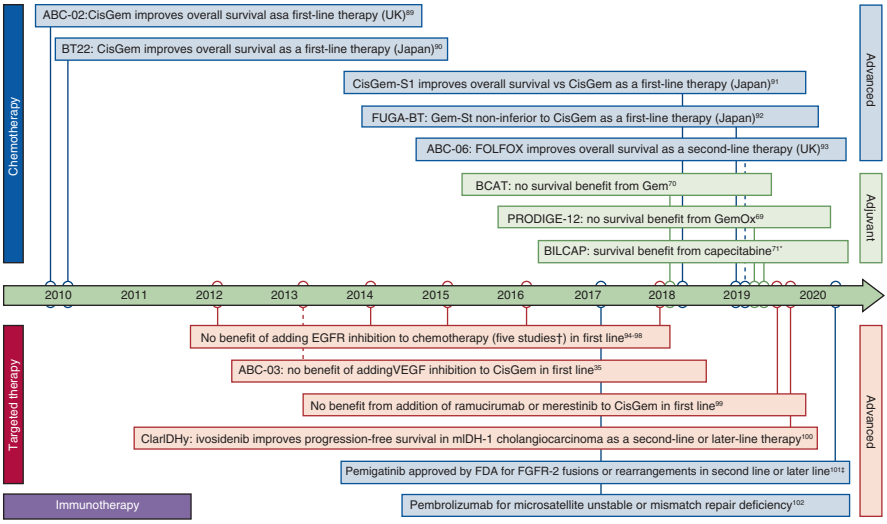

Fig. 8.5 Timeline showing drugs and trials in biliary tract cancers. (Reproduced from Valle et al. [1] with permission from Elsevier © 2021)

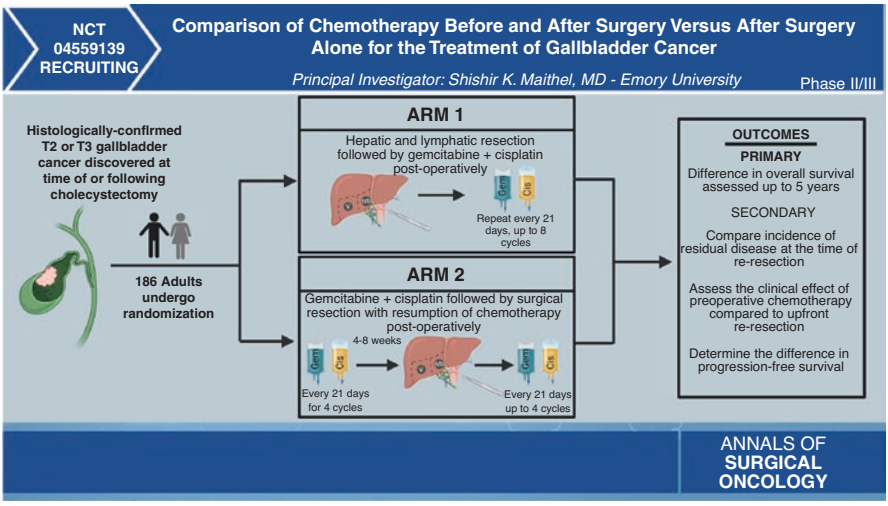

Fig. 8.6 The design of the OPT-in trial for incidental GBC. (Reproduced from [132] with permission from Springer © 2021)

8.8 Future Directions

The contemporary lack of good data on which to base current decision-making and planning for the individual patient is of concern [133]. No trials have ever been performed for a surgical technique or strategy in GBC and data from adjuvant trials are usually extrapolated by grouping all biliary tract cancers together. A predominant belief in reresection for most, if not all, patients seem to be based on a mechanistic approach that contributes to staging, but with poor data to suggest effect on survival. An increasing body of data, as well as the current TNM staging system, increasingly emphasizes biology as the determinant of survival. Thus, defining the biology of GBC from improved clinical imaging and biomarker definitions should lead to better clinical decision-making in the future. A limitation of this review is evident in the lack of high-quality data. Although large registry data point to trends, these bear the risk of resembling outdated or selective practice and not using contemporary staging standards. Thus, there is a need for improved data quality from prospective observational cohorts, imaging studies, onco-genomic profiling studies, and novel therapeutics.

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Adjuvant Therapy in Gallbladder Cancers

9

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9.1 Adjuvant Treatment

There is scarcity of literature on adjuvant treatment in GBC. Because of the low incidence of GBC and limited trials on adjuvant treatment and that too combining all biliary malignancies, it is difficult to draw conclusions on the benefits of adjuvant treatment modalities in GBC. Most GBC patients relapse distantly after complete resection and without adjuvant treatment within a span of 2 years [1–3]. Jarnagin et al. [1] did comprehensive analysis of the patterns of recurrence after surgical resection in GBC and hilar cholangiocarcinoma. The predominant pattern of recurrence in GBC was distant seen in 85% of the cases while locoregional recurrence (LRR) alone occurred in only 15% of patients. The rates of LRR in patients with microscopically involved resection margins were slightly on the higher side (20%). Approximately, 60% of the recurrences occurred within first year and 88% occurred in 2 years. Barreto et al. [3] analyzed patterns of recurrence in 163 patients of incidentally detected GBC who underwent radical re-resection. The authors concluded that most common site of relapse is distant and the presence of lymph node metastasis is the most important predictor of disease recurrence even after complete radical re-resection for incidental GBC. Based on the pattern of recurrence, it is intuitive that adjuvant treatment addressing systemic disease is likely to have impact on survival and outcome of GBC patients. The prognostic factors for OS in gallbladder cancer, based on data of 1137 patients from the Surveillance, Epidemiology and

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End Results (SEER)-Medicare, include advanced age, male sex, African American or Asian/Pacific Islander race, larger tumor size, positive regional lymph nodes, and whether patients received adjuvant chemo(radio)therapy.

There is dearth of prospective randomized trials of adjuvant therapy in GBC. Various retrospective studies and population-based databases have shown conflicting conclusions and inconsistent results. In a meta-analysis of 20 studies (which included one randomized trial of chemotherapy alone, 2 SEER analysis, and 17 institutional series) incorporating 6712 patients of GBC and bile duct cancers, adjuvant therapy resulted in non-significant ($p = 0.06$) improvement in survival as compared to surgery alone. The greatest benefit of adjuvant therapy was seen in high-risk patients (lymph node positive and R1 disease) [4]. Another meta-analysis by Ma et al. [5] which included 10 retrospective studies involving 3191 patients of GBC showed significant improvement in survival with chemotherapy as compared to surgery alone and benefit was observed mainly in node positive, margin positive and higher stage disease patients. Thus, both the meta-analysis supported the use of adjuvant therapy in high-risk patients. Adjuvant chemotherapy and adjuvant radiation (RT)/chemoradiation (CRT) and treatment guidelines are discussed below in more detail.

9.2 Adjuvant Chemotherapy

The first prospective randomized trial of adjuvant chemotherapy versus surgery alone in resected pancreaticobiliary carcinoma was published in 2002 by Takada et al. [6]. Patients were randomized to either the adjuvant chemotherapy group or the surgery-alone group. Out of 508 patients, 140 had GBC (73 in the adjuvant chemotherapy arm and 67 in the surgery-alone arm). After excluding ineligible patients, there were 69 patients in adjuvant group and 43 in surgery-alone group. The number of patients in two arms was not equally balanced. The 5-year survival and disease-free survival (DFS) of GBC patients were significantly better in the adjuvant chemotherapy group compared with surgery-alone group. The 5-year survival after curative resection was 46% in the adjuvant group and 31% in surgery alone which was not statistically significant. After non-curative surgical resection, the 5-year survival rate and DFS were significantly better in adjuvant chemotherapy group as compared to surgery-alone group. However, on intent-to-treat analysis, there was no significant difference in the median survival time between two groups (16.4 months with adjuvant and 14.1 months in surgery alone). The authors concluded that patients who received adjuvant therapy in GBC had an overall improved survival, but survival benefit was limited to the patients who underwent noncurative resections.

The second large trial PRODIGE 12–ACCORD 18 which is a multicentric, open-label, randomized phase III trial conducted in 33 centers randomized patients to either adjuvant gemcitabine and oxaliplatin chemotherapy (GEMOX) or surveillance only after surgical resection. The chemotherapy regimen consisted of gemcitabine (1000 mg/m^2 intravenously) on day 1 and oxaliplatin (85 mg/m^2 intravenously) on day 2 every 2 weekly for total of 12 cycles. A total of 196 patients were included, out of which 20% had GBC. There was no significant difference in overall survival (OS), relapse-free survival (RFS), time to definitive deterioration in health-related quality of life (HRQOL) between the adjuvant GEMOX and surveillance arms. The planned subgroup analysis did not show any favorable outcome, with respect to tumor site, stage, and lymph nodes in patients receiving adjuvant chemotherapy. Thus, the trial failed to show superiority of adjuvant GEMOX over surveillance alone in biliary tract cancers after surgical resection [7].

The third randomized trial BILCAP is a phase 3, multicentric randomized study of 447 patients across 44 centers. Patients were randomly assigned in 1:1 to eight cycles of capecitabine (1250 mg/m^2 twice daily on days 1–14 of a 3-weekly cycle) or observation. The median follow-up for all patients was 60 months. Seventy-nine (18%) patients had GBC. The primary outcome was overall survival. The median OS was not statistically different between two arms in intention-to-treat analysis (51.1 months in the capecitabine group compared with 36.4 months in the observation group, $p = 0.097$). The primary endpoint of improving OS was not met in the intention-to-treat analysis, but the protocol-specified sensitivity analysis and per-protocol analysis suggested that capecitabine can lead to improvement in OS in patients with resected biliary tract cancer in the adjuvant setting and could be considered as standard of care. The authors also concluded that adverse events or toxicities are manageable further supporting the use of capecitabine in this setting [8]. The difference in results of BILCAP and PRODIGE trial can be explained by inclusion criteria between these trials. They had enrolled patients with different biliary subtypes and proportions of risk factors. The PRODIGE 12 trial had enrolled more patients with intrahepatic cholangiocarcinoma than the BILCAP trial (44% versus 19%). Moreover, different proportions of high-risk features, such as pT3–4 tumor stage, tumor-positive regional lymph nodes, and/or positive resection margins, were included in these trials. The BILCAP trial enrolled a large proportion of patients with high-risk features than the PRODIGE 12 trial (N1, 47.0% versus 36.6%; R1-resection, 37.6% versus 12.9%, respectively) which could explain the worse median RFS and OS in the BILCAP study compared to the PRODIGE 12 trial. Various retrospective series [9–14] on adjuvant chemotherapy in GBC are summarized in Table 9.1.

Table 9.1 Retrospective series of adjuvant chemotherapy in GBC

Author	Year	Study period	Number of patients	Patient characteristics	Treatment characteristics	Regimen	Survival
Kayahara et al. [9]	2007	1988–1997	4774	All stages	Surgery—3324 adjuvant CT- 1231 (37%) Adjuvant RT 5% (effects of RT were not analyzed)	Oral anticancer drugs (details not available)	Adjuvant CT did not provide a survival benefit except in stage IVA
Park et al. [10]	2010	2000–2009	160	Particularly analyzed stage II patients	Surgery alone—91 Adjuvant therapy—69 Stage II—61 (surgery alone—18; adjuvant therapy—43) Out of 43 stage II patients, 28 received adjuvant CR, 7 received RT and 8 received concurrent CRT	5-FU-based CT: 21 patients Gemcitabine-based: 6 RT: Median dose 45 Gy Concurrent 5-FU CRT—Median dose—50.4 Gy	CT group had a better survival, but was not statistically significant
Murakami et al. [11]	2011	1990–2010	62	Adjuvant CT in stages III and IV disease	Patients with stages III and IV – 41	Ten cycles of gemcitabine plus S-1 every 2 weeks	Adjuvant CT improved survival in stages III and IV
Mantripragada et al. [12]	2016	2004–2011	4775	T2–3 or node positive nonmetastatic GBC	No adjuvant therapy—3402 Adjuvant CT: 1373 including 646 who received upfront CRT (34.3%) received radiation therapy later on	Adjuvant CT single agent in 56.9%, multiagent in 33.4%, and unspecified in 9.7% For the CRT group, median total radiation dose was 50.4 Gy	Adjuvant upfront CRT within 3 months of surgery improved OS at 2 years but the benefit was not at 5 years
Bergquist et al. [13]	2018	2004–2012	4373	T2–4	Surgery alone—3406 Adjuvant CT—967	Details not available	Adjuvant CT improved survival in T2 or greater GBC with node positive disease
Bohan et al. [14]	2021	2006–2015	5656	Stage I–III	Surgery alone—4664 Adjuvant chemotherapy—992		Adjuvant CT improved survival in node-positive (stage IIIb) patients

CRT chemoradiation, *CT* chemotherapy, *GBC* gallbladder cancer, *OS* overall survival

9.3 Adjuvant Chemoradiation (CRT)/Radiation (RT)

The benefit of adjuvant CRT or RT has not been tested in prospective randomized trials and there is lack of strong and high-quality evidence. Most of the literature is available in the form of retrospective studies. Various studies from Surveillance, Epidemiological, and End Results (SEER) database have shown that adjuvant RT provides survival benefit in node-positive or T2 or greater disease [15–17]. Wang et al. [17] in his study highlighted that benefit of adjuvant CRT can be observed in subsets of patients with T2 or N1 or greater disease. But in SEER studies, chemotherapy details are not available. On the contrary, few studies failed to show any survival benefit with RT [18, 19].

Gold et al. [20] retrospectively analyzed the outcomes of GBC patients who received adjuvant CRT after R0 resection. The adjuvant CRT regimen consisted of radiotherapy (median dose 50.4 Gy in 28 fractions) given concurrently with 5-fluorouracil chemotherapy (dose of 500 mg/m^2 given for 3 successive days during first and fifth week of radiotherapy). Out of 73 patients, 25 received adjuvant CRT. Out of 25 patients in the CRT group, 20 patients (80%) had stage II disease. Administration of adjuvant CRT showed a survival benefit in higher T stage and node-positive disease. The phase II Intergroup SWOG trial [21] evaluated the combination of adjuvant chemotherapy with four cycles of gemcitabine (1000 mg/m^2 day 1 and 8) plus capecitabine (1500 mg/m^2 per day from Day 1 to 14) every 3-weekly, followed by concurrent radiation (45 Gy to regional lymph nodes and 54–59.4 Gy to the tumor bed) and capecitabine (1330 mg/m^2 per day) in GBC and extrahepatic cholangiocarcinoma patients with stage pT2-4 or node-positive or margin-positive disease. The 2-year survival was 65% for all patients (67% and 60% in R0 and R1 patients, respectively). Kim et al. [22] in their retrospective study of 151 GBC patients evaluated the benefit of adjuvant CRT and chemotherapy by comparing with that of surgery alone. Thirty-five percent of the patients did not receive any adjuvant treatment. There were no significant differences in the incidence of LRR, distant recurrence, relapse-free survival, and OS among CRT, chemotherapy, and observation groups in patients with T2-3N0M0 stage. However, in patients with T2-3N1-2M0 stage, LRR and distant recurrence were significantly lower, and OS was significantly higher in the CRT group than those in the observation and adjuvant chemotherapy groups.

In a study of 5029 patients of GBC (stage T1- N0-1) by Mitin et al. [23] using National Cancer Database (NCDB), adjuvant treatment improved the 3-year OS (hazard ratio of 0.47 for CRT, 0.63 for RT and 0.77 for chemotherapy). Adjuvant CRT was associated with improved survival in all categories, except T1N0 and in patients with negative and positive resection margins.

In meta-analysis by Horgan et al. [4], significant benefit with adjuvant radiation was seen in R1 patients. The authors emphasized that radiation therapy should be used in patients with margin-positive disease, but its benefits in margin-negative disease remain debatable. In another meta-analysis by Ma et al. [5], subgroup analysis showed a significant improvement in survival with chemotherapy but a nonsignificant improvement in survival with RT and CRT.

In a meta-analysis of postoperative radiotherapy versus no radiotherapy in extrahepatic cholangiocarcinoma and GBC, it was found that 5-year OS was significantly higher in the postoperative radiotherapy group than in the no radiotherapy group. Also, in patients with positive lymph nodes and margins, 5-year OS was significantly higher in the radiotherapy group. Postoperative radiotherapy significantly decreased the local relapse rates, but there was no significant difference in the distant metastasis rate between the radiotherapy and no radiotherapy groups [24]. Various series using radiation or chemoradiation are summarized in Table 9.2 [15–22, 25–36].

9.3.1 Radiotherapeutic Considerations

Postoperative RT using 3DCRT or IMRT is advocated. The target volumes to be included are the tumor bed and the draining regional lymph nodes to a dose of 45–50.4 Gy at 1.8 Gy per fraction. Ben-Josef [21] advocated 45 Gy to regional lymph nodes (retro-pancreaticoduodenal, celiac, and portal vein nodes) and 54–59.4 Gy to preoperative tumor bed. This practice yielded a loco-regional relapse rate of 8% and DM rate of 44%. In another study where the above fields (localized field RT) were compared to inclusion of paraaortic lymph-nodes as well (EFRT: extended field RT) 37.5% patients developed LRR (13.3% vs. 40% in EFRT and LFRT, p = NS). The median OS was not reached (NR) vs. 42 months and the median DFS was NR vs. 30 months in EFRT vs. LFRT, respectively (p = 0.01 and 0.016). The 5-year OS was 80% vs. 42% and 5-year DFS was 80% vs. 40% for EFRT and LFRT, respectively (p = 0.01 and 0.016) [37].

9.3.2 Treatment Guidelines

ASCO guidelines recommend adjuvant chemotherapy with 6 months of capecitabine after surgical resection in GBC patients based on BILCAP study and adjuvant CRT in patients with positive resection margins [38]. NCCN guidelines recommend observation for incidentally detected T1a disease and R0 margins. Observation, adjuvant chemotherapy (gemcitabine or 5FU based), or 5FU-based CRT is advocated for patients with margin and lymph node-negative T1b or higher stage. For patients with positive margins and lymph nodes, adjuvant chemotherapy, CRT, or combined approach is recommended [39]. ESMO guidelines suggest that adjuvant therapy should be offered to patients only after a risk–benefit assessment [40–42].

9.3.3 Follow-Up

Consensus based guidelines recommend imaging every 6 months for the first two years as clinically indicated and at annual intervals with clinical review for up to 5 years. Use of CEA and CA 19–9 has been recommended as clinically indicated in

Table 9.2 Series of adjuvant radiation/chemoradiation in GBC

Author	Year	Study design	Study period	Number of patients	Treatment	Outcome
Bosset et al. [25]	1989	Retrospective	1983–1988	7	Radiation 46 Gy followed by boost of 9 Gy	Five alive with no evidence of disease
Mahe et al. [26]	1994	Retrospective	1980–1988	19	Radiation doses ranged from 30 Gy/10 fractions to 50 Gy/25 fractions	Radiotherapy was well tolerated. The authors concluded that radiotherapy could improve local control and survival
Todoroki et al. [27]	1999	Retrospective	1976–1996	Total 85 Radiotherapy—47	Intra-operative, external or intracavitary radiation therapy	5-year survival rate and local control rate was significantly higher in the adjuvant radiation group
Kresl et al. [28]	2002	Retrospective	1985–1997	21	Adjuvant CRT Median dose of radiotherapy 54 Gy with concurrent 5-FU at 500 mg/m^2 /day for 3 days during weeks 1 and 5 of radiation	Administration of adjuvant CRT resulted in favorable local control and survival rates with 5 year survival rate of 64%
Lindell et al. [29]	2003	Retrospective	Not available	Total—20 Radiotherapy—10	Intra- and postoperative radiotherapy	Actuarial 5-year survival—47% in the radiotherapy group and 13% after surgery only but was not statistically significant
Itoh et al. [19]	2005	Retrospective	1994–2004	Total—18 radiotherapy—5	Mean total dose of 45.2 Gy	5-year survival rate was not different between the surgery plus radiotherapy group versus surgery-alone group

(continued)

Table 9.2 (continued)

Author	Year	Study design	Study period	Number of patients	Treatment	Outcome
Czito et al. [30]	2005	Retrospective	1980–2003	22	Adjuvant CRT Median dose of radiotherapy 45 Gy Eighteen patients received concurrent 5-FU at 500 mg/m^2 /day in bolus for 3 days during weeks 1 and 5 of radiation or by continuous infusion at 225 mg/m^2 /day throughout the course	5-year actuarial OS—37%
Balachandran et al. [31]	2006	Retrospective	1989–2000	Total—117 Surgery alone—44 Adjuvant CRT—73	Not available	Adjuvant CRT improved survival in stage III and node-positive patients
Mojica et al. [15]	2007	Retrospective	1992–2002	Total—3187 Surgical resection—2325 Radiotherapy—395 (17% of patients who underwent surgical resection)	Not available	Adjuvant radiotherapy improved median survival in patients with regional metastasis and disease invading liver
Wang et al. [16]	2008	Retrospective	1988–2003	Total—4180 radiotherapy—760 patients (18%)	Not available	Adjuvant radiotherapy provides a survival benefit in node-positive or ≥T2 disease
Gold et al. [20]	2009	Retrospective	1985–2004	Total—73 Surgery alone—48 CRT—25	Adjuvant CRT with median dose of radiotherapy 50.4 Gy in 28 fractions concurrently with 5-FU bolus of 500 mg/m^2 for 3 days during week 1 and 5 of radiotherapy	Adjuvant CRT was associated with improved OS after adjusting for higher T stage and node positive

Kim et al. [18]	2010	Retrospective	1994–2007	Total—166 Adjuvant therapy—40 RT alone—22 Chemotherapy—1 CRT—17	Radiotherapy dose of 44 to 45 Gy 5-FU, cisplatin or capecitabine with single or combination regimen	No significant difference in disease-free survival rates between the no adjuvant therapy and the adjuvant therapy groups
Cho et al. [32]	2010	Retrospective	2001–2009	Total—100 Analyzed 68 with stage T2–3N0M0, T2–3N1M0 Adjuvant CRT—40	Adjuvant CRT- radiation dose of 45 Gy in 25 fractions with 5-FU or gemcitabine-based chemotherapy	Adjuvant CRT improved DFS and DSS in lymph node-positive cancers (T2–3N1M0)
Lee et al. [33]	2012	Retrospective	1994–2011	Total—218 Surgery alone −83 Chemotherapy—73 CRT—62	Details not available	Chemotherapy group had better survival
Müller et al. [34]	2013	Retrospective	1999–2009	46	Adjuvant CRT (radiation dose of 45–54 Gy to tumor bed and regional lymph nodes with or without concurrent 5-FU (500 mg/m^2/day by 120-h continuous infusion on days 1–5 and 29–33)	5-year OS was 51%. Adjuvant CRT was well tolerated

(continued)

Table 9.2 (continued)

Author	Year	Study design	Study period	Number of patients	Treatment	Outcome
Ben-Josef et al. [21]	2015	Prospective	2008–2012	25	Adjuvant chemotherapy with 4 cycles of gemcitabine (1000 mg/m^2 day 1 and 8) plus capecitabine (1500 mg/m^2 per day from day 1 to 14) every 3-weekly, followed by concurrent radiation (45 Gy to regional lymph nodes and 54–59.4 Gy to the tumor bed) and capecitabine (1330 mg/m^2 per day)	Treatment was well tolerated with 2-year OS of 56%
Gu et al. [35]	2018	Retrospective	2003–2013	Total—94 Surgery alone—50 CRT—44	Adjuvant CRT—Median radiotherapy dose of 50 Gy concurrently with capecitabine or S-1 or two drugs combination (oxaliplatin plus 5-FU / capecitabine / gemcitabine)	Adjuvant CRT significantly improved OS in patients with stage II to IVA
Kim et al. [22]	2019	Retrospective	2012–2017	Total—151 Surgery alone—53 Chemotherapy—39 CRT—59	Chemotherapy group—Median of 6 cycles with gemcitabine or 5 FU based CRT group—a median radiation dose of 50.4 Gy with concurrent 5FU-based chemotherapy	Adjuvant CRT resulted in significantly higher 5-year LRFS, RFS, and OS in T2–3N1-2M0 stage
S.Agrawal et al. [36]	2020	Retrospective	2007–2017	Observation:23 CT:39 CRT:80	CT: Cisplatin, gemcitabine CRT: RT 50 Gy with conc 5FU based CT	Median OS (in months): Observation:32 CT:NR CRT:46

CRT chemoradiation, *CT* chemotherapy, *DFS* disease-free survival, *DSS* disease-specific survival, *LRFS* locoregional recurrence-free survival, *RFS* recurrence-free survival, *OS* overall survival

NCCN guidelines. CA 19–9 can be used as a simple surveillance marker in such patients and a rise beyond 20 units/l should be considered for imaging to confirm or rule out recurrence as suggested in an observational study of 60 patients. The sensitivity, specificity, positive predictive value, and negative predictive value of CA 19–9 in detecting recurrence were 79.1%, 97.2%, 95%, and 87.5%, respectively. The median disease-free survival was 56 months versus 15 months ($P = 0.008$, hazard ratio (HR): 7.4 (confidence interval 1.3–40)), and the median overall survival was not reached versus 20 months ($P = 0.000$, HR: 10.7 (confidence interval 4.2–27.3)) for CA 19–9 levels less than and more than 20 ng/mL [43].

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Part III

Targeted Therapies in Gallbladder Cancer

A Look at Emerging Therapeutic Targets for Gallbladder Cancer: A Multi-Omics Approach

10

Pavan Baichan, Previn Naicker, John Devar, Martin Smith, and Ekene Emmanuel Nweke

10.1 Introduction

Gallbladder cancer (GBC) is the most common biliary tract cancer and is the fifth most common cancer of the gastrointestinal tract [1, 2]. GBC has an overall 5-year survival rate varying from 0 to 12% due to it being characteristically diagnosed at advanced stages of disease, although early detection may increase this rate to 32% [3, 4]. Genomic alterations contribute considerably to the onset of GBC [5]. Well-described genes such as *TP53* [5], *ERBB2/ERBB3* [5], *K-ras* [6], and *CDKN2 (p16)* [7] are aberrant in GBC. Data regarding the molecular changes occurring during GBC remains inadequate. Most GBC genetic studies have been conducted in populations with high incidence such as Native American, Chilean, Japanese, and Indian [8–10]. Diagnosis at a late stage of disease and aggressiveness of the cancer contribute to a poor outcome. The standard treatment options for GBC include surgery, radiation, and chemotherapy although not all patients can undergo this procedure. Chemotherapeutic treatment options for GBC are also limited (Table 10.1); however, there are currently some clinical trials investigating the use of drugs such as FOLFIRINOX [11].

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Table 10.1 List of well-described Gallbladder cancer chemotherapeutic combinations

Therapeutic	ATC drug classification	Targets	References
Gemcitabine	Pyrimidine analogue	DNA, Ribonucleoside-diphosphate reductase large subunit, thymidylate synthase, UMP-CMP kinase	[12]
Fluoropyrimidines (Capecitabine, fluorouracil, Tegafur)	Pyrimidine analogue	Thymidylate synthase, DNA, RNA	[13–15]
Gemcitabine and cisplatin (GEMCIS)	Pyrimidine analogue and platinum compound	DNA, Ribonucleoside-diphosphate reductase large subunit, thymidylate synthase, UMP-CMP kinase, DNA-3-methyladenine glycosylase, Alpha-2-macroglobulin, Serotransferrin, copper transport protein ATOX1	[16, 17]
Gemcitabine and Capecitabine (GEMCAP)	Pyrimidine analogues	Thymidylate synthase, DNA, RNA, Ribonucleoside-diphosphate reductase large subunit, UMP-CMP kinase	[18]
Capecitabine and Oxaliplatin (CAPOX)	Pyrimidine analogue and platinum compound	Thymidylate synthase, DNA, RNA	[19, 20]
Fluorouracil and Oxaliplatin (FOLFOX)	Pyrimidine analogue and platinum compound	Thymidylate synthase, DNA, RNA	[21, 22]
Gemcitabine and Oxaliplatin (GEMOX)	Pyrimidine analogue and platinum compound	DNA, Ribonucleoside-diphosphate reductase large subunit, thymidylate synthase, UMP-CMP kinase	[23]
Fluorouracil, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX)	Pyrimidine analogue, detoxification agent for antineoplastic treatment, other antineoplastic agent, and platinum compound	Thymidylate synthase, DNA, RNA, DNA topoisomerase 1, DNA topoisomerase I (mitochondrial)	[24]

There is an urgent need to assess new potential therapeutic biomarkers that could be targeted to improve treatment outcomes. In recent years, several multi-omic approaches have been utilized for the discovery of new potential therapeutic targets. These approaches include investigating the proteome, metabolome, microbiome, genome, transcriptome, and the microRNA landscape. Techniques utilized include next-generation sequencing, arrays, and mass spectrometry. In the following sections, the different emerging therapeutic targets that may prove beneficial in future treatment strategies are described (Fig. 10.1).

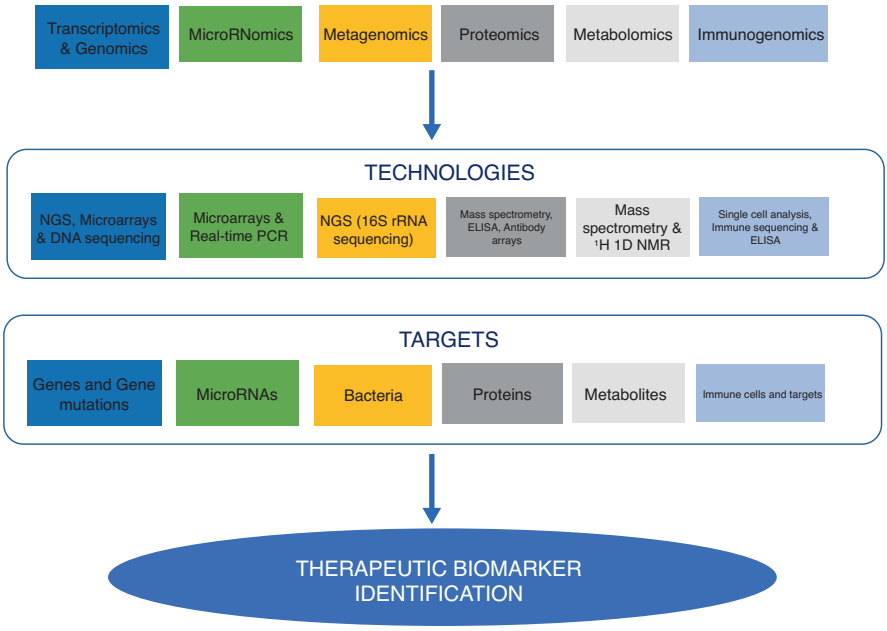

Fig. 10.1 Technologies and resulting therapeutic biomarkers for Gallbladder cancer. These potential therapeutic biomarkers can take various forms: proteins, metabolites, microbiome, microRNAs, and gene mutations

10.2 Genetic Biomarkers and Neoantigen Targets

Large-scale genomic and epigenetic studies using next-generation sequencing have helped identify gene alterations that are linked to cancer progression. Thus far, few large-scale genomic studies have focused on GBC, providing limited new information for precision therapy targets. Insights may be drawn from other genomic studies and precision therapy clinical trials of related gastrointestinal cancers. Isocitrate dehydrogenase 1 (*IDH1*) gain of function mutations and fibroblast growth factor receptor 2 (*FGFR2*) fusions are well documented genetic biomarkers linked to various cancers including bile duct cancer. Mutated *IDH1* (mIDH1) increases synthesis of 2-hydroxyglutarate, a metabolite that promotes epigenetic dysregulation [25]. *IDH1* mutations occur in up to 25% of bile duct cancer patients. Ivosidenib was shown to reduce 2-hydroxyglutarate in plasma via targeted *IDH1* inhibition and increase expression of liver-related genes in mIDH1 bile duct cancer patients [26]. Ivosidenib proved to be well tolerated in a phase-I trial of 73 patients with advanced mIDH1 bile duct cancer [27]. A global phase-III study evaluating ivosidenib patients with previously treated non-resectable or metastatic mIDH1 bile duct cancer is ongoing [28]. *FGFR2* translocations were observed in approximately 13% of bile duct cancer patients [29], resulting in FGFR2 fusions and aberrant mitogenic

signaling. Pemigatinib is approved to treat non-resectable, locally advanced, or metastatic bile duct cancer patients with *FGFR2* fusions [30]. Early phase-II trial of other targeted *FGFR* inhibitors Infigratinib [31], Derazantinib [32], and Erdafitinib [33] are underway. Other precision therapy targets being investigated for use in bile duct cancer patients' subgroups are receptor tyrosine-protein kinase *ErbB-2* (*ERBB2/HER2*) [31, 34], Serine/threonine-protein kinase B-raf (*BRAF*) [35], and tropomyosin receptor kinase (*TRK*) [36]. However, variant frequencies of these genetic biomarkers are significantly lower than that of *IDH1* and *FGFR2* in bile duct cancer patients.

Clinical trials of targeted therapies specific to gallbladder cancer are limited and often do not reach completion due to slow participant enrolment and relapse. Exome sequencing and RNA-sequencing studies of gallbladder cancer tumors help identify aberrant genes relating to disease specific pathway alterations and reveal neoantigens that could be potential therapeutic targets. A recent breakthrough study by Pandey et al. showed evidence of several predicted neoantigens that resulted from frame-shift mutations in *ELF3* [37]. The mutations were identified by performing exome sequencing on 160 GBC samples from India, Korea, and Chile. *ELF3* and *TP53* displayed the highest number of predicted high-affinity MHC class I binding neoantigens. The *ELF3* mutations significantly co-occurred with *TP53* mutations and patients displaying mutations on both proteins had an overall worse survival [37].

Neoantigen-specific activation of CD8+ T-cells (from HLA-matched healthy donor PBMCs) was assessed by measuring intracellular IFN-γ production using fluorescence-activated cell sorting. Mutant *ELF3* peptides, Y19fs, L73fs, and V345fs and mutant *ERBB2 (HER2)* peptides, S310Y and S310F were shown to activate CD8+ T-cells [37]. Transcriptome-coupled single-cell T-cell receptor (sc-TCR) sequencing was performed to confirm clonal CD8+ T-cell amplification in response to the mutant peptides. These findings suggest at the use of *ELF3*, *ERBB2,* and *TP53* neoantigen peptides as vaccine candidates. These may ultimately be used in conjunction with conventional chemotherapeutics or other targeted therapies such as checkpoint inhibitors.

10.3 MicroRNA Biomarkers in Gallbladder Carcinoma

MicroRNAs are short non-coding RNAs of length between 19 and 25 nucleotides. They function in targeting and regulating the expression of genes and thus play crucial roles in several biological processes [38]. MicroRNAs have been implicated in the progression of several cancers and shown to have therapeutic potential [39–43]. Using microarray technology, the expression of miRNAs in the blood of 40 GBC patients was compared to 40 healthy individuals and determined the significant differential expression of 11 miRNAs including miR-335, let 7a, miR-202, miR-187, and miR-21 [44]. In a similar study, miRNA signatures from tissue samples were profiled and shown that 24 miRNAs were differentially expressed and associated with poor outcomes. In this study, miR-145-5p, a tumor suppressor, was

determined to be the most under-expressed and activated the STAT1 signaling pathway [45]. The expression of miR-155 was evaluated in GBC patients compared to those with pancreaticobiliary maljunction using real-time PCR and found to be significantly upregulated and associated with poor prognosis and metastasis [46]. The overexpression of miR-155 led to increased cellular growth and proliferation suggesting its role in GBC progression. miRNA 335-5p, was downregulated in GBC patients correlating with lymph node and liver metastasis, clinical stage and histological grade [47].

Using microarray technology, miR-145, miR-143, mi1–133, and miR-1 were significantly downregulated and demonstrated to have tumor suppressive functions. Their target genes shown to be linked to pathways involved in cell growth, adhesion, and migration [48]. Among these miRNAs, increased miR-1 and miR-145 were shown by *in vitro* assays using the gallbladder cancer cell line, NOZ, to inhibit cell viability. miR-26a which directly targets *HMGA2*, a protein highly expressed in cancer, was determined to be downregulated in GBC [49]. In a similar study, miR-135a-5p was shown to be downregulated in GBC tissues and impede cellular proliferation indicating its potential as a therapeutic target [50]. Conversely, miR-182 is significantly overexpressed in GBC and is induced by TGF-β resulting in the promotion of cell proliferation migration, invasion, and metastasis [51].

Kitamura and colleagues profiled 368 miRNAs and observed the dysregulation of 21 miRNAs in GBC and their expression was inhibited by PCI-24781, a known repressor of cancer cell growth [52]. Overexpressing the potential tumor suppressor, miR-136, in both *in vitro* and *in vivo* models resulted in reduced cellular proliferation by suppressing the *MAP2K4* pathway [53]. One study found that miR-145 was downregulated in GBC and increasing its expression in GBC cells (GBC-SD) reduced cell growth and induced apoptosis by targeting *DFF45* [54]. *DFF45* functions in cancer initiation and progression by regulating apoptosis [55]. MicroRNA-218 was demonstrated as a therapeutic target for cancer including gallbladder cancer by inhibiting cellular proliferation, invasion, and migration [56–58].

10.4 Biomarkers in the Microbiome

The gut microbiome has diagnostic, predictive, and therapeutic utility in the management of GBC [59]. Analysis done by 16S rDNA sequencing showed that biliary duct cancers including GBC had an abundance of *Pseudomonadaceae* and *Bifidobacteriaceae* families with some of their members suggested to be of predictive value [60]. Analysis of bile obtained from GBC and cholelithiasis patients demonstrated that *Escherichia coli*, *Fusobacterium nucleatum,* and *Enterobacter* spp. were predominantly present in GBC [61]. A recent study conducted metagenomics sequencing on GBC and chronic calculous cholecystitis patient samples also showed the predominant abundance of *Enterococcus faecium* and *Peptostreptococcus stomatis* [62].

Salmonella typhi, a typhoid-causing bacteria that is found and infects the intestinal gut, has been associated with GBC. One study analyzed tissues, bile, and serum of GBC patients compared to those with gallstones and found a significant positive

association between *Salmonella typhi* and GBC [63]. This association has also been observed in other similar studies conducted in countries such as Chile [64, 65].

Of interest, the *Helicobacter* spp. also shows promise as predictive and therapeutic targets in gallbladder diseases and hepatopancreaticobiliary cancers [66]. For example, *Helicobacter pylori* has been linked to cholecystitis, a gallbladder disease that increases the risk of GBC [67–69].

10.5 Proteomic Biomarkers

Abnormal expression of key proteins involved in cancer has been known for decades; despite this, proteomic biomarker research for cancer only began within the last 15 years with the advent of sufficient proteomic technologies such as protein microarrays and mass spectrometry (MS). Currently, there are over 20 FDA-approved/cleared protein biomarkers for a variety of cancers [70]. Specifically in GBC, protein biomarkers have the potential to provide diagnostic, prognostic, and therapeutic utility [71, 72]. Several protein biomarkers have been identified over recent years. S100A10 is a protein which is involved in multiple functions such as enzyme activation, phosphorylation, and calcium homeostasis [72]. This protein forms a complex with annexin A2 (ANXA2) which acts as a plasminogen receptor and is involved in cancer progression via migration and metastasis. The ANXA2-S100A10 complex binds plasminogen, which induces a signaling cascade which ultimately results in degradation of the ECM allowing for increased migration and metastasis [73]. Various studies have showed that interference with S100A10 functioning via peptides, antibodies, and small molecule inhibitors has been effective in reducing cancer potency in cellular models [73]. Therefore, S100A10 overexpression in GBC may have potential and value as a new diagnostic and therapeutic protein biomarker [72].

Additionally, ANXA4 was also identified using 2-dimension electrophoresis as well as Tandem-MS. ANXA4, also known as Annexin IV, is found on the membrane surface and potentially acts a regulator of membrane fusion and possesses structural properties to induce ion channels [74]. ANXA4 has been described to be upregulated in various other cancers, including cancers of the colon, pancreas, and gallbladder [74]. Immunohistochemical staining demonstrated abundant ANXA4 expression in GBC tissues when compared to normal tissues. High ANXA4 expression was also associated with increased lymph node metastasis, invasion depth, and TNM staging. It was observed that knockdown of ANXA4 in mice models inhibited GBC tumor growth via the NF-κβ signaling pathway [75]. Therefore, ANXA4 could serve as an important prognostic biomarker and possibly therapeutic target in GBC tumor initiation and progression.

Prosaposin (PSAP) and transgelin (TAGLN) have been shown to be upregulated and downregulated in GBC, respectively. These proteins have been identified using iTRAQ-based quantitative proteomics using high-resolution mass spectrometry [76]. PSAP is a lysosomal protein which is localized to the membrane and is suggested to be a secretory protein. PSAP has also been demonstrated to be upregulated in other cancers such as prostate and breast cancer and promotes growth via the MAPK

signaling pathway [77, 78]. PSAP showed a strong presence in GBC tissue, was found to be upregulated by 2.7-fold, and predominantly localized to the cytoplasm. Elevated PSAP levels has been suggested to increase cancer cell growth and survival and thus potentially serve as a GBC biomarker [76]. TAGLN is an actin stress fiber-associated protein and is known to be elevated with differentiation. In several cancers such as breast, colon, prostate, and gallbladder cancer, it is downregulated by oncogenic Ras. TAGLN is suggested to be to be an early marker for transformation due to reduced levels disrupting normal actin structure leading to invasive properties [76].

Another protein involved in GBC progression is pleckstrin-2 (PLEK2) that was determined to be upregulated in GBC tissues when compared to cholelithiasis tissues and is correlated with high TNM stages and liver invasion [79]. It was discovered that PLEK2 dysregulation also modulates epithelial-to-mesenchymal transition (EMT) markers and remodels cellular morphology inducing aberrant migration, invasion, and metastasis. PLEK2 interacts with the EGFR/CCL2 signaling pathway by interacting with EGFR inducing inhibition of EGFR ubiquitination resulting in consistent activation. This interaction promoted migration, invasion, and liver metastasis through the EMT process [79].

Cyclooxygenase-2 (COX2), another potential therapeutic biomarker, is involved in various cancer progression mechanisms such as positive growth regulation, tumor development, and vascularization. A link between COX2 overexpression and cancer has been established in gallbladder, colon, breast, urinary bladder, and pancreatic cancers [71, 80]. Cytoplasmic staining for COX2 is frequently seen in chronic inflammation diseases, such as gallstone disease (a pre-malignant phase for GBC) [81]. COX2 is highly expressed at the invasive tumor front (ITF), which is the highest proliferating portion of tumors is indicative of poor prognosis suggesting its role as a viable protein biomarker for GBC [81].

One of the most frequently dysregulated and mutated in cancer is p53 leading to a disruption of its function. This protein functions as a tumor suppressor and over 50% of human cancers have mutated p53 expression [71, 82–84]. The prognostic significance of overexpressed p53 has been reported in several malignancies including GBC. Studies have shown that p53 expression levels increase with grade of tumor, from well-differentiated to poorly differentiated [85]. It is important to note, p53 functions as a tumor suppressor so its overexpression does not correlate with tumorigenesis. This is due to the fact that p53 becomes dysfunctional even though expression levels are elevated when compared to normal or chronically inflamed tissues.

There are many pathways, which contribute to the dysregulation of cellular processes resulting in tumorigenesis. These pathways have been the target in an attempt to treat many cancer types, along with the dysregulated and dysfunctional proteins which function within these pathways. The extracellular signal-regulated kinase (ERK) 1/2 plays a role in the MAPK/ERK signaling pathway which is responsible for proliferation and anti-apoptosis in cancer cells [71, 86]. ERK1/2 is activated via phosphorylation and is significantly upregulated in GBC. High expression of p-ERK/1–2 indicated low grade differentiation and was further increased in late stages of GBC correlating with increased tumor size, lymph node involvement, and local invasion [86].

10.6 Other Potential Biomarkers: Metabolites

Metabolomics is the study of small molecules (i.e., metabolites) in the body. Metabolites include small molecule substrates, intermediates, and products of cellular metabolism [87]. Metabolomics is fast becoming a useful technique for the identification of cancer biomarkers [88, 89]. Using nuclear ^{1}H 1D nuclear magnetic resonance (NMR), various metabolites were identified in patients suffering from cholecystitis, an inflammation of the gallbladder which is a risk factor of GBC [90]. These metabolites included acetate, alanine, histidine, lactate, glutamate, formate, tyrosine, lipid, glutamine, and 1, 2-propanediol, which are involved in amino acid metabolic pathways and have been linked to the disease. Metabolomics biomarkers may prove useful as early diagnostic and therapeutic biomarkers of GBC [91], however, further studies are necessary to assess the sensitivity and specificity of such biomarkers in clinical settings [91].

10.7 Emerging Targets and Therapies: The Role of the Immune System

Targeted therapies may include those that are directed toward specific molecules (i.e., "molecular targets") such as genes, RNA sequences, peptides/proteins, and metabolites that are involved in cancer progression and those that target cancer cells specifically or disrupt cellular processes that promote cancer progression with minimal side effects (such as immunotherapy). Immunotherapy is an emerging type of cancer treatment that assists the immune system in destroying cancer cells. A prominent example of immunotherapy are immune checkpoint inhibitors such as programmed death 1 (PD-1) protein inhibitors. PD-1 is a co-inhibitory receptor on the surface of activated T-cells (and other immune cells) that regulates T-cell activity. The PD-1 receptor attaches to programmed death ligand 1 and 2 (PD-L1 and PD-L2) present on tumor and/or stromal cells, resulting in immune suppression [92, 93]. Monoclonal antibodies have been shown to be effective in inhibiting the interaction between PD-1 and its ligands, potentiating antitumor activity [94, 95]. Pembrolizumab is a humanized monoclonal antibody that targets PD-1 and has shown to have lasting antitumor activity in advanced biliary tract cancer patients, independent of PD-L1 expression levels [96, 97].

10.8 Conclusion and Future Perspectives

The upward trend of incidence and mortality of gallbladder cancer observed worldwide calls for a better way to diagnose and treat the disease. This requires a better understanding of the molecular underpinnings surrounding its induction and progression. The emergence of an array of new technologies allows for the continued deciphering of the molecular architecture of GBC and consequently the identification of therapeutic biomarkers of GBC. This might require a multi-omics approach

as we have discussed in this chapter (Table 10.2). To strengthen the utility of these potential biomarkers, future strategies might have to combine various biomarkers. These biomarkers might be targeted alone or in combination. For example, concomitant targeting a protein biomarker and bacteria may increase efficacy in GBC treatment. Similarly, the emergence of immunotherapy may further provide treatment avenues that are effective and with little side effects.

Table 10.2 Emerging and some current therapeutic biomarkers for gallbladder cancer

Type of biomarker	Name of target	Function of target	References
Protein	ANXA2-S100A10 complex	Migration and metastasis	[73]
	ANXA4	Lymph node metastasis and TNM staging	[75]
	PSAP	Cellular growth and survival	[76]
	TAGLN	Invasion	[76]
	PLEK2	TNM staging and liver invasion	[79]
	COX2	Vascularization	[80]
	p53	Tumor suppressor	[82–84]
	ERK1/2	Anti-apoptosis and proliferation	[86]
Genomic/ neoantigens	IDH1	Promotes epigenetic dysregulation	[25]
	FGFR2	Aberrant mitogenic signaling	[29]
	ErbB-2 (ERBB2/HER2)	Aberrant proliferation	[31]
	BRAF	Cellular proliferation and differentiation	[35]
	TRK	Survival and differentiation	[36]
	ELF3	Cellular differentiation	[37]
MicroRNA	miR-145-5p	Tumor suppressor	[45]
	miR-155	Cellular growth and proliferation	[46]
	miRNA 335-5p	Cellular growth and proliferation	[47]
	miR-145	Tumor suppressor	[48]
	miR-143	Tumor suppressor	[48]
	miR-133	Tumor suppressor	[48]
	miR-1	Tumor suppressor	[48]
	miR-26a	Cellular differentiation	[49]
	miR-135a-5p	Cellular proliferation	[50]
	miR-182	Anti-apoptotic	[51]
	miR-136	Tumor suppressor	[53]
	miR-218	Anti-proliferation	[56, 58]
Microbiome	*Pseudomonadaceae*	All upregulated in GBC	[60])
	Bifidobacteriaceae		[60]
	Escherichia coli		[61]
	Fusobacterium nucleatum		[61]
	Enterobacter spp.		[61]
	Salmonella typhi		[63]
	Helicobacter spp.		[67–69]

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MicroRNA and Their Role in Carcinoma Gallbladder

11

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11.1 Introduction

MicroRNA (miRNA) technology is a novel and exciting development in cancer diagnostic and therapeutic and may potentially address shortcomings related to the management of gallbladder cancer in the present era. Gallbladder cancer (GBC) is an aggressive malignant disease and carries an extremely poor prognosis. Advanced disease corresponds to dismal outcomes. Patients usually have no specific presenting symptoms, and thus the common presentation in these patients is with late-stage disease. Conventional chemotherapy and radiation have not shown much improvement in terms of survival and quality of life. In cases with lesions confined to the gallbladder mucosa, the 5-year survival rate is reported to be 32% while for advanced lesions; 1-year survival rate is only a 10% [1]. Surgery is the sole therapeutic modality which maybe curative. Unfortunately, curative surgery is limited to early-stage gallbladder cancer. Early detection of gallbladder cancer and addition of novel therapeutic modalities form the cornerstones of improving outcomes in these patients. Emerging evidence links miRNA as a potential marker of early gallbladder cancer as well as a therapeutic target in cancer. The characterization of alterations of miRNA in GBC pathogenesis, and its prognosis is evolving.

11.2 What Are MicroRNAs?

MicroRNAs (miRNAs) are conserved short (18–25 nucleotides in length) noncoding RNAs which bind and regulate mRNA translation into proteins. These endogenous molecules are ubiquitous in plants, animals, and viruses and act as

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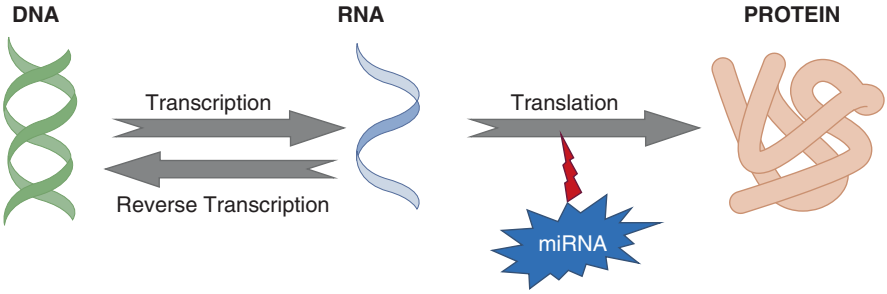

Fig. 11.1 Role of miRNA in central dogma

master regulators of gene expression regulating about 60% of human genes [2–4]. A total of 10,000 plus miRNAs have been identified till date [4–6]. Mis-expressed miRNAs have found to be associated with several pathological conditions [7, 8]. miRNAs are transcribed from the non-coding intron regions in DNA and negatively regulate translation of mRNA transcribed from the coding exon regions in DNA (Fig. 11.1).

miRNAs function as negative regulators of post-transcriptional gene expression. It binds to the 3′untranslated region (UTR) of target mRNAs, and destabilizes/degrades transcriptionally or inhibiting them translationally (or both) [9, 10]. Furthermore, miRNAs also bind to 5′UTR region, coding region, and gene promoter regions. miRNAs bind to target mRNAs through imperfect pairing; thus it regulates the expression of multiple genes [11]. Also, one gene can interact with multiple miRNAs [12].

11.2.1 miRNA Synthesis

miRNA synthesis involves three main steps:

1. Transcription,
2. Nuclear processing and export to the cytoplasm,
3. Cytoplasmic processing.

11.2.2 miRNA Transcription

Akin to mRNA transcription, miRNAs are also transcribed in the nucleus of the cell from DNA. miRNAs are first transcribed as precursor miRNA (pri-miRNA) by RNA polymerase II. RNA polymerase II also transcribes messenger RNA (mRNA). Pri-miRNA is a stable stem-loop which includes a hairpin structure and may be more than 1000 nt in length. One of the two strands of the pre-miRNA hairpin ultimately becomes the future mature miRNA.

11.2.3 Nuclear Processing and Export

Pri-miRNA is recognized and cleaved by microprocessor complex (double strand specific ribonucleases Drosha and Pasha) with the release of a hairpin shaped pre-miRNA with length varying from 60 to 100 nt [13, 14]. Pre-miRNA is exported out of the cytoplasm via the exportin-5 (XPO5) complex.

11.2.4 Cytoplasmic Processing

Subsequently, per-miRNA is cleaved by Dicer-TRBP (TAR RNA-binding protein)-PACT (or PRKRA) complex producing 20–24 nt miRNA duplexes in which one of the strands is the future miRNA [2]. These miRNA duplexes, known as miRNA: miRNA* duplex, become associated with argonaute (Ago) proteins to comprise RNA-induced silencing complex (mi-RISC) function [15, 16]. Either 5′ or 3′ end of pre-miRNA becomes associated with Ago proteins, with propensity for binding for strains with the least stable pairing at 5′ end. Next, RISC is released, coupled with degradation of one strand (passenger strand) from miRNA:miRNA duplex, while the other strand (guide strand miRNA) remains associated with RISC in a complex known as mature miRNA. The mature miRNA complex interacts with and regulates the target genes [17].

Furthermore, non-canonical miRNA biogenesis pathways exist. These pathways describe differing combinations of the proteins involved in the canonical pathway, mainly Drosha, Dicer, exportin 5, and AGO. The non-canonical miRNA biogenesis may be grouped into Drosha-independent and Dicer-independent pathways [14]. Drosha-independent pathway products resemble Dicer substrates. Examples of such pre-miRNAs are mirtrons and 7-methylguanosine (m^7G) capped per-miRNA. Microns are RNAs produced from the splicing of introns of mRNA during mRNA processing. These nascent RNAs are exported to the cytoplasm through exportin 1 without the need for Drosha cleavage (Fig. 11.2).

Conversely, Dicer-independent miRNAs are produced by Drosha from short hairpin RNAs (shRNA). These pre-miRNAs are insufficient in length to be Dicer-substrates, and thus require binding to AGO for further maturation [18]. This in turn enhances loading of the entire pre-miRNA into AGO2 and consequently AGO-dependent slicing of the 3p strand. The 3′-5′ trimming of the 5p strand completes the maturation of DICER independent miRNAs [19].

11.2.5 Mechanism of miRNA-Mediated Gene Silencing

The miRNA-induced silencing complex (miRISC) includes the guide miRNA strand and AGO. miRNAs binding occurs in a sequence specific manner at the 3′ UTR of target mRNAs resulting in mRNA deadenylation and decapping, thereby inducing translational repression and arrest. miRNA binding may also occur at 5′ UTR, coding sequence and within promoter regions. The binding of miRNAs to 3′

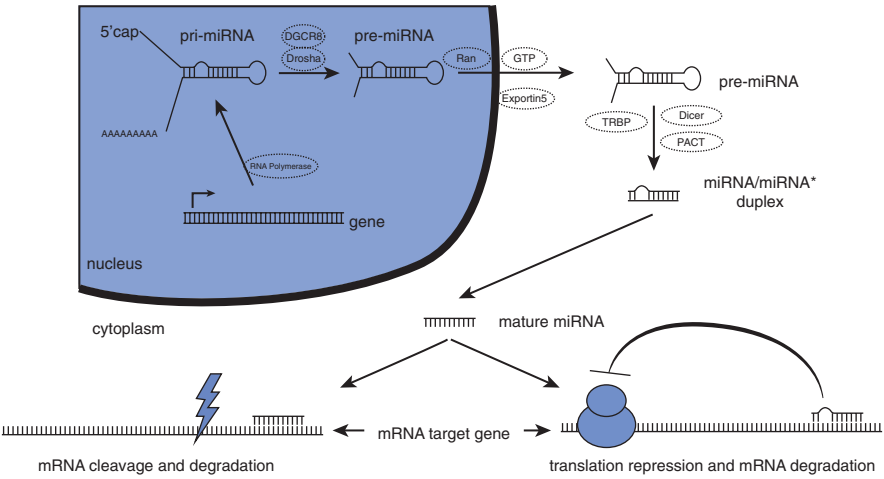

Fig. 11.2 miRNA biogenesis

UTR, 5′ UTR, and coding regions produce translational silencing effects on gene expression, whereas miRNA binding with promoter region induces transcription. Further studies are required to fully elucidate the functional significance of different modes of interaction. MiRISC targets its mRNA based on complementary sequences on the target mRNA, known as the miRNA response elements (MREs). MRE complementarity determines further slicing or translational inhibition of target mRNA, as previously discussed. Fully complementing miRNA and MRE interaction induces endonuclease activity of AGO leading to mRNA cleavage. However, majority of miRNA and MRE interactions are only partially complementary since most MREs contain mismatches to their guide miRNA, thereby inhibiting AGO endonuclease activity. This type of interaction leads to mRNA silencing and translational arrest.

The formation of a silencing miRISC recruits GW182 family of proteins; GW182 provides scaffolding needed for recruitment of effector proteins, such as poly(A)-deadenylase complexes PAN2-PAN3 and CCR-NOT, following miRNA-target mRNA interaction. Target mRNA poly(A)-deadenylationis initiated by PAN2/3 and completed by CCR4-NOT. Further decapping occurs facilitated by decapping protein 2(DCP2) and associated proteins, followed by 5′-3′ degradation by exoribonuclease 1 (XRN1) [19] (Table 11.1).

The mechanism of miRNA regulation is under control of various nuclear and cytoplasmic factors under three main steps, transcription, biogenesis, and binding at target sites. Transcription of miRNA is under the control of regulators (p53, E2F, or cMyc) with oncogenic or tumor suppressor functions. Epigenetic modifications at the promoter and genomic changes like chromosomal rearrangements and mutation or SNP causes miRNA aberrations. Biogenesis and maturation of miRNA is under control of various enzymes or co-regulators such as exportin 5, Drosha, Dicer, TRBP, Ago2, and RISC, which are frequently deregulated under various pathological conditions [2].

Table 11.1 Known miRNA and their site of modulation

miRNA	Target
miR-1[a]	VEGF, Snail2, Slug, Notch signaling
let-7	EGFR, TSP1, TIMP-1, IRS2, IGF-1R, INSR, HMGA2, Cyclin A, CDK1, CDK2, Cyclin D, cMyc, Cdc34, RAS, Mmp11, HMGA2, Egfr, FAK, IGFR
let-7a	cMyc
let-7a	Caspases-3/7
let-7c	BCL-XL
let-7d	Twist1
let-7f	THBS
let-7g	BCL-XL
miR-9	VEGF, MMP, Snail1, E-cadherin, NFkB
miR-10	VEGF
miR-10a	Caspases
miR-10b	HOXD10, KLF4
miR-13a	GAX, HOXA5
miR-15	Cyclin E, Cyclin25A, CDKs, Cdc27, APC
miR-15/16	CDK2/4/6
miR-15a	BCL-2, MCL1
miR-15a-16-1	Cyclin D, TPI1, AldoA
miR-15a/b	VEGF
miR-15b[a]	BCL-2, BCL-W, BMI2
miR-16	VEGF, FGFR, VEGFR, G0/G1, Cyclin E
miR-16-1	BCL-2, MCL1
miR-17[a]	VEGFR
miR-17-3p	LDH-A
miR-17-5p	VEGF, TIMP-1, GPI, Bim, P21, HBP1
miR-17-92	VEGFR, JAK, PTEN, THBS, TSP1, PTEN, G1/S (Akt pathway), Rb, P21, P27, p57, p19, p16, PTEN
miR-17/20	Cyclin D, E2f, IL8, IL8, CXCL1, CK8
miR-18a	VEGF
miR-19	Bim
miR-19a	GLUT1, Citrate synthase, Cyclin D
miR-19a/b	TSP1
miR-19b	GLUT1, Citrate synthase
miR-20a[a]	FAS/DR4,5
miR-20a/b	VEGF
miR-20b	HIF-1
miR-21[a]	PTEN, PDCD4, Sprouty1, JAG-1/ DLL4, GLUT3, GLUT1, PKM2, LDHB, LDH-A, Hexokinase 1, AKT, PTEN, HIF-1alpha, G1/S, Cyclin25A, CDKs, PTEN, Smad, Fas L, TRAIL, TGFβ, MMP2, MMP10, TIMP, TPM1, PDCD4, RECK, BMD6, PTEN, MARCKS, Cdc25A, BTG2, Mapsin, Her2
miR-23	TGFβ
miR-23a	SMAD4, XIAP
miR-23a/b	Myc, Apaf-1

(continued)

Table 11.1 (continued)

miRNA	Target
miR-23b	Notch signaling, c-MET, POX/PRODH, Proline oxidase
miR-24[a]	HNF-alpha/stat3, Cyclin A, CDK1, CDK2, Cyclin B, XIAP, CDK2/4/6, E2f, P19, P16, CMyc, FAF1
miR-24-2c	BCL-2
miR-24a	Caspases-9
miR-25	P53, Bim
miR-26a[a]	HGF, Smad-1/4, PDHX, Cyclin D, Cyclin E, PTEN
miR-27	APC
miR-27a/b[a]	Apaf-1
miR-27b	THBS, TSP1
miR-29	P53, PI3K, CDC42, p85α, BCL-2, MCL1, Wnt/β-catenin signaling
miR-29a	TTP
miR-29a/b	MCT1, MTC1
miR-29b[a]	VEGF, MMP, insulin, ketoacid dehydrogenase complex NMN, NNMT, MMP2, MMP9
miR-29c[a]	Cyclin E
miR-30	JAG-1/DLL4, P53
miR-30a	Snail1
miR-30d	P53
miR-30e[a]	NF-kb
miR-31	VEGF, P19, RHOA, Integrin α5
miR-32	SLC45A3, Bim
miR-33	P53
miR-33a	SREBP-1/2
miR-33a/b	ABCA1, ABCG, NPC1 CROT, HADHB, PRKAA1, IRS2, SIRT6;/AMPK
miR-33b	SREBP-1/2, cMyc
miR-34	G0/G1, P21, BCL-2
miR-34a[a]	Hexokinase 2, Hexokinase 1, PCTP, LIPA, GSS, ACSS1, SCD1, AKT2, SIRT1, P53, Cyclin E, E2f, CMyc, CDK2/4/6, Snail1, CD46, c-MET
miR-34a-c	Cyclin D
miR-34c	BCL-2
miR-92	PTEN, Bim
miR-92-1	VHL, HIF-1alpha
miR-92a	Integrin alpha5
miR-92b	P27, p57, p19, p16
miR-93	GLUT4, E2F1, P21
miR-99a[a]	PKM2
miR-99a/b	TGFβ
miR-100[a]	PI3K, mTOR, Cyclin25A, CDKs, PLK1, cdc25c, CDK1-cyclin B1 complex
miR-101[a]	VEGF, MCL1, EZH2
miR-103	Acetyl Co A, Insulin
miR-103/107	Caveolin-1
miR-106a[a]	GLUT3, Rb
miR-106b	ABCA1, P21, E2F1, P21
miR-106b-25	TGFβ

Table 11.1 (continued)

miRNA	Target
miR-107	HIF-1, Acetyl Co A, Insulin, CDK2/4/6
miR-122	PKM2, TPI1, AldoA, Agpat1, Cidec, Stard4, CAT-1, P53, BCL-W
miR-122a	Citrate synthase
miR-124	PKM2, SLC16A1, MCT1, MTC1, Snail2
miR-124a	CDK2/4/6, Notch signaling
miR-125	PKM1, Hexokinase 2, E2f
miR-125a[a]	Cyclin A, CDK1, CDK2
miR-125b[a]	VEGF, HER2/3, PCTP, LIPA, GSS, ACSS1, SCD1, AKT2, p53, Cyclin25A, CDKs, Bak, P53
miR-126	VEGF, EGF, PIK3R2, ANGPT, PI3K, PTEN, IRS1, PKM2, PI3K
miR-128	BAX
miR-128a	E2f, Wee1, FADD
miR-128b	EGFR
miR-129	CDK2/4/6
miR-130	PPARγ
miR-130a[a]	FGF, VEGF
miR-130b	GLUT1
miR-132	FGF, VEGF, PTEN,
miR-132/212	Rb
miR-133	GLUT4, Caspases-9
miR-133a	BCL-XL, RHOA
miR-133a/b[a]	PKM2
miR-133b	BCL-W, MCL1
miR-135a[a]	cMyc
miR-137	PKM2, CDK2/4/6
miR-138	GLUT1, Hexokinase 1, P53, ZEB1/ZEB2, SOX4
miR-143[a]	Hexokinase 2, AKT, PI3K, BCL-2, RAS
miR-143/145	KLF4/5
miR-144	Caspases
miR-145	MMP
miR-145	CMyc
miR-146	SMAD
miR-146a	FAS/DR4,5
miR-146a,b	NFkB
miR-146b-5p[a]	SMAD
miR-148a[a]	Citrate synthase, BCL-2
miR-148b	Citrate synthase
miR-149	E2f, E2F1
miR-150	GLUT1, GLUT4
miR-152	Citrate synthase
miR-153	BCL-2, MCL1, Snail1, ZEB1/ZEB2
miR-155[a]	VEGF, AT1R, Hexokinase 2, Wee1, Caspases-3/7, FADD, TGFβ, RHOA
miR-175p	E2F1
miR-181	P21, Notch signaling

(continued)

Table 11.1 (continued)

miRNA	Target
miR-181a	Bim, BCL-2
miR-181b	BCL-2
miR-183	IDH2
miR-185	SREBP-1/2, RHOA
miR-186	Caspases- 10/8
miR-191	VEGF
miR-192	P53, TGFβ
miR-193b	Cyclin D, MCL1
miR-194	P53, P21, BMI1
miR-194/215	P21
miR-195	TPI1, CAB39, Cyclin D, Cyclin E, E2f, CDK2/4/6, Wee1, BCL-2, NF-kb
miR-195-5p[a]	GLUT3
miR-196	VEGF
miR-196b	FAS/DR4,5
miR-199	HER2/3
miR-199-3p	PI3K
miR-199a	VEGF, mTOR, HIF-1alpha
miR-199a-3p	ApoE, DNAJA5
miR-199a-5p	GLUT1, ApoE, DNAJA4
miR-199b	JAG-1/ DLL4
miR-200	VEGFR, PGI, Snail2, ZEB1/ZEB2, Slug, Notch signaling
miR-200a	Wnt/β-catenin signaling
miR-200b	ETS
miR-200c	FAF1
miR-200s	GPI
miR-203	Surviving, Snail1, Snail2, Slug
miR-204	BCL-2, TGFβR2, Slug
miR-204/211	Snail2
miR-205	ACSL1, BCL-W, ZEB1/ZEB2
miR-206	VEGF
miR-210	EphrinA3, Cox10, SDH, ISCU 1/2, GPD1L, HIF-1alpha, E2f
miR-212	PED
miR-214	VEGF, PTEN, PTEN, P53, Twist1
miR-215	P53, ZEB1/ZEB2, TGFβ
miR-216a	PTEN, SMAD
miR-217	PTEN, SMAD
miR-218[a]	BMI1, surviving
miR-221	PI3K
miR-221-222	TIMP, PUMA, G1/S (Akt pathway), P27, p57, p19, p16, PTEN, TRPS1, ESR1
miR-223[a]	GLUT4, FOXO1, cMyc, E2f
miR-224	API-5, Smad
miR-290	Rb
miR-296	VEGF, HGS
miR-299-5p[a]	Citrate synthase

Table 11.1 (continued)

miRNA	Target
miR-301a	GLUT1
miR-302a	Cyclin D
miR-302b	GPI
miR-320	IGF-1, PFKP/m
miR-326	PKM2
miR-322/424	Cyclin25A, CDKs
miR-328[a]	CD45
miR-330	E2f
miR-331	E2f
miR-335	SOX4, TNC, SP1
miR-340	PKM2
miR-342	SREBP-1/2
miR-351	ANGPT
miR-352-5p	VEGF
miR-361-5p	VEGF
miR-365[a]	BAX
miR-365-2	BCL-2
miR-371-373	Wnt/β-catenin signaling
miR-372	CDK2/4/6, P21, Wee1, NF-kb
miR-373	NF-kb
miR-373/520c	TGFβR2, CD44
miR-375	LDHB, PCTP, LIPA, GSS, ACSS1, SCD1, AKT2, insulin
miR-378	ERRγ, GABPA, ESRRG, Caspases-3/7
miR-380	P53
miR-421	Citrate synthase
miR-424	VEGF, FGFR, VEGFR, HIF-1alpha
miR-429	cMyc
miR-449a	Cyclin D, Cyclin25A, CDKs
miR-449a/b	Cyclin25A, CDKs
miR-451	CAB39
miR-483-3p	PUMA
miR-486	PTEN
miR-491	BCL-XL
miR-494	Citrate synthase
miR-503	FGF, VEGF
miR-504	P53, P21
miR-512-3p	c-FLIP
miR-516-3p	Wee1
miR-519c	HIF-1
miR-520	PFKP/m, NF-kb, TGFβ
miR-532-5p	GLUT1
miR-580	Twist1
miR-590	Fas L, TRAIL
miR-593	PLK1, cdc25c, CDK1-cyclin B1 complex

(continued)

Table 11.1 (continued)

miRNA	Target
miR-608	BCL-XL
miR-629	HNF-alpha/stat3
miR-644a	GAPDH
miR-661	Snail1, StarD10, Nectin-1
miR-708	ZEB1/ZEB2
miR-758	ABCA1
miR-1285	P53
miR-1291	GLUT1, NMN, NNMT
miR-1296	Mcm2
miR-1908	ApoE, DNAJA4
miR-bart5	PUMA

[a] Role implicated in carcinoma gallbladder

11.3 MicroRNA and Cancers

The role of miRNAs is well established as both tumor suppressor and oncogenes, thereby regulating cancer progression. These miRNAs are known as "oncomiRs" [9]. Oncogenic miRNAs act directly on transcripts with pro- apoptotic or anti-proliferative functions [20]. Tumor suppressor miRNAs suppress the expression of oncogenes and/or genes promoting cellular proliferation [21]. Circulating and tissue miRNA profiles are being utilized as diagnostic and prognostic biomarkers and therapeutic targets for cancer [22–24]. In addition, tumor-based miRNA signatures were suggested to identify tissue of origin of cancer [25, 26]. In general, miRNAs are involved in transcriptional regulation of important genes that control key signaling pathways involved in apoptosis, cellular proliferation, angiogenesis, and regulation of the microenvironment (Fig. 11.3).

miRNAs regulating cancer progression are known as "OncomiRs" [9]. Several miRNAs have now been identified to play important roles as either tumor suppressors or oncogenes. Oncogenic miRNAs act through mRNA transcripts with pro-apoptotic or anti-proliferative functions [20]. Tumor suppressor miRNAs suppress cellular proliferation by regulating the expression of oncogenes [21] (Table 11.2).

11.3.1 miRNA and Cell Cycle Regulation

miRNAs control cell cycle by targeting cell cycle regulatory genes or indirectly by targeting signaling pathways [25–28]. Cyclins (cyclin A, B, D, E) and cyclin dependent kinases (CDK 2, 4, 6) promote the cell cycle with Retinoblastoma protein inactivation and E2F transcription factor activation [29–31]. Conversely, the INK4 (p16, p15, p18, and p19) and Cip/Kip (p21, p27, and p57) families suppress the cell cycle by activating CDK inhibitor [32–35].

Oncogenic miRNAs, such as miR-17-92, cause cell cycle progression by targeting its negative regulators [36]. Unregulated miR-221/222 and miR-21 has been shown to

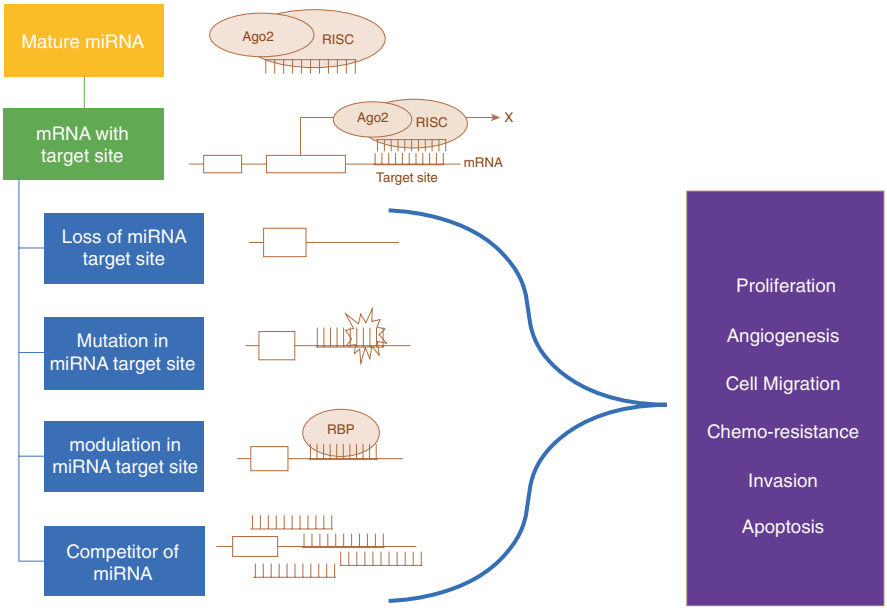

Fig. 11.3 Role of miRNA in cancers

Table 11.2 Targets for miRNA modulation

Specific action	Target
Angiogenesis	
A negative regulator of VEGF signaling	Sprouty1
Angiogenesis inhibitor	GAX, HOXA5, TSP1
Cell survival pathway	IGF-1
Degrade ECM	MMP
Degrades VEGFR2/antiangiogenic	HGS
Determine formation of arteries or veins	EphrinA3
Growth factor promoting angiogenesis signaling	EGF
Inhibit MMPs	TIMP-1
Involved in JAK/STAT signaling	JAK
Mediate cell–matrix and cell–cell interactions	Integrin alpha5
Mediate TGF-beta signaling promoting angiogenesis	Smad-1/4
Mediates cell-to-cell and cell-to-matrix interactions	THBS
Notch signaling and vascular development	JAG-1/ DLL4
Promote angiogenesis	Cox10
Regulate angiopoietin-2	PDCD4,
Regulate PI3K/Akt/VEGF signaling	PTEN,
Transcription control of VEGF	E2F1
Transcription factor for VEGF, HGF, VEGFR, MMPs	ETS
Transcription factor regulating various gene expression	KLF4/5
Transcriptional activation of VEGF	HIF-1

(continued)

Table 11.2 (continued)

Specific action	Target
Upregulate VEGF	AT1R
Vessel-destabilizing controlling vessel regression	ANGPT
Apoptosis	
Activate anti-apoptotic proteins/ apoptosis inhibitor	NF-kb
Activate transcription of pro-apoptotic Bcl-2/ apoptosis inducer	P53
Activates caspase and mediate extrinsic death signaling	FADD
Activates extrinsic death signaling	FAS/DR4,5
Binding to their receptor, induces death receptor signaling	Fas L, TRAIL
Blocks apoptosis by interacting with procasp-3, casp-8, or by inducing cell cycle arrest	P21
Cellular destruction/pro-apoptotic	Caspases-3/7
Cellular destruction/pro-apoptotic	Caspases-9
Downregulate Bcl-E|, Bcl-2, while upregulate Bax/apoptosis inducer	Smad
Form apoptosome after binding to cyt c and pro-caspase-9	Apaf-1
Govern MOMP/pro-apoptotic	Bak
Increase mitochondrial outer membrane permeability/pro-apoptotic	BAX
Induce cytochrome c release/pro-apoptotic	PUMA
Inhibits active caspase 9/anti-apoptotic	Surviving
Inhibits caspase 3,7,9/ anti-apoptotic	XIAP
Inhibits the assembly of DISC/anti-apoptotic	c-FLIP
Initiator caspases, activates casp 3, 6, 7/pro-apoptotic	Caspases- 10/8
Interact with other members of the BCL-2 protein family/ pro-apoptotic	Bim
Mediates programmed cell death	FAF1
Regulate various protein in apoptotic pathway/apoptosis regulator	E2F1
Suppresses E2F1-induced apoptosis/apoptosis inhibitor	API-5
Cell cycle	
Activate cyclin D, CDK4/6 and cdc25A (oncogene)	Cdc34
Activate p21 (tumor suppressor protein)	P53
Activation of CDKs	CDK2/4/6
G2/M transition	PLK1, cdc25c, CDK1-cyclin B1 complex
Inhibits cdc2 and CDK2	P21
Initiation of genome replication	Mcm2
P53 upregulation	CDC42, p85α
Prevents the activation of cyclin E- CDK2 or cyclin D-CDK4complexes (negative regulator of cell proliferation)	p19
Regulate G1/S and G2/M transition	PI3K
Regulate M/S phase	Cyclin A, CDK1, CDK2, cyclin B, cyclin D
Suppress CDK1-cyclinb1 complex	Wee1
Suppress cell cycle (tumor suppressor protein)	Rb
Target cell cycle proteins for degradation	Cdc27, APC
Tumor suppressor gene	PTEN

Table 11.2 (continued)

Specific action	Target
Metabolism	
Amino acid metabolism	CAT-1
AMPK signaling	CAB39
Cholesterol metabolism.	ABCA1
Glucose metabolism	AldoA, citrate synthase, GAPDH, GLUT1, GLUT3, GLUT4, GPI, Hexokinase 1, HEXOKINASE 2, IDH2, ISCU 1/2, LDH-A, LDHB, PDHX, PFKP/m, PGI, PKM1, SDH, SLC45A3, SMAD4, TPI1
HIF1/MYC signaling	cMyc
Insulin signaling	Caveolin-1
Lactate metabolism	Insulin
Lipid metabolism	ACSL1
Nicotinamide metabolism	NMN, NNMT
P53 signaling	BMI1
PI3K/Akt/mTOR signaling	AKT
Warburg effect	ERRγ, GABPA, ESRRG
Metastasis	
Activation of MAPK pathway	RAS
Activation of Wnt/β-catenin signaling	HBP1
Cell–cell and ECM interactions	Integrin α5
Degradation of TNF-α, Cox2, IL-3, IL-8, VEGF	TTP
Downregulation of E-cadherin	Snail1
EMT-invasion-metastasis	TRPS1, ESR1
Endothelial activation and mesenchymal invasion	RHOA
Epithelial marker	E-cadherin
Induces metastasis by MMP activation	IL8
Invasion	
Invasion, migration	HOXD10, KLF4
Involved in Wnt/β-catenin signaling	APC
Mediate TGF-β signaling	SMAD
Mediate TGF-β signaling	
Metastasis-angiogenesis	ApoE, DNAJA4
MMP inhibitor	TIMP
Promote EMT	Notch signaling
Promote invasion via PI3K/AKT or Rho signaling	CD44
Regulate Ezh2 and EMT metastasis	SOX4
Regulate snail expression and promote metastasis	BMI1
Tissue remodeling, migration and invasion	MMP2, MMP10, MMP9
Transcription repression of E-cadherin	EZH2
Upregulation of ZEB1 and 2	NFkB

promote G1/S transition [37], while overexpression of miR-16 and mir-34 family miRNAs may lead to G0/G1 arrest. The let-7 and miR-15 families which are major tumor-suppressor miRNAs are frequently lost or downregulated in various cancers [26, 38]. Furthermore, recent studies have demonstrated a complex interaction between miRNAs and several transcription factors (p53, cMYC) governing the cell cycle [39, 40].

11.3.2 miRNA and Apoptosis

miRNAs have been shown to modulate the extrinsic and intrinsic apoptotic pathway, by regulating the expression of pro-apoptotic and anti-apoptotic protein. In general, pro-apoptotic miRNAs (mir-15, mir-16, let-7f, mir-34, mir-1, mir-101, mir-29) target anti-apoptotic genes or negative regulators of apoptosis while anti-apoptotic miRNAs (mir-21, mir-133, mir-17-92, mir-206, mir-143, mir-145, mir-155, mir-221/222) target pro- apoptotic genes or positive regulators [41–43].

P53, an important player of apoptosis, is negatively regulated by mir-125b and mir-380-5p while miR-29 family members were identified as positive regulators by targeting upstream CDC42 and p85α [44]. In addition, mir-10a, let-7a, mir-144, mir-133, mir-24a, and mir-155 were shown to affect caspases activation with consequent diminished apoptosis [43, 45, 46].

11.3.3 miRNA in Local Spread and Metastasis

miRNAs, collectively termed as "MetastamiR," play significant roles in metastasis by regulating the expression of different genes involved in various steps of metastasis such as cancer cell detachment, invasion, and migration [47–50]. mir-200f and mir-203 are well-known epithelial markers that are associated with metastasis when overexpressed by targeting ZEB1/2 and Snail1/2 expression [51, 52]. mir-221/222, mir-103/107, mir-27, mir-9, mir-155, mir-81a, and mir216a/217 are EMT inducer, while mir-30a, mir-34a/b/c, mir-124, mir-203, mir-145, mir-204/211, mir-138, mir-215, mir-708, and mir-205 are EMT inhibitors [53]. Furthermore, miR-143, miR-29b, miR-206, mir-340, miR-218, mir-491-5p, miR-338-3p, let-7, miR-31, mir-21, mir-181, and mir-22/222 regulate extracellular matrix remodeling through modulation of matrix metalloproteinases in cancer [54].

MiR-10b, mir-21, mir-520c, and mir-373 were reported as pro-metastatic miRNAs [55], while let-7, mir-126, mir-335, mir-206, and mir-31 were found to be anti-metastatic miRNAs [56]. Downregulation of the miR-200f, miR-148a miR-148b, and miR-9 family, and upregulation miR-210 is specific to metastatic cancers [57].

11.3.4 miRNA and Angiogenesis

Several angiomiRs targeting angiogenesis have been also identified [58]. Specifically, miR-17-92 cluster, miR-27b, miR-126, miR-130a, miR-210, miR-296, mir-21,

mir-31, let-7f, and mir-378 have pro-angiogenic function and promote tumor angiogenesis, while miR-221 /miR-222, miR-320, mir- 26a, miR-15, miR-16, miR-20a, and miR-20b are anti-angiomiRs [36, 59–61].

miRNAs also regulate endothelial cell (EC) function and vascular development [59, 62–65]. ECs demonstrated high expression of miR-21, let-7f, miRNA-23-24 cluster, mir-15b, mir-16, mir-100, miR-126, miR-221/222, and miR-17-92 cluster [36, 66]. miR-126 was suggested to be an EC specific miRNA that promotes angiogenesis response to VEGF and bFGF [67].

11.3.5 miRNA and Cancer Metabolism

miRNAs have been established as master supervisors of energy metabolism including carbohydrate, lipid, insulin, protein, and nucleic acid metabolism [68–70]. Since identification of miRNAs as "oncomiRs," ongoing research has demonstrated their dysregulation in pathological states, notably cancer. With evolving research and advancements in diagnostic technology, miRNAs for specific cancer types have been identified and continue to evolve. However, further studies are needed for their characterization as biomarkers and progression to clinical practice is anticipated owing to the astronomical growth in the field of miRNA technology since the first miRNAs were identified two decades ago.

Further miRNA, as a viable therapeutic target is a fascinating area of cancer therapy research with overwhelming expectations because:

1. miRNAs small molecules composed of known and conserved sequences;
2. it can target multiple genetic pathways and regulate wide array of biological process;
3. the potential targets of a particular miRNAs can be predicted by using bioinformatical tools; such as miRanda (http://www.microRNA.org), microCosm (previously known as miRBase targets, http://www.mirbase.org), Targets can (http://www.targetscan.org), or PicTar (http://pictar.mdc-berlin.de);
4. miRNA expression is frequently dysregulated in cancer pathogenesis; and.
5. the cancer phenotype, aggressiveness and response to therapy may be modified by targeting miRNA expression [29, 71–73];
6. Complete regression to normal cellular phenotype has also been postulated, potentially curing cancer.

11.3.6 Role of miRNA in Gallbladder Cancer

miRNAs in gallbladder cancer pathogenesis are either oncogenic miRNAs or tumor suppressor miRNAs. miRNA alterations and their correlation to gallbladder cancer pathology, prognosis, response to therapy continues to evolve and further studies are required before miRNAs become established in routine clinical practice. Furthermore, miRNAs as therapy for gallbladder cancer has limitations as therapeutic delivery of targeted miRNAs continues to be a challenge in the present era.

11.3.7 miRNAs Overexpression in GBC: Oncogenic miRNAs

Mir-155, mir-182, and mir-20a are reported as onco-miRNAs in gallbladder cancer. miR-155 overexpression is associated with aggressive behavior such as the presence of lymph nodal involvement, metastasis, and angio-invasion. Furthermore, gallbladder cancer cell lines transfected with miR-155 inhibitors have demonstrated significant decrease in cellular replication and cancer growth. Conversely, cells transfected with miR-155 showed increased cell proliferation. The modulation of the miR-155 level adduces a prognostic marker and therapeutic target for gallbladder cancer [44].

Mir-20a, another oncomiRNA was found to be highly expressed in gallbladder cancer cells and is associated with significant propensity for local invasion, distant metastasis, and poor prognosis. Patients with high miR-20a expression demonstrate a worse overall survival. Upregulation of miR-182 expression promotes cell migration and metastasis, while downregulation inhibits TGF-β-induced invasion. CADM1 has been identified as a target gene of miR-182, both in vivo and in vitro that is negatively regulated by mir-182 [74].

11.3.8 miRNAs Downregulated in GBC: Tumor Suppressive miRNAs

Tumor suppressive miRNAs in GBC include mir-34a, miR-335, miR-135-5p, miR-26a, miR-1, miR-145, and mir-146b-5p. Downregulation of miR-335 is associated with aggressive tumor behaviors such as higher grade, advanced pathologic T stage, clinical stage, lymph node metastasis, and shorter overall survival compared to GBC without miR-335 suppression [75]. Another tumor suppressor miRNA, mir-34a, is found to be significantly suppressed in GBC compared to normal peritumoral gallbladder tissue. miR-135a-5p and miR-26a have been shown to significantly influence GBC cell proliferation. miR-135-5p expression was correlated with histologic grade and cell cycle arrest in the G 1/S phase. Mir-26a downregulation in GBC has been reported to be associated with high histological grade and cellular proliferation [76].

Decreased expression of mir-146b-5p has also been reported in GBC tissue and correlates with carcinoma size [47]. Overexpression of miR-146b-5p was found to inhibit cell growth through enhanced apoptosis, G1 phase arrest and modulation of EFGR function [47] (Fig. 11.4) (Table 11.3).

11.3.9 Current Considerations in miRNA Therapeutics

Over the last two decades, hundreds of different miRNAs and their targets have been identified. The field of miRNA research is still nascent and awaits further discoveries and finesse before mi RNA therapy becomes a wide-reaching reality.

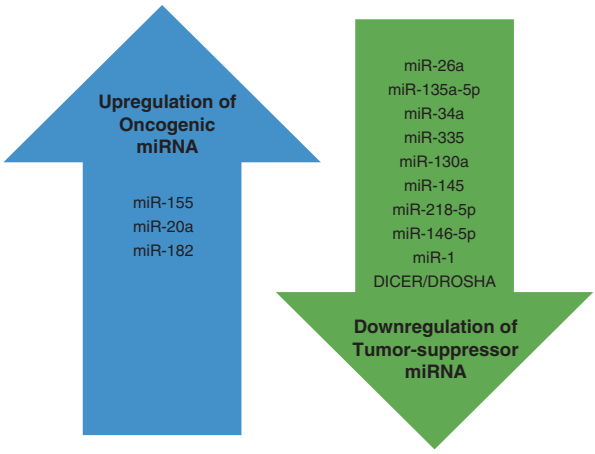

Fig. 11.4 Dysregulation of miRNA in gallbladder cancer

Table 11.3 Association of miRNA and GBC

Parameter	MiRNA associated
Carcinoma size	mir-146b-5p
TNM staging	mir-187, – mir-143, mir-202, mir-335
Histologic - grade	mir-335, hsa-mir-135a-5p, mir-26a
Progression	mir-146b-5p
Lymph node metastasis	mir-155
Metastasis	mir-187, – mir-143, mir-202, mir-335, mir-182
Poor survival/prognosis	mir-335, mir-34a, mir-20a, mir-155

Growth of the miRNA field over the past 20 years has been astronomical, with identification of hundreds of different miRNAs and their targets. The field though is still relatively new, and there is much to be discovered and finessed before miRNA therapy becomes a wide-reaching reality. Although there are still challenges to develop safe and effective miRNA therapeutics, the large body of research occurring in this field continues to improve development of miRNA clinical trials.

11.3.10 Inhibition of miRNA

miRNAs are increased in many different diseases and inhibition of these miRNA would be beneficial to prevent or reverse disease progression; for example, miR-21 [2] is overexpressed in many cancers. Due to the large number of miRNAs which are overexpressed in many diseases, gene therapy targeting overexpressed miRNAs with miRNA has become a major area of interest. There are several different methods that are currently being explored to inhibit miRNA binding to targets. These include miRNA sponges, antisense antagomers and small molecule inhibitors.

11.3.11 Replacement of miRNA

While the majority of miRNA therapeutic research is focused on miRNA inhibition, miRNA replacement therapy is the other modality of miRNA therapeutics. In most cancers, the tumor repressors let-7 [48] and miR-34 [13] are also decreased. In Alzheimer's disease miR-107 is decreased at early time points and therefore might be a good target for replacement [14] and in hypertrophic cardiomyopathy, miR-451 is decreased [15]. Replacement of these miRNAs by their mimics could reverse disease states through re-inhibition of their target genes and normalization of cell processes and division.

11.3.12 Delivering microRNA

One of the major issues with gene-based therapy is the delivery of the therapeutic to the correct place without its degradation in the blood stream or excretion through the kidney. As miRNAs are small and charged molecules, they are water soluble and may be injected intravascularly or subcutaneously. However, they are very quickly degraded and excreted via the kidney [49]. Modified miRNAs are more stable and have decreased clearance; however, they may still not always be accessible to the target cell (Fig. 11.5).

There are two main strategies for delivery of miRNA to the target tissue; local delivery and systemic delivery.

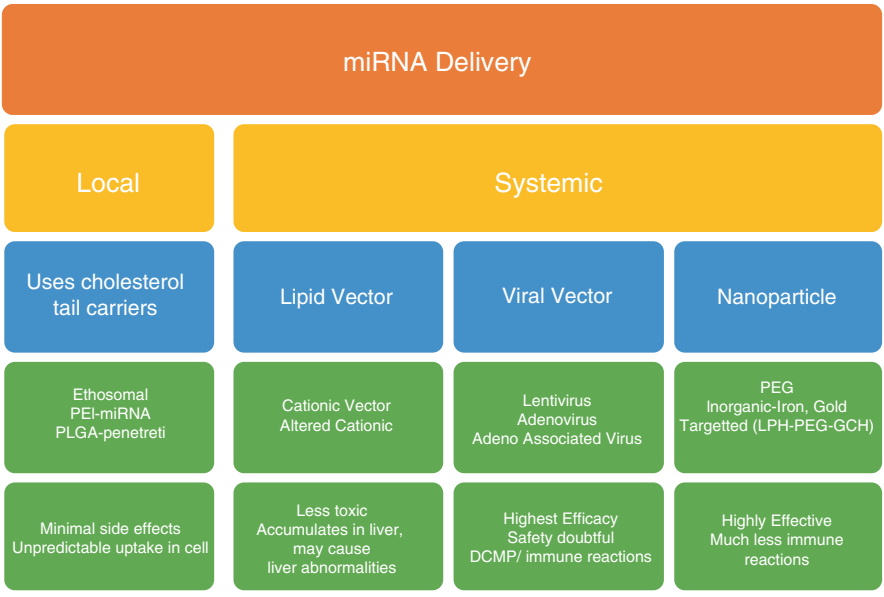

Fig. 11.5 Delivery systems for miRNA

11.3.12.1 Local Delivery Methods

Local delivery of modified miRNA has a much lower side effect profile compared to systemic delivery. However, not all tissue targets are compatible with local delivery. One modality for topical delivery is siRNA in an ethosomal carrier system [18]. Polyethyleneimine/miRNA complexes have also been used to locally deliver unmodified miR-145 and 33a in animal models of cancer [50]. Also in cases of B cell cancers, nanoparticles like PLGA-penetratin are locally injected to deliver a miR-155 inhibitor in vivo, thereby preventing progression [50].

11.3.12.2 Systemic Delivery Methods

Systemic delivery methods utilize vectors for modified miRNA delivery. The pharmacokinetics is much more complex as the vector and miRNA travel through the blood stream and are taken up by the target tissue from blood stream. Other issues such as the size of the delivery vector, the charge, and the safety of the vector need to be considered when designing vectors.

11.3.12.3 Viral Vectors

Viral vectors have the highest efficacy for delivering miRNA into cells; however, their safety remains a controversial issue. Lentiviral, adenoviruses, and adeno-associated viruses (AAV) have all been utilized in animal models to determine safety, efficiency, and off target effects of miRNA delivery. Lentiviruses are less safe than adenovirus or AAV as they can integrate their genome with the cells increasing the risk of causing cancer. AAV2/9 vectors have also shown promise in delivering miRNA, especially to the heart, as cardiac cells have a greater affinity for AAV9 vectors compared to other tissue types [51].

11.3.12.4 Lipid Vectors

Lipid delivery vectors are bilayers of lipid that contain the miRNA and protect it from degradation by nucleases, endosomal, and lysosomal degradation. Negatively charged hydrophilic MiRNA bind to cationic liposomes forming stable complexes. The positive charge also aids uptake into the negatively charged cell membrane [25]. Altered cationic liposomes, such as DOTMA, spontaneously form liposomes around miRNA have also been successful in delivering miRNA to cells, with decreased toxicity and better delivery profile compared to unaltered cationic liposomes [27].

Neutral liposomes are less toxic than cationic liposomes and do not form aggregates in biofluids, but they have decreased transfection rates in vitro and have less loading capacity [25].

11.3.12.5 Nanoparticles

Nanoparticle and nanopolymer delivery systems for gene therapy have been advancing. Several different kinds of vectors have been used in vivo for miRNA delivery, including altered PEG, inorganic nanoparticles, and nanoparticles with targeting abilities [32].

11.4 Conclusion

miRNA technology is a rapidly evolving field with prospective advantage in the diagnosis and therapy for myriad diseases, including cancer. Since the discovery of first miRNA and elucidation of mechanism of RNA silencing, over 10,000 miRNAs have been identified. Elucidation of the role of these miRNAs in normal cellular functioning and regulation, alteration in pathological states, modalities to regulate these miRNAs in vitro and in vivo models and advances in systemic and local delivery systems has progressed at a remarkable pace. The clinical use of miRNA as diagnostic biomarkers and targeted therapy in cancers, including gallbladder cancer may soon be a wide-reaching reality, as technological advancements evolve.

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Gallbladder Cancer: Epigenetic Landscape, Targeted Therapy, and Prospect of Epitherapy

12

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12.1 Introduction

Gallbladder is a part of the biliary tract. Cholelithiasis, cholecystitis, and gallbladder cancer (GBC) have been identified as gallbladder disorders. Currently, GBC is considered as the most common biliary tract malignancy with poor prognosis and the sixth most common gastrointestinal malignancy worldwide [1]. Development of cancer depends upon the interaction between the genome, epigenome, and the environmental factors. The suggested environmental and clinical risk factors that may likely predispose susceptible individuals to biliary tract cancers (BTC) or gallbladder tumorigenesis are several, including infectious agents (e.g., liver flukes, Clostridium, typhoid, *Helicobacter pylori* infection, etc.), clinical issues (e.g., hepatobiliary stone, gallstone, gallbladder polyps, congenital biliary cysts, etc.), gender, age, alcohol, smoking, obesity, diet, reproductive factors, and exposure to certain chemicals [2–6]. Globally, about 19.3 million new cancer cases and approximately 10.0 million cancer deaths are estimated for 2020 [7]. As per GLOBOCON, the newly registered gallbladder cases were 115,949 and the mortality rate was 84,965 in the world in 2020 [7]. Gallbladder cancer is a rare malignant tumor with wide variations in occurrence [8]. It is a life-threatening cancer with an average survival of about 6 months, while the 5-year survivorship rate is only 5% [9, 10]. Globally, the incidence of gallbladder cancer varies from region to region. Because of geographical location and ethnicity, the incidence of gallbladder cancer is diverse. It is far more common, for example, in areas of Chile where rates have been observed as

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high as 27/100,000 in women and in northern India, it is 21.5/100,000 [11]. Besides, high incidence of GBC is reported in the regions of South America, India, and Pakistan. Elevated cases of gallbladder cancer are also seen in East Asia and Eastern Europe [12]. India features high incidence of GBC in North, North-East, Central, and Eastern India, and much less frequent in South and West India [13]. Gallbladder cancer occurs more frequently in women compared to men [11, 14]. Patients with gallbladder cancer (GBC) quite often do not show specific diagnostic symptoms, hence, remains a late-stage disease, where radical surgery becomes the only effective treatment. However, most cases (about 90%), which come to medical attention, are not even resectable due to lymph metastasis (about 50%) or have spread to other organs or tissues as well, requiring alternate therapeutic strategy to reduce the risk and enhance the survival of the patient [15]. Thus, till today, early diagnosis and successful treatment of GBC have remained a major challenge.

Epigenetics is a major contributing factor in cancer development. These factors include methylation of DNA, modifications of histones (e.g., methylation, acetylation, phosphorylation, sumoylation, etc.), tumor suppressor or oncogenic role of small non-coding RNAs like microRNAs, long non-coding RNAs, circular RNAs, etc. Several protein coding genes are known to participate independently or through various molecular pathways (which normally control different cellular functions), contributing to the process of tumorigenesis, if their normal expression is dysregulated, often behaving as tumor suppressors or oncogenes (Fig. 12.1) [16]. MicroRNAs negatively regulate protein coding genes, either by binding imperfectly to their 3′ untranslated regions or rarely to the 5′ UTRs or by regulation at translational level. Binding to these regions results in regulation of gene expression at transcriptional level. Several factors such as location, abundancy, and the affinity of complementation of miRNA with their target mRNAs is highly dynamics [17]. Another important class of non-coding RNAs is long non-coding RNA which are also complex and diverse and is responsible for regulating numerous cellular processes including RNA processing, chromatin modification, and gene transcription [18].

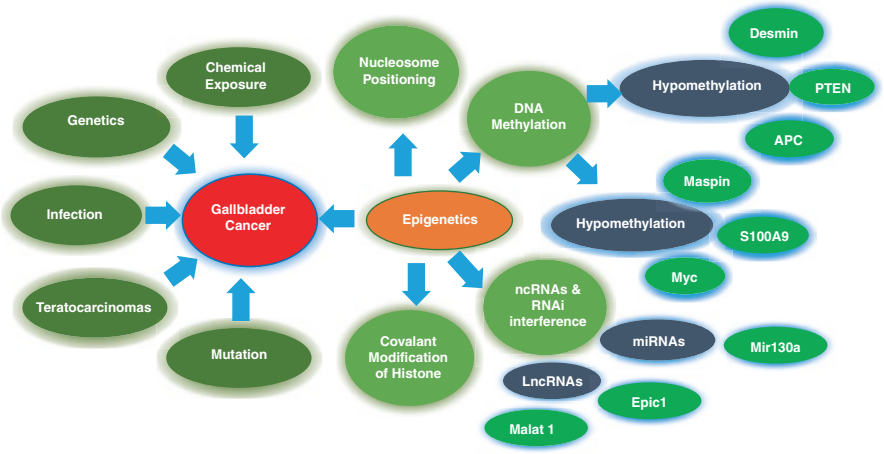

Fig. 12.1 Genetic and epigenetic factors involved in GBC

Cancer, in general, is a consequence of both genetic and epigenetic dysregulation. While genetic alterations are permanent and heritable, epigenetic alterations are reversible but, heritable. Reversibility of the epigenetic changes in gene expression makes it amenable to effective therapeutic interventions. Several drugs have been developed or in process of development, targeting the epigenetic regulators, readers, writers, and erasers, in the treatment of different cancers [19, 20]. Many of them are under clinical trials at different stages, while some of them have been approved by US-FDA for clinical applications, mostly in cancers of hematological origin. The properties of solid tumors or cancers of other tissue types, including lungs, gastrointestinal system, pancreas, prostate, biliary tract system, breast, etc., make them somewhat difficult for targeted therapy applied on hematological malignancies. The biliary tract cancers (BTC) account for about 3% of all gastrointestinal malignancies. BTC includes intrahepatic cholangiocarcinoma (IHCC), extrahepatic cholangiocarcinoma (EHCC), ampulla of Vater cancer (AVC), and gallbladder cancer (GBC). Some of the molecular targets in these cancers are very specific in their functions and, therefore, may be exploited for targeted therapy of these cancers, including GBC [21].

So far, no early diagnostic biomarkers are known for GBC with higher specificity and sensitivity. The large number of studies made on the genetic and epigenetic regulation of GBC during the last few decades have identified and predicted several potential diagnostic/prognostic biomarkers, including both coding and non-coding genes, acting individually or being part of different signaling pathways. Many of these genes are still under laboratory or preclinical tests in GBC and yet to reach to clinical trial. It is important to note that some of these genes are common between GBC and several other cancers, regulating related cellular functions, and are currently being targeted for drug development and or under clinical trials. While most of the efforts on targeted therapy in GBC are based on mutations in the target genes or pathways, epigenetic targeting of these genes is still in its infancy. Since, in cancer, epigenetic alterations are widespread in the patient genome, it is likely that some of these targets are also epigenetically modified in GBC. Thus, selection of patients and targeting epigenetic regulators as well as the concerned genes/pathways by evolving appropriate therapeutic strategy may lead to a positive clinical response. However, there are some problems to be resolved for the success of epigenetic therapy. Epigenetic events are widely distributed in both normal and cancer cells. While certain epigenetic alterations are unique and play major role in some cancers, in normal cells usually they are compensated, therefore, important, and unique alterations need to be identified first. So far, epigenetic therapy has been found more promising in cancers of hematological origin than solid tumors. Solid tumors are composed of both malignant and non-malignant cells, including stem cells. These stem cells cause recurrence and develop resistance to chemotherapy, while epigenetic agents (e.g., DNMT and HDAC inhibitors) can reverse it by reprograming of stem cells [22–25]. However, combination therapies have shown promise in solid tumors in pre- and early clinical investigations [20].

The focus of this chapter is to provide an overview of the epigenetic mechanisms, biomarkers, the concept of targeted therapy, current status of development

of drugs targeting the epigenetic regulators (writers, readers, and erasers), implications of targeted therapy, and future prospects of epigenetic therapy in gallbladder cancer.

12.2 Epigenetics: Definition, Concept, and Mechanism

C. H. Waddington first came up with the concept of epigenetics [26]. Waddington mentioned the role of epigenetics in embryo development in his definition. However, the definition evolved over time as a result of its participation in various biological processes. Epigenetics focuses on the processes that control activating and deactivating genes. Regulating gene expression entails several different types of epigenetic mechanisms, such as DNA methylation, histone modifications, chromatin remodeling, X chromosome inactivation, and RNAi mediated interference. These processes, which control how genes work, are complex and multi-faceted. Inhibition of tumor suppressor gene expression by methylation of the 5' gene promoter (hypermethylation) in many cancers is a well-known epigenetic phenomenon. In contrast, hypomethylation results in oncogenic activation and chromosome instability. Significantly, unlike mutational events where genomic changes are permanent and heritable, epigenetic modifications are reversible and heritable, without directly impacting and modifying the DNA sequence [27].

12.2.1 Epigenetic Regulation of Gene Expression and Cancer

DNA methylation is the most common epigenetic modification, which occurs when a methyl group (CH_3) is added to the cytosine-C5 position in the CpG dinucleotide [28]. In human genome, it is distributed in diffused form throughout as well as in local accumulation. Approximately 80% of CpGs exhibit diffused distribution in repetitive DNA sequences, which usually remain heavily methylated, while other CpGs are always unmethylated in a healthy tissue [29]. The methylation of promoter CpGs has been found to exert a critical role in the regulation of gene expression, genomic imprinting, and inactivation of the X-chromosome in females and in tumorigenesis of many cancers. These CpG-rich sequences are present in various regions of the human genes, mainly located around the transcription start sites of a promoter and less frequently in first exon or in the intronic regions of the gene [30]. The chemical modifications including DNA methylation, histone acetylation, and histone methylation control the accessibility of chromatin to transcription factors or other DNA binding proteins. Cancer cells often exhibit extensive alterations of DNA methylation, with DNA hypermethylation at CpG-rich sites and genome-wide hypomethylation. The aberration of DNA methylation is associated with both the silencing of tumor suppressor genes and activation of oncogenes. In a physiologically normal cell, DNA methylation, nucleosome remodeling, and covalent modifications of histone, all together, involve in gene silencing, referred to as heritable gene silencing and occurs mostly at the start site of genes [31]. The different enzymes that take part in these modifications include DNA methyltransferases

(DNMTs), histone deacetylases (HDACs), and histone methyltransferases (HMT) [32]. As part of the normal functioning of the body, silencing is a critical phenomenon for development and differentiation. Malfunctioning of these enzymes is one of the causes that leads to diseases, like cancer [33].

Numerous non-coding RNAs show altered expression, with some miRNAs being upregulated and several other being downregulated in GBC. According to their functional role these can be categorized as oncogenes or tumor suppressors. The interaction of miRNAs, long non-coding RNAs, and coding RNAs together could be affected by the epigenetic mechanism, such as DNA methylation, histone modification, and inactivation of DNA repair genes by histone acetylation, which may lead to the development and progression of GBC.

12.2.2 Epigenetic Regulation of Coding Genes and Biomarkers in Gallbladder Cancer

In gallbladder cancer, the available information on abnormal methylation of genes leading to cancer is extremely limited in comparison to most other. Extensive investigations are on globally on the role of DNA methylation in GBC. Methylation-specific PCR (MS-PCR) is performed on a regular basis to determine the state of methylation of genes in gallbladder cancer [34]. Gallbladder cancer is comprised of three principal phases of methylation: gallstone, dysplasia, and gallbladder cancer [35]. With increased tumor growth, hypermethylation of genes involved in Wnt signaling, hedgehog signaling, and tumor suppression have been reported. Methylation is also measured using EPIC and Mass Array methods [35]. In an earlier study, methylation of about 4, 85, 577 CpG sites was studied using HumanMethylation450 BeadChip Array (Illumina Inc.), which identified seven significantly hypermethylated genes and 61 significantly hypomethylated genes in gallbladder cancer in north-central Indian population [36]. Validation of these genes is expected to determine and predict their likely potential as diagnostic/prognostic epigenetic biomarkers for GBC. Altered methylation status of certain tumor suppressor genes, such as p16 (cyclin-dependent kinase inhibitor 2A), p73 (Tumor protein73), APC (Adenomatous polyposis coli), hMLH1 (MutL homolog 1), in samples of chronic cholecystitis and gallbladder cancer in the Chilean population is reported [37]. Apart from the above, hypermethylation of CDH13 (Cadherin 13), CDH1 (Cadherin 1), RUNX3 (Runt-related transcription factor 3), APC (Adenomatous polyposis coli), P16INK4A (cyclin-dependent kinase inhibitor 2A), and HPP1 (hyperplastic polyposis 1) has also been observed in the patients with gallbladder cancer [38, 39]. Methylation status of the PTEN (Phosphatase and tensin homolog) gene promoter was investigated using methylation-specific PCR, which revealed significant hypermethylation of the gene in patients with advanced gallbladder cancer [40]. Epigenetic inhibition of APC (adenomatous polyposis coli) in advanced gallbladder cancer was observed in the patients from north-central India and China [41, 42]. Certain genes, like MASPIN (mammary serine protease inhibitor), are found hypomethylated (MS-PCR) in gallbladder cancer and are correlated with poor patient outcomes [43]. A recent study showed hypermethylation of Desmin promoter in gallbladder cancer and was

suggested to serve as biomarker for gallbladder cancer [44, 45]. Overexpression of LSD1 (Lysine specific histone demethylase 1) was also found to promote development of gallbladder cancer and may be a predictor of deterioration in prognosis [46]. EZH2 (enhancer of zestehomologe 2) is a histone methyltransferase and is responsible for the transcriptional repression by trimethylation of H3K27 [47]. A brief description on the functional significance of a few important genes associated with gallbladder cancer and their role in other cancers is given in Table 12.1.

12.2.3 Epigenetic Regulation of Non-coding Genes and Biomarkers in Gallbladder Cancer

RNA, initially known as a mediator of information between DNA to protein, recent proofs showed that it plays important roles in various biological processes of the cell. miRNA is a type of endogenous, single-stranded, non-coding small RNA that remains highly conserved during evolution [48, 49]. They can reduce the expression of gene via mRNA degradation or repression of translation [50, 51]. Human transcriptome consists of various types of ncRNAs, which perform number of functions. From the recent studies it is now confirmed that the epigenetic modification is regulated by non-coding RNAs and the regulation of target gene expression is achieved by chromatin remodeling through modification of histone [52].

12.2.3.1 MicroRNAs

MicroRNAs (miRNA), originally discovered in *Caenorhabditis elegans* are small, evolutionary conserved, single stranded, non-coding RNA molecules that bind to 3′ or 5′ end of target mRNA to prevent protein production. Around 30% of protein-coding genes are shown to be regulated by miRNA [53, 54]. They contribute a percentage of 1–5% of the human genome. The biogenesis of miRNA is a multi-step complex process, results in producing a stem loop structure of approximately 70nucleotide in the nucleus called as pre-miRNA, which is excised from the primary miRNA by a ribonuclease enzyme called Drosha. Translocation of pre-mRNA into the cytoplasm is carried out by a Ran GTP Exportin-5 nuclear export factor. In the cytoplasm with the help of another ribonuclease called Dicer, pre-miRNA is further cleaved into an RNA duplex of approximately 22-nucleotide [55]. It further interacts with the protein of Argonaute family present in the RISC complex (RNA induced silencing complex). One of the stable single strands of the miRNA duplex complex called mature strand remains associated with the RISC complex and leads the complex to bind with the 3′ UTR (not always) of the target mRNA, while the other strand is directed to degradation. Lastly, miRNA exerts its function either by cleavage of mRNA or translation repression depending upon the complementary between the binding sequence of miRNA: mRNA [50, 51, 56, 57]. Several miRNAs are now known to play significant role in GBC, regulating various target genes, acting as tumor suppressors or oncogenic. In Table 12.2, a brief description on the molecular characteristics and functions of some important microRNAs involved in gallbladder carcinoma is given.

Table 12.1 Brief overview of genes involved in GBC and their methylation status

S. no.	Gene	Gene functions	Chromosomal location	Methylation frequency	Methylation status (GBC)	Involvement in other cancers	References
1.	APC 1A	Tumor suppressor, cell adhesion, migration, transcriptional activation, and apoptosis	5q22.2	96% GBC and 80% GSD	Hypermethylation	Colorectal cancer	Sparks et al. [282] and Tekcham et al. [280]
2.	PTEN	Tumor suppressor	10q23.31	30% GBC and 22.86% GSD	Hypermethylated	Prostate cancer, kidney cancer	Tekcham et al. [40], Wise et al. [283] and Que et al. [284]
3.	MASPIN	Tumor suppressor	18q21.33	71% GBC and 51.43% GSD	Hypermethylated	Breast cancer, NSCLC, Oral squamash cell cancer	Tekcham et al. [280]; Baghel et al. [43]
4.	THBS1	Platelet aggregation, angiogenesis, and metastasis	15q14	52% in GBC and 28.57% in GSD	Hypermethylated	Glioblastoma, colorectal cancer, liver cancer, breast cancer, prostate cancer	Tekcham et al. [280]
5.	Myc	Protooncogene, cell cycle progression, cellular transformation, and apoptosis	8q24.21	–	Hypomethylation	Lung cancer	Ishak et al. [132]
6.	UHRF1	P53 dependent DNA checkpoint	19p13.3	–	–	Breast cancer, cervical cancer, gastric cancer, colorectal and liver cancer	Qin et al. [285]
7.	SHP1	Cell growth, differentiation, mitotic cycle, and oncogenic transformation	12q13.31	80% GBC	Hypermethylated		Takahashi et al. [38]

(continued)

Table 12.1 (continued)

S. no.	Gene	Gene functions	Chromosomal location	Methylation frequency	Methylation status (GBC)	Involvement in other cancers	References
8.	3-OST2		16p12.2	72% GBC	Hypermethylated	Breast cancer, colon cancer, and lung and pancreatic cancer	Takahashi et al. [38]
9.	CDH13	Negative regulator of growth during neural differentiation	16q23.3	44% GBC and 8% CC, 75%	Frequent methylation		Takahashi et al. [38]
10.	BMP3		4q21.21	–	Hypermethylated		Zhang et al. [199]
11.	CDH1	Tumor suppressor, proliferation, invasion, and metastasis	16q22.1	38% GBC and 12% CC	Frequent methylation	Hepatocellular carcinoma, Epithelial ovarian cancer	Takahashi et al. [38], Zhang et al. [286] and Thakur et al. [287]
12.	RUNX3	Tumor suppressor	1p36.11	32% GBC	Hypermethylated	Kidney cancer, gastric cancer, colorectal cancer	Takahashi et al. [38], Kim et al. [288], Liu et al. [289] and Zheng et al. [290]
13.	APC	Tumor suppressor, cell adhesion, migration, transcriptional activation, and apoptosis	5q22.2	30% GBC 27%	Hypermethylated	Colorectal cancer, gastric cancer	House et al. [37], Takahashi et al. [38], Zhang et al. [162] and Zhou et al. [305]
14	RIZ1	Tumor suppressor	1p36.21	26% GBC	Hypermethylated	Liver cancer, glioma	Takahashi et al. [38] and Zhang et al. [162]
15.	$P16^{INK4A}$	Tumor suppressor	9p21.3	24% GBC and 4% CC	Hypermethylated	Ovarian cancer, breast cancer	Takahashi et al. [38]
16.	SEPT9	Tumor suppressor, cytokinesis, and cell cycle control	17q25.3	–	Hypermethylated	Biliary tract cancer, cervical cancer colorectal cancer, glioma	Jiao et al. [291], Branchi et al. [292], Sun et al. [167] and Zhang et al. [199]

17.	BLU	Tumor suppressor	3p21.21	26%	Hypermethylated		Riquelme et al. [293]
18.	RASSF1A	Tumor suppressor	3p21.21	4%	Hypermethylated	Breast cancer, colorectal cancer	Riquelme et al. [293]
19.	SEMA3B	Tumor suppressor, apoptosis	3p21.31	96%	Hypermethylated	Lung cancer, endometrial cancer, hepatocellular cancer, ovarian cancer	Riquelme et al. [293]
20.	DAPK1	Tumor suppressor	9q21.33	61%	Hypermethylated	Bladder cancer, gastric cancer, hepatocellular carcinoma	García et al. [140]
21.	DLC1	Tumor suppressor	8p22		Hypermethylated	Colorectal cancer	García et al. [140] and MannaVola et al. [294]
22.	RARβ2	Embryonic morphogenesis, cell growth, and differentiation	3p24.2	44%	Hypermethylated	Prostate cancer, thyroid cancer	García et al. [140]
23.	MGMT	Cell death, mutation, and cancer	10q26.3	30%	Hypermethylated	glioblastoma, breast cancer, gastric cancer, ovarian cancer	García et al. [140]
24.	P15	Tumor suppressor	9q21.3	21.7%	Hypermethylated	Breast cancer	García et al. [140]
25.	P16	Tumor suppressor	9p21.3	26.8%	Hypermethylated	Lung cancer, cervical cancer	García et al. [140]
26.	P73	Tumor suppressor	1p36.32	28%		Lung cancer	Boominathan et al. [295]
27.	WIF1	Tumor suppressor	12q14.3	–	Hypermethylated	Pancreatic cancer, colorectal cancer, colon cancer	Huang et al. [194]

Table 12.2 List of microRNAs identified in GBC (and other cancers)

S. no	miRNA	Chromosomal location	Functional characteristics	Interactions	Other cancer	References
1.	miRNA-1	20q13.33	Tumor suppressor	VEGF-A and AXL	Cervical, prostate, lung squamous cell carcinoma	Letelier et al. [296]
2.	miRNA-20a	13q31.3	Oncogenic	Smad7	Colorectal, lung, gastric, and neuroblastoma	Chang et al. [218]
3.	miRNA-21	17q23.1	Oncogenic	PTEN	Colorectal, pancreatic, and breast cancer	Peng et al. [297]
4.	miRNA-26a	3p22.2	Tumor suppressor	HMGA2	Osteosarcoma, nasopharyngeal cancer	Zhou et al. [298]
5.	miRNA-30b	8q24.22	Tumor suppressor	Semaphorin-6B (SEMA6B)	Hepatic, gastric, breast, and bladder cancer	Cui and Bian [138]
6.	miR-33a	22q13.2	Tumor suppressor	Interleukin-6 (IL-6)	Osteosarcoma, lung, and cervical cancer	Mingdi et al. [299]
7.	miRNA-34a	1p36.22	Tumor suppressor	PNUTS	Colorectal, breast, and liver cancer	Jin et al. [81]
8.	miRNA-122	18q21	Oncogenic		Cholangiocarcinoma, prostate, and thyroid	Li et al. [300]
9.	miRNA-135a-5p	3p21.2	Tumor suppressor	VLDLR	Ovarian, thyroid, glioblastoma, head, and neck squamous cell carcinoma	Zhou et al. [301]
10.	miRNA-130a	11q12.1	Tumor suppressor	HOTAIR, cMyc	Hepatocellular carcinoma	Ma et al. [302]
11.	mirRNA-125b	11q24.1	Tumor suppressor		Breast cancer, lung cancer	Yang et al. [303]
12.	miRNA-143	5q32	Tumor suppressor		Gastric, colon, and colorectal	Li et al. [300]
13.	miRNA-145	5q32-33	Tumor suppressor	STAT-1, DNA fragmentation factor (DFF)	Prostate, bladder, colon, ovarian, and esophageal	Letelier et al. [296]
14.	miRNA-146b-5p	10q24.32	Tumor suppressor	EGFR	Thyroid	Cai et al. [165]
15.	miRNA-155	21q21.3	Oncogenic		Esophageal and breast cancer	Kono et al. [304]
16.	miRNA-182	7q32.2	Oncogenic	CADM1	Cervical, colorectal cancer	Zheng et al. [199]
17.	miRNA-187	18q12.2	Oncogenic		Gastric, colorectal, prostate, and breast	Li et al. [300]
18.	miRNA-218-5p	4p15.31	Tumor suppressor	BMI1, CCT1	Gastric and lung cancer	Sekine et al. [306]

12.2.3.2 Long Non-Coding RNAs

The lncRNAs are long RNA transcripts of up to 200 nucleotides that do not encode any proteins. Several biological phenomena, like regulation of expression of coding and non-coding genes, imprinting of genomic loci and allosteric regulation of enzymatic activity is performed by lncRNAs [58, 59]. As they are transcribed by RNA polymerase II and are often 5′-capped, spliced, and polyadenylated at their 3′ tail, refers very similar to mRNA. Also, in contrast to mRNA, they are less conserved at primary nucleotide sequence, but are more conserved than neutrally evolving genetic elements [60, 61]. In general, the lncRNAs can be divided into five major categories, which are sense lncRNAs, antisense lncRNAs, bidirectional lncRNAs, intronic lncRNAs, and intergenic lncRNAs. They can suppress gene expression by following different approaches, like alteration in recruitment of transcription factors [62], alteration of histone modifications [62, 63], and reduction of chromatin accessibility [64, 65]. Some lncRNAs control the expression of nearby genes by affecting their transcription and chromatin structure in Cis manner, while others function away from their loci acting in transform. Their functions can be of a structural or regulatory nature and, they can function as a part of regulatory processes which involve transcript at different level of transcription and translation, as well as signaling pathways. As they have specific expression patterns in diseases like cancer they are being used as biomarker in various targeted developing strategies. Our understanding on the role of lncRNAs in GBC is poor as compared to many other cancers. Although the current list of identified lncRNAs in GBC is limited to very few members only, the field is growing rapidly. Table 12.3 provides a summary of the molecular characteristics and functions of some important lncRNAs in GBC.

12.2.4 DNA Methylation Screening Techniques

MS-PCR (methylation-specific PCR), the most common and routinely used technique for investigating DNA methylation [66]. It detects methylation without any specific enzyme, which is susceptible to methylation, like methylation-sensitive restriction enzymes [67]. Bisulphite sequencing for DNA methylation is a widely used tool for understanding epigenetic regulation [68]. Long-read nanopore sequencing is used to determine the methylation level of CpG sites and the haplotype [69]. Next-generation sequencing and micro-array technologies are also used to evaluate large methylome fractions [70]. Methylation-sensitive high-resolution melting (MS-HRM) is a quick way to diagnose imprinting and clinically validate the findings of whole epigenome studies [71]. MeDIP and MBDCap are the most cost-effective and efficient methods to study DNA methylation in a locus or the entire genome [72]. Recent developments in high throughput technology has provided a better insight into the global or genome-wide methylation pattern in cancer tissue using HumanMethylation450 BeadChip (Infinium) methylation microarray (450K) and Methylation EPIC BeadChip (Infinium) microarray (850K) developed by Illumina Inc., USA.

Table 12.3 List of lncRNAs identified in GBC (and other cancers)

LNCs	Regulation	Nature	Other cancer	Effect	Reference
EPIC1	Upregulation	Oncogenic	Colon cancer, GBC	Tumor cell proliferation	Wang et al. [307]
CCAT1	Upregulation	Oncogenic	Colon, GBC	Lymph node invasion and poor survival	MZ et al. [308]
LINC00152	Upregulation	Oncogenic	Hepatocellular carcinoma, gastric, colon, GBC	PI3K/AKT pathway activity	Cai et al. [196]
PAGBC or (LINC01133)	Upregulation/ downregulated	Oncogenic/tumor suppressive	Colorectal, non-small lung cancer, GBC	Cell proliferation, migration, and invasion	Wu et al. [195]
MALAT1	Upregulation	Oncogenic	Biliary tract cancer, GBC	correlated with an advanced tumor stage and a poor prognosis	Wu et al. [170]
ANRIL	Upregulated	Oncogenic	breast, gastric, lung, cervical, and bladder cancer	Increase proliferation of cell and inhibits apoptosis	Zhang et al. [309]
H19	Upregulated	Oncogenic	gastric, breast and ovarian cancer	cell proliferation and invasion	Liu et al. [310]
LET	Downregulated	Tumor suppressive	Gastric, cervical hepatocellular	Inhibits cell proliferation and regulates apoptosis	Wu et al. [195]
MEG3	Downregulated	Tumor suppressive	Gastric, hepatocellular carcinoma	Regulate apoptosis and cell proliferation	Zhang et al. [311]
GCASPC	Downregulated	Tumor-suppressive	Hepatocellular carcinoma	Regulate pyruvate carboxylate dependent cell proliferation	Ma et al. [312]

12.3 Therapeutic Approaches in GBC: Epigenetics Regulators, Inhibitors, and Targeted Therapy

12.3.1 Epigenetic Regulators

Epigenetics involves covalent modifications of DNA/RNA and histones in chromatin. The type of modifications, mainly include DNA methylation, histone methylation and acetylation. Other types of modifications are phosphorylation, ribosylation, sumoylation, etc. These modifications regulate transcription by preventing or allowing accessibility to various protein factors, including transcription factors. The regulators of epigenetic modifications are defined as "writers, readers, and erasers," which perform specific functions and makes the epigenetic modifications reversible. "Writers" are enzymes that add modifications (e.g., methylation and acetylation) to the nucleic acids or histones in chromatin. "Readers" are proteins that identify specific epigenetic marks. They select chromatin remodelers and non-coding RNAs, which are involved in downstream processes and control all these events. "Erasers" are those factors that can remove the modifications. These epigenetic modifiers (enzymes or chromatin binding proteins) work in coordination and regulate gene expression, maintaining cellular structure and functions. The functions of these regulators can be modulated by small molecule inhibitors. Several epigenetic drugs or inhibitors of DNA methyltransferase (DNMT) and histone deacetylase (HDAC) are currently being developed to target the writers, readers and erasers. Combinations of these inhibitors or with chemotherapies, immunotherapies and or targeted therapies may prove more effective and are being evaluated in many cancers, particularly in hematological cancers through preclinical or clinical trials (Figs 12.2 and 12.3) [20, 73–77].

The Epigenetic writers catalyze active (e.g., acetyl group) and repressive (e.g., methyl group) marks (chemical groups) to DNA or histones in chromatin, which include DNA methyltransferases (DNMTs), histone acetyltransferases (HATs), and histone methyltransferases (HMTs). Drugs against some of the DNMTs, like EZH2

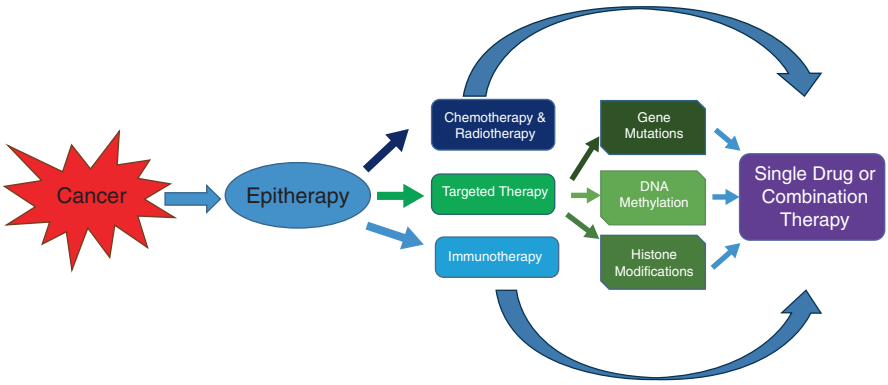

Fig. 12.2 Diagrammatic presentation of various steps in epigenetic therapy

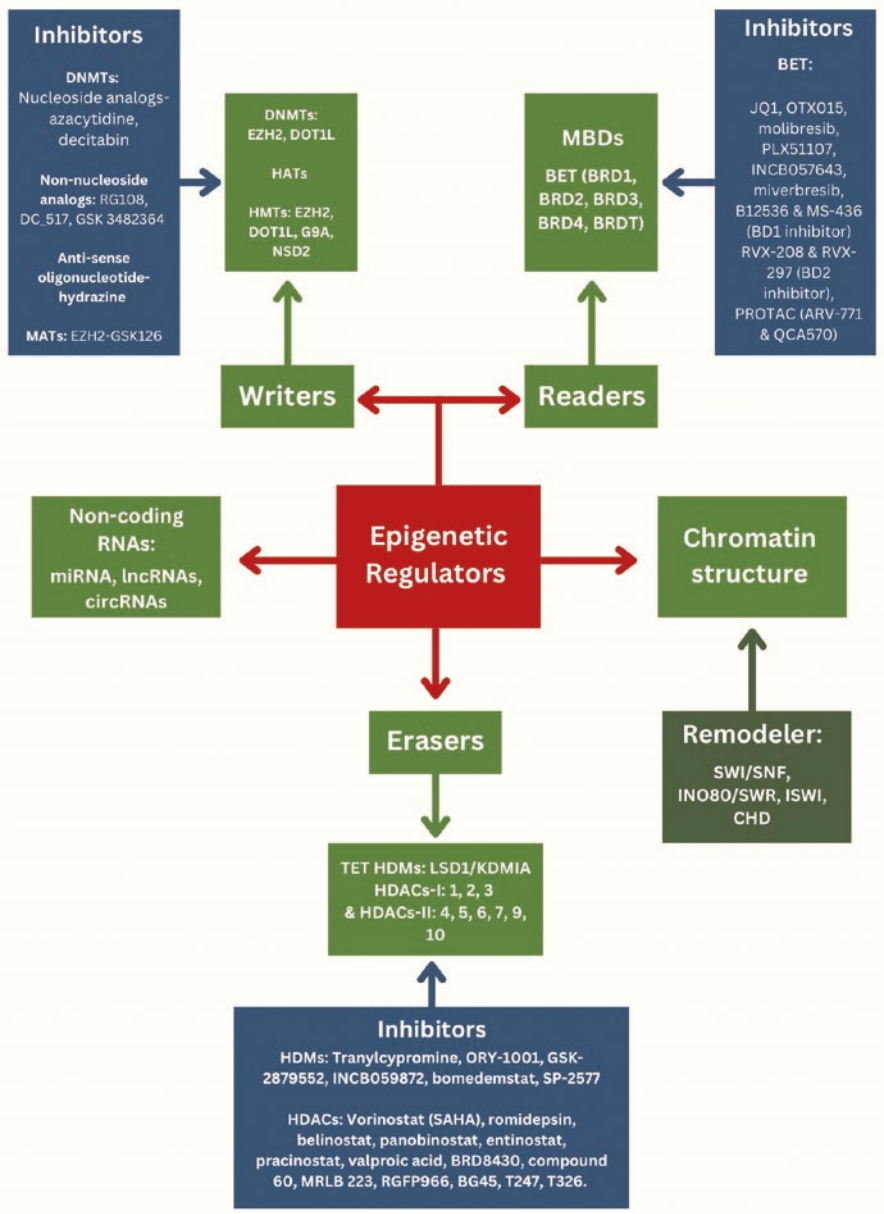

Fig. 12.3 The epigenetic regulators: writers, readers, erasers, non-coding RNAs and their inhibitors (for more details, see text)

(enhancer of zeste homolog 2) and DOT1L (disruptor of telomeric silencing 1-like) have been developed and are currently being tested through clinical trials for their efficacies.

Among the DNMTs inhibitors, the 5-Azacytidine or azacitidine, a ribonucleoside analog and 5-aza-20-deoxycytidine or decitabine, a deoxyribose analog, are most recognized. They are incorporated into DNA, become covalently bound to DNMTs and prevent transfer of methyl group, inducing DNA demethylation and proteasomal degradation of DNMTs, followed by reactivation of silenced genes. Both are FDA approved drugs for AML, CMML, and MDS [78–82]. In contrast, the non-nucleoside analogs, such as RG108, DC_517, and GSK3482364 are devoid of adverse effects and directly target the catalytic site of DNMT enzymes, inhibiting DNA methylation [83]. In addition to these analogs, the antisense oligonucleotides, like MG98 (inhibit DNMT1 translation), inhibit DNA methylation by blocking the transcription of individual DNMT [83–86].

Histone acetyltransferases (HATs) inhibitors are reported to block the catalytic activity of HATs in many cancers. Even though these inhibitors are not exclusive to HATs, they are still considered promising for cancer treatment. For example, bisubstrate inhibitors have been found to selectively inhibit HATs p300 and PCAF, facilitating re-expression of tumor suppressor genes [87, 88]. Curcumin, a natural product is found to inhibit HATs, which, in turn, inhibit histone H3 and histone H4 acetylation by p300 and CBP, leading to inhibition of proliferation and induction of apoptosis in cancer cells [89]. There are some other HATs inhibitors also known to inhibit acetylation, such as garcinol, anacardiac [90, 91], and isothiazolones [92].

Several pharmacological inhibitors have been developed to target histone methyltransferases (HMTs), like EZH2, DOT1L, G9A, and NSD2. EZH2 is known to cause H3K27 trimethylation (H3K27me3), and if inhibited, could reduce tumor growth. Several small molecule inhibitors, such as GSK126 or GSK2816126) that mimic adenosyl methionine (SAM), are found to act as competitive inhibitors of EZH2S, which upon binding to EZH2 inhibit its methyltransferase activity [33, 93–96].

The Epigenetic readers identify specific post-translational modifications, like methylation or acetylation on the DNA or histones in chromatin. For example, methyl-CpG-binding domains (MBDs) recognize methylated CpGs, bromodomain-containing proteins recognize acetylated histone residues and chromodomains recognize methylated lysines in histone. Among the pharmacologically most investigated family of readers is the BRD and extraterminal domain (BET) family, a key regulator of tumorigenesis [97, 98]. Several drugs, e.g., JQ1, OTX015 (MK-8628), molibresib (GSK525762), PLX51107, INCB057643, and mivebresib, etc. inhibiting functions of BET family members, the bromodomain-containing proteins 2, 3, 4, and t (BRD2, BRD3, BRD4, and BRDt), have been developed. Some are currently under preclinical and clinical investigations also [99–102].

The Epigenetic erasers are proteins that modulate gene expression by removing DNA or histone modifications, such as methylation and acetylation, as opposed to the functions of writers. Among the most investigated erasers are ten-eleven translocation (TET) enzymes, histone demethylases (HDMs), and HDACs. There are two classes of HDACs, class I (HDAC 1, 2, 3, and 8) and class II (HDAC 4, 5, 6, 7, 9, and 10), all of which are being targeted for drug development [103]. Inhibitors, specific to these erasers, like lysine-specific demethylase 1 (LSD1 or KDM1A, demethylates H3K4me1/2 and H3K9me1/2, 59 [104] and HDAC inhibitors are currently being investigated for their potential and specificity. A few FDA approved

drugs against HDAC are vorinostat (SAHA), romidepsin (FK228), belinostat (PXD101), and panobinostat (LBH589) [105]. Clinical investigations are also in progress validating the potential and specificity of drugs against HDMs (e.g., tranylcypromine or TCP, ORY-1001 or RG-6016, GSK-2879552, INCB059872, and IMG-7289 or bomedemstat) [87, 106–110].

12.3.2 Combination Therapy

Epigenetic regulation is a multi-step process that calls for therapeutic approaches combining two or more therapies for better outcome. Their synergistic role may lead to inhibition of tumor growth, re-induction of tumor suppressor genes, etc. Targeted therapies often cause resistance because of genetic aberrations or altered transcriptional regulation, which can be reversed to normal by combined treatment with specific epigenetic agents, for example, resistance to kinase inhibitors can be overcome by inhibition of HDAC [20, 111–113].

Rational drug designing and combining epigenetic drugs with chemotherapy, targeted therapy, and or immunotherapy may have significant antitumor effect and can also reduce cytotoxicity and drug resistance. DNA methylation ($C^{me}pG$ doublets) and histone (lysine) acetylation are closely associated, particularly in hpermethylated and poorly acetylated heterochromatin regions, affecting gene expression. Low doses of DNA demethylation inhibitors enhance reactivation of silent genes that follows HDAC inhibition. Preclinical studies in certain cancers have shown encouraging results of combined treatment of DNMT (e.g., azacitidine, decitabine, and hydralazine) and HDAC (e.g., pracinostat, valproic acid, entinostat, and vorinostat) inhibitors leading tore-expression of genes, induction of cell death, differentiation, and growth arrest [114–117]. Combination of epigenetic drugs with immunotherapies (e.g., immune checkpoint therapy) was found to enhance antitumor immune responses in cancer cells inhibiting checkpoint molecules. Epigenetic reprogramming, e.g., inhibiting DNMTs, HDAC, EZH2, and LSD1, induce a number of immunomodulatory activities, including upregulation of tumor antigens, and PD-1 ligands [118–123].

While epigenetic therapies have proved quite promising in hematological malignancies, and approved by regulatory bodies, like US-FDA, their efficacies in solid tumors, including GBC, have still remained a challenge. Selection of patient group, optimization of dose, and trial strategy, etc. could be the key elements of its success. However, uses of combination therapies in solid tumors are expected to yield better results.

12.3.3 Targeting DNA and Histone Modifications

12.3.3.1 Targeted Therapy in GBC

The concept of targeted therapy in cancer evolved basically to regulate the expression (block or activate) of specific genes or their products (RNA/protein) whose role in the process of carcinogenesis (e.g., cell proliferation, differentiation, migration,

angiogenesis, etc.) was established [124, 125]. Several small molecule drugs and antibodies have been developed, which upon entering the cell or by binding to the cell membrane receptors or their ligands, inhibit the target protein/enzymes, regulate cell proliferation or apoptosis, or mediate angiogenesis and immune response in tumor cells [126–129].

The appropriate choice of targeted therapies in Advance Biliary Tract Cancers (ABTCs) has been majorly based on high throughput technologies, like NGS. Most of the therapeutic strategies in ABTC have been developed based on common genetic mutations in target genes, varying between IHCC (intrahepatic cholangiocarcinoma), EHCC (extrahepatic cholangiocarcinoma), and GBC [130–132] and have been considered as potential diagnostic/prognostic biomarkers, some of which are currently under clinical trials.

In GBC, several intracellular signaling pathways are known to be aberrantly regulated. Several drugs or small chemical molecules have been created to intervene the specific pathway or specific targets in the respective pathway. Some of these targets, currently under clinical investigations, are mostly growth factor receptors like human epidermal growth factor receptor 2 (HER2), growth factor receptor tyrosine kinases (RTKs), vascular endothelial growth factor receptor (VEGFR) and molecules from apoptosis and cell signaling pathways like programed death receptor (PD1), phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR), and RAS/BRAF/MEK/MAK. We have briefly described here the molecular functions and ongoing pre-clinical investigations/clinical trials on these targets in various cancers to understand the prospect of targeting them for epigenetic therapy of GBC [27, 133–142].

HER2 or ErbB2 is a member of HER family of cell surface receptors. It has a transmembrane tyrosine kinase domain and can activate several signaling pathways following binding with EGF.49 [143]. HER2 is known to be over expressed in several cancers, including breast, colon, lung, stomach, and biliary tract cancers (BTC), either due to activated mutation or by epigenetic regulation [139, 140, 144–146].

The members of VEGF (includes VEGF-A, B, C, D, and PLGF or placenta growth factor) and VEGFR (includes RTK members VEGFR 1-3 and non-tyrosin kinase co-receptors neuropilin-1 and 2) families play significant role in vascularization in tumor angiogenesis [147–150]. In GBC, the serum level of VEGF is found to be higher. In GBC, VEGF is suggested to promote angiogenesis, cell proliferation and invasion, but inhibit apoptosis [120, 151], however, suggested that VEGF not alone but, together with increased expression of VEGF-A and estrogen receptor 1 (ER1), is a poor predictor of GBC prognosis and, hence, can serve as biomarker for GBC with therapeutic implications.

HER1 or EGFR (also termed as ErbB1 or erythroblastic leukemia viral oncogene homologue) is a member of RTKs (receptor tyrosine kinases) involved in multiple signaling pathways, including (MAPK)/ERK, (PI3K)/PTEN/AKT, SRC, PLC-γ1-PKC, JNK, and JAK-STAT [152, 153]. It plays significant role in cancer proliferation, angiogenesis, cell motility, adhesion, and metastasis [153, 154]. EGFR is found to be mutated in various cancers, resulting in its constitutive expression, which makes it a potential diagnostic/prognostic biomarker as well as a therapeutic

target [31, 155–158]. Significantly increased expression of EGFR has been reported in many cancers, including non-small cell lung cancer (NSCLC), BTC, and gallbladder cancer [159–165]. EGFR promotor was observed to be hypermethylated in various cancers, like gastric cancer, CRC, NSCLC, lung adenocarcinoma, and GBC [27, 134–137]. In GBC, EGFR is reported to be regulated by miRNA-146b-5p, which acts as a tumor suppressor [166].

MAPK (RAS/RAF/MEK/ERK) pathway is an important signaling pathway essential for various cellular activities. In many cancers, including GBC, it is often found to be dysregulated. In cancer, the RAS/RAF/MEK/ERK pathway play active role in proliferation, differentiation, cell cycle progression, apoptosis, survival, metastasis, metabolism, angiogenesis, etc. [167–169]. Apart from mutations in the members of MAPK pathway that activate tumor formation, understanding the role of epigenetics in this process is the focus of current investigations targeting epigenetic therapy.

Investigations on the epigenetic regulation of the pathway revealed that the non-coding RNA, MiR-663a, disrupts MAPK/ERK pathway by regulating the expression of EMP3, which, in turn, suppresses GBC progression [170]. However, the long non-coding RNAs, MLAT1, SLC25A22, and miR-101 activate MAPK/ERK, inducing cell proliferation and metastasis in GBC [171–173]. Likewise, several miRNAs targeting KRAS, an oncogene, are found downregulated in various cancers, acting as tumor suppressors, while some miRNAs are upregulated due to mutations in KRAS, behaving as oncomiRs [174–176].

PI3K/AKT/mTOR pathway is suggested to be significantly involved in growth, mobility, differentiation, metabolic activity, and apoptosis in cancer cells [177–180]. This pathway is reported to be significantly upregulated in breast cancer [181], NSCLC [182], gastric cancer [183], hepatocellular carcinoma [147], colorectal cancer [184], pancreatic cancer [185], cholangiocarcinoma [186], and GBC [187]. It is significantly involved in the initiation and progression of GBC [180, 188, 189]. Dysregulation in the PI3K/AKT/mTOR generally occurs due to PI3KCA mutation, PTEN downregulation, overexpression of phosphorylated AKT or mTOR [190–192]. In many cancers, including GBC, PTEN promotor is observed to be hypermethylated, resulting in its significantly reduced expression [40, 189–192]. Members of ErbB family (ERBB1-4 or HER1-4) regulate PI3K-Akt and MAPK pathways. mTOR, a ser/thr kinase, is downstream to ErbB-PI3K-AKT pathway and its inhibition has antiproliferative effect in GBC cell lines [171, 193–195].

Recent investigations have demonstrated significant role of non-coding RNAs in the regulation of PI3K/AKT/mTOR pathway. It is suggested that lncRNA HGBC, stabilized by HuR, regulates miR502-3p/SET/AKT, leading to induction of cell proliferation, migration, and invasion [183]. In another study, lncRNA PAGBC, stabilized by PABPC1 was suggested to act as a miRNA sponge, and regulate tumor growth and metastasis by activating PI3K/AKT/mTOR pathway [196]. Similarly, lncRNA LINC00152, induced by Specificity protein 1, was also found to regulate growth and metastasis in GBC by activating PI3K/AKT pathway [197]. It was demonstrated that miR-143-3p inhibits PI3K/AKT pathway via targeting ITGA6 and suppress growth and angiogenesis of GBC [66]. It is found that upregulation of PI3K/AKT/mTOR or its deregulation by aberrantly expressed miRNAs affect

tumorigenesis. For example, in hepatocellular carcinoma (HCC), induced by mTOR, a total of 16 miRNAs are differentially expressed (13 downregulated and 3 upregulated) and 13 lncRNAs (12 upregulated and 1, HULC, downregulated) impacting HCC progression [198, 199].

These investigations have, thus, identified several potential therapeutic targets for GBC as well. Several inhibitors, like A66, Wortmannin, and LY294002, have been developed against the identified targets of PI3K/AKT/mTOR pathway, which inhibit proliferation, migration, and invasion of GBC [200–202]. Drugs like rapamycin, RAD001, and AZD8055 have been shown to block mTOR, resulting in the inhibition of growth and migration of GBC cells [189].

Combined therapy of mTOR inhibitor INK-128 and HDAC inhibitor JNJ-26481585 has been shown to suppress cancer growth, both in vitro and in vivo. Use of these inhibitors along with gemcitabine has synergistic effect on suppressing GBC growth and metastases, suggesting their therapeutic potential. Thus, mTOR activation may be useful both as prognostic biomarker as well as identifying patients who can be benefited most from mTOR inhibitors [199].

PD-L1 (programed death ligand-1)/PD-1 (Programed cell death protein-1). PD-L1 or CD274 (cluster of differentiation 274) or B7-H1 (B7 homolog1) is a type 1 transmembrane protein, while PD-1 is expressed on various activated immune cell types, including T cells, B cells, macrophages, etc. PD-L1 interacts with its receptor PD-1, inhibiting T cell activation. The expression of PD-L1 is normally absent on the cell surface of most human tissues, however, becomes upregulated in many cancers [203, 204]. Significant mutational load in these genes in GBC has been found [205–208]. However, the mechanism how these proteins function in GBC development is still not very clear. Treatment with PD-1/PD-L1 inhibitors (e.g., nivolumab) has been found to be a promising and safe targeted therapy [209–212]. Combination therapies using anti-PD-L1/PD-1 checkpoint agents, chemotherapy and targeted therapies are also in clinical practice [213–219]. The epigenetic agent guadecitabine that targets DNMT in combination with Durvalumab, an anti-PD-L1 antibody, is currently in the first phase of clinical trial in liver, pancreatic, bile duct or gallbladder cancer (ID: NCT03257761) [20]. PD-L1 gene promotor methylation has been observed in various cancers, such as CRC and prostate cancer [220, 221]. Combination therapy of epigenetic modifiers, like DNMT or HDAC inhibitors and anti-PD-L1/antiPD1 inhibitors may prove to be more effective [221]. The expression of PD-L1 is also regulated post-transcriptionally by various epigenetic modifications, including promotor DNA methylation, histone methylation/acetylation [204].

The PD-L1 mRNA has potential binding sites in its 3′UTR for various microRNAs, such as miR-513 and miR-155 [222, 223], which are required for IFNγ induced PD-L1 signaling. In addition, a number of other miRNAs are also identified to regulate the expression of PD-L1, e.g., miR-15, miR-16, miR-17, miR-34, miR-93, miR-106b, miR-138, miR-140, miR-142-5p, miR-152, miR-193a, miR-197, miR-200, miR-217, miR-424 (322), miR-513, and miR-570 [204]. This regulation may be lost if mutation or truncation occurs in the binding sites in 3'UTR of the target genes [224].

Role of circular RNAs (circRNA), another class of non-coding RNAs, in the regulation of PD-L1 expression has also been implicated. The circRNAs bind and inhibit specific miRNAs, disrupting their regulatory functions. For example, circRNA hsa-circ-0020397 binds with the tumor suppressor miR138 and downregulate it in CRC, consequently upregulating PD-L1 expression [163]. Specific miRNAs have been reported regulating PD-L1 expression in different cancers, e.g., miR-570, miR-34a, and miR-200 in prostate, miR-152 and miR-200 in gastric, miR-138-5p in CRC, and miR-142-5p and miR106-b in pancreatic cancers [221, 225–228]. Indirect regulation of PD-L1 expression by miRNAs (e.g., miR-20b, miR-21, and miR130b) suppressing the negative regulators of PD-L1 (e.g., PTEN) has also been reported [229].

C-mesenchymal–epithelial transition factor (MET) encodes tyrosine kinase receptor of hepatocyte growth factor (HGF). It acts as an oncogene. Binding of MET with HGF consequentially induce PI3K/AKT, RAS/RAF/MEK/ERK, and Wnt/b-catenin signaling, regulating proliferation, metastasis, and drug resistance [6, 230–232]. The expression of MET is found to be high in cancers of liver, pancreas, breast, gastric, GBC, etc. [211, 233–242]. Use of MET inhibitors in GBC treatment is currently in clinical trial phase [243].

The expression of MET is epigenetically regulated via epigenetic modifications, like DNA methylation and histone acetylation [244–246]. In several cancers it is found to be overexpressed due to hypomethylation and acetylation, e.g., pancreatic ductal adenocarcinoma (PDAC) [246]. Its expression may also be regulated indirectly through methylation of its regulators [243]. About 30 miRNAs are known to be involved in its translational regulation [247]. MET may also regulate the expression of certain miRNAs, acting as an inhibitor [248].

TP53 is known as a tumor suppressor gene, exhibiting frequent mutation in several cancers. It is widely believed to function in DNA damage response, cell cycle arrest, and apoptosis [249, 250]. Its expression is also found specific to a particular ethnicity [251–254]. Overexpression and high mutation frequency makes it a potential cancer biomarker for various cancers, including GBC [255, 256]. It is known to be an independent factor for the poor prognosis of GBC [255]. While limited reports are available on the epigenetic regulation of TP53, DNA methylation has been demonstrated in both in vitro and tissue samples [257, 258].

CDKN2A/B (cyclin-dependent kinase inhibitor A/B). It is reported to inhibit CDK4 and CDK6 and cause cell cycle arrest at the G1/S phase. Like TP53, it is also found to be frequently mutated in GBC, which could be a cause of GBC pathogenesis [259, 260]. Its function, thus, makes it a potential target for GBC therapy [132, 226]. The tumor suppressor protein $p16^{INK4a}$, encoded by CDKN2A or multiple tumor suppressor 1 (MTS1), is found inactive in many cancers [226]. CDKN2A gene promotor is found to be hypermethylated in many cancers more often than CDKN2B [261–264]. Inhibition of DNMT1 caused upregulation of p16INK4a, which is an inhibitor of CDK4 [262, 265]. DNMT3b is also found to downregulate p16INK4a expression through DNA methylation [262, 265].

12.3.4 Targeting Non-Coding RNAs

Studies on small non-coding RNAs, including micro-RNAs (miRNAs), long non-coding RNAs (lncRNAs), small interfering RNAs (siRNAs), small nucleolar RNAs (snoRNAs), and piwi-interacting RNAs [266], carried out during the last two decades have demonstrated their significant role in the regulation of gene expression. These non-coding RNAs are involved in the post-transcriptional regulation of various target genes, including those involved in epigenetic events [267, 268]. MiRNAs can either directly target the epigenetic machinery affecting expression of tumor suppressors or oncogenes or they themselves behave as tumor suppressors (downregulated) or oncogenic or oncomiRs (upregulated) [16, 176, 269, 270]. OncomiRs suppress the expression of tumor suppressor genes [271], while tumor suppressor miRNAs (e.g., let-7and miR-34) repress the expression of oncogenes, such as KRAS. They are suggested to mediate the process of carcinogenesis, including proliferation, invasion, and chemotherapy resistance, in turn, serving as biomarkers or new therapeutic targets in epigenetic therapy of various cancers [272–274]. These miRNAs can be inhibited by modified antisense oligonucleotides and can have potential in developing novel therapies [275]. Similarly, development of tumor suppressive mimetic miRNAs (mimic) can also have implications in therapeutic significance. In GBC, several miRs and lncRNAs, such as miR-125b-5p, miR-122, miR-223, miR-31, miR30a-5p, lncRNA-HGBC, lncRNA PVT1, and lncRNA GBCDRlnc1 are currently under clinical validation for their therapeutic potential [19, 183, 196, 198, 200, 201, 276–281]. Identification of miRNAs with active involvement in a cancer can have implications in improving patient selection for targeted molecule, developing more effective therapeutic agents, and using them as potential early diagnostic biomarkers [280]. Currently, several phase-I clinical trials are undergoing using miRNA or lncRNA therapy against specific oncogenes [7, 198, 281].

The above discussed markers are only a few of those being targeted in various cancers and have the potential to be prospective molecules for therapeutic targets in GBC as well, either as mono-therapy or in combination therapy. To achieve best result, recommendations are being widely made to use combination therapies targeting different key pathways, which may have synergistic efficacy and minimal toxicity. Development of tumor immune therapy is currently an emerging strategy suggested to have better efficacy in the treatment of different cancers, including GBC.

12.4 Future Scope of Epitherapy in GBC and Conclusion

Gallbladder cancer is a relatively rare cancer and, hence, has received little global attention to investigate the genetic, epigenetic and therapeutic intricacies as deeply as several other cancers, like breast, prostate, lung, gastric, etc. Patients with gallbladder cancer do not have specific symptoms at the beginning, so they often lack the possibility of optimal treatment. Thus, it is necessary to identify potential

biomarkers for the diagnosis and prognosis of patients with gallbladder cancer. Since epigenetics has a reversible mechanism in nature, markers based on epigenetics are now considered as the primary markers for cancer. A factor that may restrict the elaboration of such indicators or biomarkers is the inconsistent geographic distribution of the disease. Variable population data revealed the absence of a universal epigenetic biomarker. Hence, further work is required to identify population-specific epigenetic markers in susceptible populations to enhance patient survival. Moreover, it is possible that a panel of several genes, like those mentioned above, in combination, may act as a successful biomarker in disease diagnosis and or prognosis. Though, most of the markers projected above and discussed under targeted epigenetic therapy are showing better promise or being studied at the level of clinical trials in various other cancers, their roles are similar in GBC. This provides a rationale to propose and target them for therapeutic test in GBC also. For a successful effort in developing effective epitherapy for GBC, collaborations among various investigators and clinicians working on GBC in diverse susceptible global populations are essential to develop more effective therapy against GBC, in order to enhance the patient survival and reduce the mortality.

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Targeted Treatment of Gallbladder Cancer

13

Amol Patel and Vivek Hande

13.1 Introduction

The targeted treatment with tyrosine kinase inhibitors (TKIs) or various monoclonal antibodies has changed and improved the spectrum of treatment of multiple cancers in addition to conventional cytotoxic chemotherapy agents. The first approved targeted monoclonal antibody treatment is trastuzumab in ERBB2 positive metastatic breast cancer in 1998. Trastuzumab has made a big impact in the care of breast cancer patients, both in adjuvant and metastatic settings. Trastuzumab saved countless lives and will keep saving cancer patients in the future [1]. Over the last three decades, biliary tract cancers (BTCs) are treated as one group comprising of intrahepatic, extrahepatic cholangiocarcinoma, and gallbladder cancer (GBC). Gemcitabine- and platinum-based combination therapy remained the treatment of choice over these years [2]. Recent advances in understanding of molecular mechanisms of cancers have improved our understanding of BTCs. The seminal work by Hiromi Nakamura et al. on genomic characterization of biliary tract cancers revealed that these diseases are genomically different [3]. Molecular signatures differ as per site [4–7] (Fig. 13.1). The ERBB2 amplification is a characteristic of GBC as compared to cholangiocarcinoma. Similarly, FGFR2 rearrangement is commonly seen in extrahepatic cholangiocarcinoma. Here, we discuss available literature on targeted treatment of GBC.

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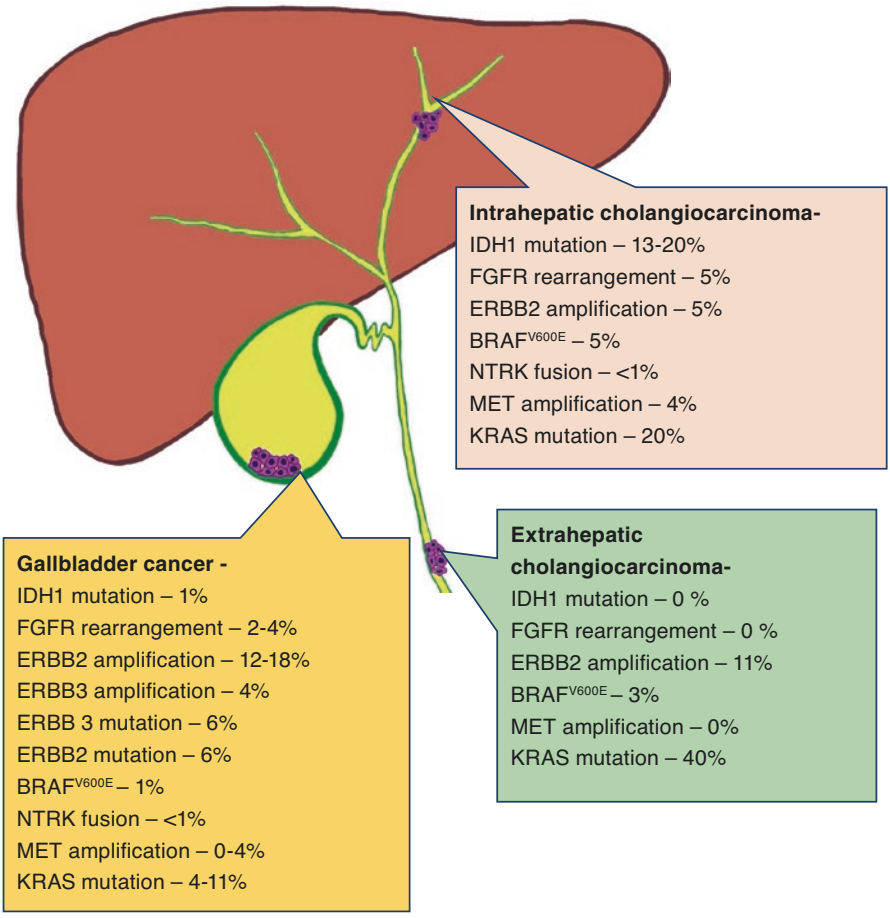

Fig. 13.1 Molecular targets in biliary tract cancers

13.2 Treatment Category

13.2.1 Adjuvant

There is no data available for any available targeted therapy in GBC in spite of enrichment of molecular targets. After the BILCAP trial, NCCN guidelines included single agent capecitabine for adjuvant chemotherapy in resected biliary tract cancer [8]. This is the only treatment available in adjuvant setting. However, in practice the combination of gemcitabine and platinum doublet is used by many institutes.

13.2.2 Neoadjuvant

There are no approved targeted therapies in neoadjuvant setting [9].

13.2.3 Metastatic or Palliative

The majority of published literature is in advanced and metastatic stage. As per paradigm of evolution of drugs in oncology, the newer experimental drugs are studied in metastatic setting first. The GBC has been studied extensively for aberrant genetic pathways. However, the present literature is largely limited to case reports and case series. The available evidence is narrated in the following paragraphs.

13.3 Targeted Therapy—Epithelial Growth Factor Receptor (EGFR/HER1)

EGFR expression is 100%, 52%, 38% in intrahepatic cholangiocarcinoma, extrahepatic cholangiocarcinoma, and GBC cell lines, respectively [10]. Anti-EGFR antibodies are approved in head and neck cancer and left sided colon cancer. The anti-EGFR antibodies, Cetuximab and Panitumumab, have been studied in cholangiocarcinoma. The combination of cetuximab and GemOx was studied in a phase 2 clinical trial with negative results [11]. There was no benefit of adding the cetuximab to chemotherapy.

13.3.1 Erlotinib

Erlotinib reversibly inhibits EGFR (HER1) tyrosine kinase pathway, which inhibits the downstream pathway and arrest cell proliferation. It has high affinity for del19 and exon 21 mutation in lung cancer and used as first generation TKI in lung cancer. Somatic mutations have been studied in EGFR gene in biliary tract cancer in exon 19, exon 20, exon 21. No mutations were seen in exon 18 [12]. In BTCs, erlotinib was first studied in 2005 by Philip PA et al. in phase 2 study as a single agent in patients who were treated with one prior systemic or locoregional therapy [13]. In this study of 42 patients, 16 (38%) patients had gallbladder cancer. The partial response was seen in 3 (8%) patients and lasted for 14 months. 17 (43%) patients had stable disease for a median time of 4.4 (range: 2–20) months. The median overall survival (OS) was 7.5 months. The EGFR expression was graded on IHC and was categorized from 0 to 3+. EGFR expression did not predict response to Erlotinib therapy. Erlotinib is used as third line treatment option in GBCs.

In a phase 3 Korean trial, the combination of Gemcitabine-Oxaliplatin was studied with and without Erlotinib in advanced or metastatic BTCs by J Lee et al. [14].

In this trial, the median progression free survival (PFS) was 5.8 months in combination group versus 4.2 months in the group without erlotinib (HR-0.80, CI 0.61–1.03, $p = 0.087$). The objective response was 29% versus 15% ($p = 0.005$). The median OS was similar in both groups 9.5 months versus 9.5 months (HR-0.93, CI 0.69–1.25, $p = 0.611$). In the subgroup of cholangiocarcinoma, the median PFS was significantly longer (5.9 versus 3 months, $p = 0.049$). The GBC patients were 47 (35%) in Gemcitabine-Oxaliplatin group and 35 (26%) in Erlotinib with chemotherapy arm. Distribution was not balanced in randomized groups. In subgroup analysis, HR for GBC was 0.99 (0.63–1.58), meaning the combination did not favor the therapy. The study was further limited by molecular analysis of KRAS mutation in only 60 patients. Six patients had KRAS mutation and only three responded to Erlotinib combination, further failing to derive any conclusion. The study by Lee et al. is conducted 10 years ago, recent positive study with improved outcomes of TKI plus chemotherapy in treatment of EGFR mutated metastatic non-small cell lung adenocarcinoma, has reemphasized the concept of similar molecular based studies in advanced BTCs and can be explored in the future clinical trials [15].

13.3.2 Gefitinib

A good response and prolonged PFS was (were) reported in a patient of GBC with EGFR exon 20 T790M mutation when gefitinib was combined with Gemcitabine-Oxaliplatin chemotherapy [16].

13.4 BRCA 1 & 2

Olaparib has been used in BRCA mutated ovarian cancer in maintenance and recurrent settings. The somatic BRCA 1 & 2 mutations are common in gallbladder cancer and seen in 24% of the cases [17]. The germline mutations in Portuguese families are rare [18]. Indian data on germline BRCA 1 & 2 is not available. Response to Olaparib was reported in GBC in germline mutated BRCA1patient [19]. The role of PARP inhibitors in BRCA 1 & 2 mutated (germline and somatic) remains to be elucidated.

13.5 ERBB2 (HER-2-Neu) Amplification

The GBCs are enriched in ERBB2 amplification in up to 10–18% in advanced cases [17, 20, 21]. In GBC cell lines the response to trastuzumab and neratinib has been demonstrated earlier [22]. Milind Javle et al. studied the role of targeted therapy in GBC [23]. In this retrospective study, the first proof of activity of trastuzumab in GBC was established. In a cohort of nine patients, the median duration of response to targeted therapy was 40 weeks (range: 8–168 weeks). We did the prospective study of second line trastuzumab based chemotherapy in advanced GBC and proof

of activity of trastuzumab and/or Lapatinib was established [24]. The multi-institutional data from north India of trastuzumab combined with chemotherapy versus chemotherapy alone in 38 patients, showed the median progression free survival of 9.7 months versus 4.1 months, respectively [25]. Similarly, the OS was 14 versus 6 months in chemotherapy plus trastuzumab versus chemotherapy alone. Overall response rate of 71% is encouraging. In this study, 21 and 10 patients received trastuzumab with chemotherapy in first and second line, respectively [25]. This is the largest data available in abstract form from India till date. There is enough evidence of role of trastuzumab in HER-2-neu amplified GBC to carry forward the research in phase 2 and 3 studies. Phase 2 trials are recruiting patients in India [26].

13.6 $BRAF^{V600E}$ Mutation

Around 5% of intrahepatic cholangiocarcinoma carry $BRAF^{V600E}$ mutation. The frequency of this well-established molecular driven gene is very rare in GBCs and intrahepatic cholangiocarcinoma [27, 28]. We have no patient of this mutation in our study [24]. In Chinese patients, 3% of GBC had $BRAF^{V600E}$ mutation, the publication was in abstract form [29]. Dabrafenib and Trametinib combination is approved in $BRAF^{V660E}$ mutated malignant melanoma in adjuvant and metastatic setting, anaplastic thyroid cancer, and non-small cell lung carcinoma. This combination has been studied in various other malignancies [30, 31]. Response to this combination has been reported in intrahepatic cholangiocarcinoma [32]. In the NCI-MATCH trial sub-protocol H, 04 patients of cholangiocarcinoma with $BRAF^{V600E}$ mutation were included, who had progression on standard first line chemotherapy. Three patients had partial responses with progression free survival of 9.1, 12.8, and 29.4 months [33]. In ROAR phase 2 basket trial, 43 patients of advanced biliary tract cancers, overall response rate with above combination was 51%, again only one patient with GBC is included in this study and majority were intrahepatic cholangiocarcinoma [34]. In our prospective study of 50 patients of GBC, only one patient had BRAF amplification, whether it will respond to targeted therapy is a matter of active research [24]. In GBC, the role of Dabrafenib and Trametinib still remains to be established.

13.7 FGFR Gene Rearrangement or Fusion

In 2020, the first targeted therapy got FDA approval for use in biliary tract cancers is pemigatinib at a dose of 13.5 mg orally (2 weeks on, 1 week off), along with companion diagnostic test Foundation Medicine CDx [35]. Pemigatinib is a selective inhibitor of the Fibroblast growth factor receptor gene. Abou-Alfa G et al. lead the study FIGHT 202, 107 patients received pemigatinib after failure of at least one line of systemic therapy [36]. Median progression free survival was 7.2 months and objective response rate of 35.5%. Three patients had complete responses and 35 patients had partial responses. These responses were seen exclusively in FGFR2

fusion or rearrangement. There were no responses seen in patients with other or no FGF/FGFR alterations, highlighting the importance of testing and treating patients with only FGFR2 fusion or rearrangement. Hyperphosphatemia was the most common adverse effect (60%) and hypophosphatemia occurred in 23% of the patients. Nine percent patients discontinued treatment and no death was attributed to pemigatinib as assessed by the investigators. Pemigatinib is not available in India at present.

13.8 Vascular Endothelial Growth Factor Receptors (VEGFR)

Bevacizumab—In a decade-old phase 2 study, Bevacizumab was studied in combination of Gemcitabine and Oxaliplatin. This cohort included ten patients of GBC in a total of 35 patients. The median PFS was 6 months. Combination therapy was well tolerated with grade 3 and 4 neutropenia and it was seen that 20% and 14% had hypertension, neuropathy and deranged liver functions. FDG-PET response predicted PFS and OS in biliary tract cancers [37]. This encouraging PFS should have been tested in phase 3 design. Bevacizumab was also studied in combination with Erlotinib. In this study of 49 patients, 12% had partial response with median OS of 9.9 months. Four patients suffered from grade 4 toxicities [38].

Apatinib is a selective VEGFR-2 blocker, and it has been approved in some countries in gastric and GE junction adenocarcinoma in third line setting. In a retrospective study of 21 patients (five patients of GBC), partial response was seen in 3 (14%) and stable disease was seen in 12 (57%) patients. It highlights the activity of VEGFR 2 blockers in biliary tract cancers [39, 40].

13.9 Isocitrate Dehydrogenase 1 (IDH1)

IDH1 mutation inhibits HNF-4-alfa and stop hepatocyte differentiation and leads to biliary carcinogenesis [41]. The IDH1 inhibitors are approved in refractory and treatment ineligible acute myeloid leukemia. In 13% of patients with intrahepatic cholangiocarcinoma, IDH1 mutation is seen which can be targeted by oral ivosidenib. In phase 3 trial of ivosidenib in advanced intrahepatic cholangiocarcinoma, progression free survival was 2.7 months in ivosidenib vs. 1.4 months in placebo (HR-0.37, CI 0.25–0.54, p one sided <0.0001) [42]. The median OS in ivosidenib and placebo was 10.8 months and 9.7 months, respectively. Crossover was permitted in this trial. Objective response was 2% and 51% had stable disease in the ivosidenib arm. 30% of patients in ivosidenib arm had serious adverse events like hyperbilirubinemia, QT prolongation, pleural effusion. Authors concluded that there is feasibility and clinical benefit of targeting the IDH1 mutated cholangiocarcinoma.

13.10 NTRK Gene Fusion

The NTRK fusion is rare (<1%) in BTCs [43–45]. Tumor agnostic effect of Entrectinib has been studied in various cancers and has shown durable responses, promising PFS and OS in various cancers [46, 47]. Similarly, Larotrectinib has shown efficacy in these tumors [48]. These NTRK fusion inhibitors have shown intracranial responses similar to extracranial response rates.

13.11 Immunotherapy

13.11.1 Nivolumab Plus Ipilimumab

The CA209–538 trial is a multicenter, phase2, three cohort study of Nivolumab plus Ipilimumab in rare cancers [49]. Nivolumab Ipilimumab combination was administered for four doses and subsequently only Nivolumab was continued till progression, or for maximum duration of 2 years or unacceptable activities. The sub-protocol of advanced BTCs included 39 patients who received at least one line of systemic therapy. Thirteen patients were of GBCs. The objective response rate was 23% and disease control rate was 44%. The overall response rate in intrahepatic cholangiocarcinoma was 31%. No response was seen in extrahepatic cholangiocarcinoma. The duration of response ranged from 2.5 to > =23 months. All patients had microsatellite stable disease. 15% patients had grade 3 or 4 immunological toxicities without any treatment related death.

13.11.2 Nivolumab

Nivolumab was studied in advanced refractory BTC as a single agent in a phase 2 clinical trial in Korea. In the intention to treat analysis, median progression free survival was 3.68 months and median OS was 14.24 months. The objective response rate was 22% in investigator assessed analysis and 11% in independent review analysis. This study established the activity of immunotherapy in BTCs [50].

13.11.3 Pembrolizumab

Case reports of impressive outcomes of pembrolizumab in 2 patients of advanced BTCs have been reported. Pembrolizumab was administered at half the dose of recommended dosing and both patients did maintain the response for 2 years [51]. The role of predictive biomarkers for response to immunotherapy remains uncertain [52]. PDL1 expression of more than 50% has been used in lung cancer and used as a guidance to single agent Pembrolizumab. The PDL1 expression is variable in BTCs and it is 23% in GBCs and at cut off of >50%, it is 7.5% [53].

13.11.4 Combination of Immunotherapy and Chemotherapy

The IMMUNOBILPRODIGE 57 trial is a safety study of durvalumab, tremelimumab, and paclitaxel combination in advanced biliary tract carcinoma. There is an increased rate of anaphylactic reactions in this study [54].

13.12 Cancer Vaccine

Cancer vaccine is still in the conceptual stage, holding a promise for prevention and treatment of GBC. The recent seminal work of A Pandey et al. on mutated ELF3 gene has highlighted the potential of future development of vaccine against GBC [55]. The frameshift deletions in E74 like Transcription factor (ELF3) gene leads to neoantigen production which in turn leads to activation of CD8+ T cells. ELF3 mutations were less common in Indian (7%) patients as compared to Korean (31%) and Chilean (22%). Though this pivotal work is limited by small numbers, it is an intriguing work for basic and immunological researchers to take a lead.

13.13 Conclusions

The role of targeted therapy in gallbladder cancer is evolving. Gallbladder cancer is enriched with genomic alterations which can be targeted. Early data is promising. BTCs differ genomically in respect to sites. Further trials should consider these diseases differently as gallbladder cancer for better representation and outcomes. Understanding the resistance mechanisms and designing the trials with molecular inputs is the key to rapid success in future.

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Molecular Pathways in Gallbladder Cancer as Potential Therapeutic Target

14

Sameer Gupta, Puneet Prakash, Vijay Kumar, Arun Chaturvedi, and Shashwat Tiwari

14.1 Introduction

Gallbladder cancer (GBC) is a common malignancy in Northern India though globally the disease has considerable geographic differences in the incidence and etiology [1, 2]. Besides gallstones, which are found in nearly 80% of GBCs, anomalous pancreatico-biliary junction (APBJ) (relatively frequent in East Asia) is known to play an important role in the development of GBC [3, 4]. The disease usually presents in advanced stage and carries high mortality. There has been considerable progress in the treatment of GBC, however, we seem to have reached a plateau in terms of success achieved by the conventional treatments comprising radical surgery, radiation and chemotherapy.

Although considerable progress has been made in understanding of genetic changes involved in the etiopathogenesis of several human tumors, there have been relatively limited advancements in our understanding of molecular changes involved in the development of GBC. Because only a small fraction of patients with cholesterol gallstones develops GBC, it is important to identify the genetic and molecular factors that induce progression from cholelithiasis to GBC. The molecular pathways of GB carcinogenesis, disease progression, and metastasis are entirely untapped areas for new drug development. Better understanding of these molecular pathways can not only help in identification of novel markers to help in early diagnosis of disease, but also in defining targets for development of new generation of targeted anti-cancer drugs.

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This chapter will aim to define main molecular changes and pathways involved in GB carcinogenesis and progress made in targeting the key pathways as a therapeutic modality. We will also try to highlight the increasing understanding of immune-based therapies and their application in treatment of GBC.

14.2 Gallbladder Carcinogenesis Pathways

There are two main pathways of GB carcinogenesis, one functional and other anatomical. Both of these pathways play an important role in the pathogenesis of GBC in different parts of the world. In southeast Asia, for example, where gallstones are more common, functional pathway due to chronic irritation of the gallbladder secondary to the gallstones result in the development of dysplastic mucosa, which later progresses to GBC.

The second, less common pathway to the development of GBC, is because of an anomalous pancreato-biliary junction [5]. This is a congenital abnormality, which is commonly detected in East Asia (Korea and Japan) [6]. The most distinctive molecular feature of APBJ-related GBC is its association with a relatively high frequency of KRAS mutations (15–58%) at codon 12 even in early stages of disease [5]. P53 mutations are a late or tumor-specific change, whereas in cholecystitis-associated GBCs they represent an early change. Thus, it is a biologically different disease having specific genomic signatures.

GBC shows a very strong predilection for cholelithiasis, female gender, age over 65 years and presence of TP53 mutations early on during the multistage progression of dysplastic mucosa into frank invasive malignancy of the gallbladder [7]. The biology of the GBC occurring in younger patients is strikingly different from that occurring in the elderly in the sense that it is less commonly associated with gallstones and shows the presence of KRAS mutation and late occurrences of TP53 mutations [8].

More commonly GBC arises in the metaplastic epithelium accumulating the genetic insults, thus resulting in dysplasia and carcinoma in situ [9]. Pathologic studies have shown that most invasive GBCs are associated with dysplasia and carcinoma in situ. Adenomas are rarely found in the context of such dysplastic changes. Thus, based on present evidence, a metaplasia-dysplasia—carcinoma in situ—carcinoma sequence seems to be the more prevalent route over an adenoma-carcinoma sequence in GBC [9].

14.3 Molecular Changes/Mutations Involved in Gallbladder Carcinogenesis

Many molecular changes involved in GB carcinogenesis have been identified, however, the complex interplay of these mutations which aid the continued growth of cancer, poor response to conventional chemotherapeutic drugs and exact role in pathogenesis, is still not completely understood (Fig. 14.1).

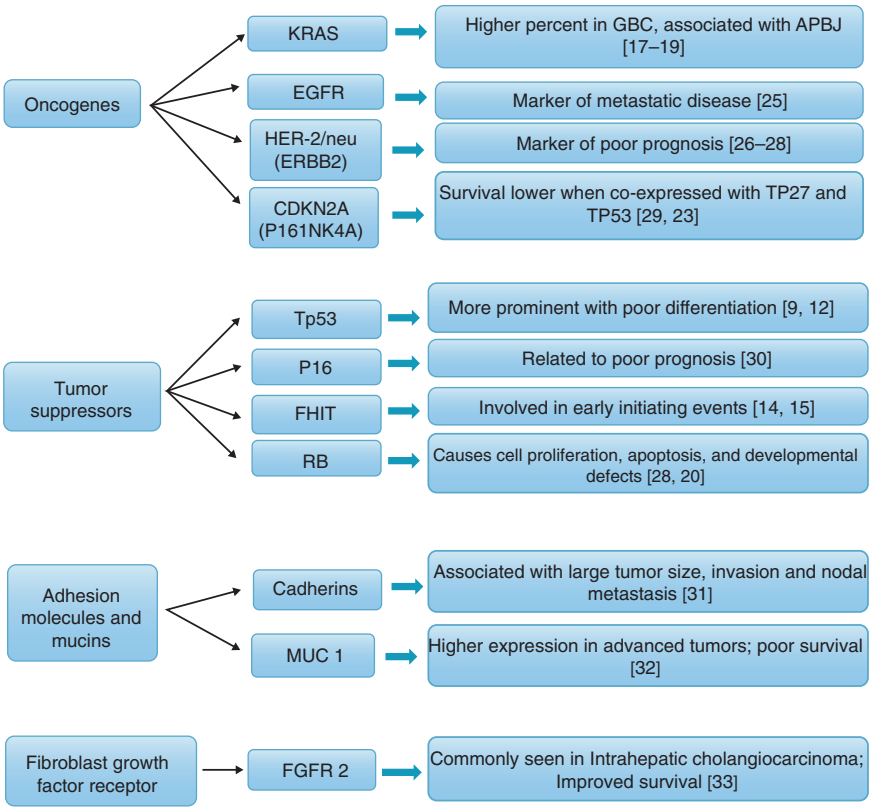

Fig. 14.1 GBC carcinogenesis: commonly affected family of genes

14.3.1 Tumor Suppressor Gene (TP53 Mutations)

TP53 tumor suppressor gene plays a key role in maintaining the genomic integrity and is described as the most common cancer related genetic abnormality, occurring in more than 50% of human tumors [10]. Higher mutation frequency and over-activation of p53 protein has tumor-promoting effect that allows inappropriate proliferation of genetically damaged cells and correlates with an inferior survival in GBC [11]. Of the 393 codons present in the TP53 gene, mutations are especially frequent in the middle region comprising of exons 5–85. TP53 allelic loss is one of the earliest events detected in the sequential pathogenesis of GBC, starting in epithelia that have a normal histological appearance [10]. Although the reported frequency of p53 immunostaining in GBC varies widely (ranging from 35% to 92%), most studies report a frequency greater than 50% [12]. TP53 mutations commonly associated with GBC are missense mutations, causing over-production of non-functional protein with an increased half-life, leading to increased expression of non-functional p53, which can be detected by immunostaining [9]. Nearly half of

the TP53 mutations in GBC occur in exon 5 [13]. Study by Singh et al. in North Indian GBC patients showed that p53 mutation can act as an independent prognostic factor for GBC [14].

14.3.2 HER-2/Neu

Besides the proven prognostic and therapeutic implication of HER-2/neu overexpression in breast cancer, HER-2 gene overexpression has also been observed in multiple other cancers such as stomach cancer, colon cancer, lung cancer, and biliary tract cancer. Kiguchi et al. first reported that overexpression of HER-2 in the basal layer of biliary tract epithelium in transgenic mice leading to the development of gallbladder adenocarcinoma at age of 3 months. In human GBC, HER overexpression was found between 9.8% and 12.8% of GBC patient population [15].

14.3.3 EGFR Mutations

EGFR activation triggers multiple intracellular downstream signaling cascades, including ERK/MAPK, PI3K-AKT, SRC, JNK, and JAK-STAT pathways thus mediating cancer proliferation, angiogenesis, cell motility, adhesion, and metastasis [16]. Enhanced activation of EGFR in gallbladder cancer tissues firmly correlated with inferior prognosis of these patients [17]. A recent study reported low rate of EGFR associated somatic mutations (2.5–3.9%) in patients with gallbladder cancer [18].

14.3.4 VEGF Mutations

There are five distinct VEGF family members in a mammal: VEGF-A (also referred to as VEGF), VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PLGF). VEGF-A, VEGF-B, and PLGF are mainly involved in angiogenesis, while VEGF-C and VEGF-D regulate lymph angiogenesis. VEGF-A and VEGF-B have a strong ability display to interact with VEGFR1 and VEGFR2 expressed on vascular endothelial cells and vascular smooth muscle cells [19]. Sun XN in a series of 84 patients reported a high expression of VEGF-A (53.6%) and its positive correlation with cancer progression, metastatic disease, and histological differentiation; VEGF-A overexpression serving as an independent prognostic factor of survival in GBC [20]. A recent study led by Xu et al. observed that VEGF-A was remarkably increased in the serum of GBC patients and stimulated angiogenesis, invasion and cell proliferation while inhibiting the apoptosis in GBC cells [21]. Combined high expression of VEGF-A with Estrogen Receptor-1 (ER-1) predicted poor prognosis for GBC patients, suggesting that VEGF-A combined with hormone receptor ER may provide a biomarker for GBC prognosis [22]. The elevated serum VEGF-C was positively correlated with decreased OS and increased lymph node metastasis [23].

14.3.5 Mitochondrial DNA Mutations

Mitochondrial DNA mutations in human tumors have been assumed to result from the action of reactive oxygen species produced during inflammation [24]. Recently, mutations of the mtDNA D310 sequence at the displacement loop have been detected as relatively frequent and early events in the sequential pathogenesis of GBC, being found in epithelia with normal appearance in chronic cholecystitis cases [25]. The finding that molecular abnormalities characteristic of GBC occur in chronic cholecystitis is powerful evidence that a common inflammatory condition is a precursor to GBC.

14.3.6 Fragile Histidine Triad (FHIT) Gene Mutation

Epigenetic inactivation of tumor suppressor genes SEMA3B and FHIT in gallbladder cancers have been described by Witsuba et al., suggesting a potential role in gallbladder cancer pathogenesis [26]. High incidences of mitochondrial DNA (mtDNA D310) mutation (46–57%) [13], p53 overexpression, and FHIT loss of expression (55–58%) [25] have been observed in histologically normal and dysplastic epithelium adjacent to GBC arising in the setting of chronic inflammation.

14.3.7 Proto-Oncogene KRAS Mutations

KRAS mutations are described less commonly in GBC associated with gallstones while they are frequent and early events in GBC associated with congenital abnormality of the pancreatic bile-duct junction (APBJ) [27]. CTNNB1 (β-catenin)-mutated adenomas represent a relatively low malignant potential, whereas KRAS-mutated dysplastic lesions associated with inflammation (such as in cases of APBJ) may have a greater malignant potential [28]. Although a low incidence (0–10%) of KRAS mutations has been described in most studies of GBC in western countries, studies from Japan are more variable, with reported incidences from 0 to 59%. Majority of KRAS mutations (50–80%) have been reported in GBC associated with APBJ, indicating that reflux of pancreatic juice has a role in the occurrence of these mutations [5, 29]. Similar to other human cancers, almost all the KRAS mutations reported in GBC most commonly occur at codon 12 resulting in continuous and inappropriate growth signals [30]. KRAS mutations seem to be cancer specific and are seldom, if ever, detected in pre-neoplastic tissue seen in GBC associated with gallstones.

14.3.8 DNA Damage Repair (DDR) Pathway Mutations

DDR pathway can execute full repair or elimination of damaged cells, essential for maintaining genomic stability and protect host organisms against possible

carcinogenesis. There are four major DDR pathways identified in the cells, for example, base excision repair, nucleotide excision repair (NER), double strand break repair, and mismatch repair (MMR) [30]. Reported incidence of DNA repair mutations (MSH6, BRCA1, BRCA2, ATM, MLH1 or MSH2 genes) was found to be 13% in intrahepatic cholangiocarcinoma (IHCCA), 26% in Extrahepatic cholangiocarcinoma (EHCCA), and 6% of GBC cases in 321 BTCs who underwent mutational profiling [31]. A study carried out by Chae et al. showed that mutation of DDR gene was detected in 62.5% of patients with biliary tract cancer (including 20.2% of GBC patients) and mutation in DDR pathway genes were found to be associated with greater median OS (21.0 vs. 13.3 months) and median PFS (6.9 vs. 5.7 months) in biliary tract cancer that were managed with first line platinum-based chemotherapy. Thus, mutations in DDR genes may serve as predictive biomarkers for the response to DNA repair inhibitors such as platinum-based chemotherapy, in patients with BTC [32]. The subset of cancers with mismatch repair (MMR) system defects is very sensitive to programed cell death protein 1 (PD-1) blockade using checkpoint inhibitor agents like pembrolizumab [33].

14.3.9 PI3K/AKT/mTOR Pathway Mutations

The PI3K/mTOR signaling cascade is involved in multiple physiological cellular processes including mobility, cell growth, metabolic activity, differentiation, and programed cell death and may be a potential molecular target for GBC cell growth and survival. A study documented the rate of activating mutations in PI3K to be approximately 12.5%, exclusively in GBC [34]. PIK3CA with E545K mutation was observed in ~5.9% of GBC patients, with poor prognosis [18]. Expression profiling of BTC compared with normal biliary epithelium has identified AKT/mTOR signaling components as being upregulated, including the potential drug target insulin-like growth factor 1 receptor (IGF1-R) [34, 35]. Epigenetic alteration of PTEN also promotes the development of gallbladder cancer as approximately 30% of gallbladder cancer patients display hypermethylated PTEN promoter [36].

14.3.10 Methylation and Gallbladder Cancer

Hyper-methylation in gene promoter regions is a common epigenetic mechanism for the inactivation of tumor suppressor genes. Multivariate analysis found Methylguanine-DNA-Methyltransferase (MGMT) gene to be an independent prognostic factor for survival, representing the important role of epigenetic process in gallbladder carcinogenesis [37]. Study by Letelier et al. showed that promoter methylation of specific genes like CDH1, CDKN2A-p16, REPRIMO (tumor suppressor gene family) and UCHL1 (also known as PGP9.5) may have an important role in gallbladder carcinogenesis [38]. Analysis of the methylation frequencies in GBCs

compared with the findings in chronic cholecystitis specimens demonstrated a group of eight genes (3-OST-2, CDH13, CDH1, RUNX3, APC, RIZ1, P16INK4A, and HPP1) that showed specificity for GBC, suggesting that they play an important role in GBC pathogenesis. Of these genes, the gene with the highest frequency (72%) of methylation in GBC was 3-OST-2. This gene encodes an O-sulfotransferase that is involved in the final modification step of glycosaminoglycan chains of heparan sulfate proteoglycans (HSPGs). HSPGs are known to play major roles in cell growth, adhesion, and migration by interaction with a wide range of growth factors, morphogens, cytokines, and extracellular matrices [39].

14.4 Evolving Landscape of Molecular Targeted Therapy for GBC

The survival in patients with unresectable biliary tract cancer has been shown to differ by the tumor type owing to different carcinogenic pathways and distinct chemo-sensitivities demonstrated by each of them. There is a long felt need for effective chemotherapy to improve the survival of patients with biliary tract cancer, especially GBC as most of these patients have advanced/metastatic disease at the time of diagnosis, making them unsuitable for any surgical intervention. Even patients undergoing curative surgery have frequent recurrences and need effective adjuvant therapy.

Chemotherapy regimens of FOLFOX (5-fluorouracil and oxaliplatin), CAPOX (capecitabine and oxaliplatin), GC (gemcitabine and cisplatin), and GEMOX (gemcitabine and oxaliplatin) are amongst the various chemotherapy regimens being used for palliative or Adjuvant treatment for GBC [40].

More recently, based on results of BILCAP trial, National Comprehensive Cancer Network (NCCN) has recommended two options for GBC treatment: single-agent therapy employing fluoropyrimidine or gemcitabine-based treatment, and multi-agent therapy including oxaliplatin, cisplatin, and capecitabine [41, 42].

The need for targeted therapy has arisen from the poor results and toxicity of conventional chemotherapy. The molecular genomics revolution, which has changed the paradigm of treatment in many cancers, has led to novel therapeutic approaches for biliary tract cancers as well. Several advanced technologies including proteomics, transcriptomics, and next-generation sequencing has heralded a new era of use of novel therapeutic approaches like targeted therapy, nanoparticle-based delivery systems, and immunotherapy in various cancers.

Trials of targeted therapy for GBC have focused on major molecular pathway involved in carcinogenesis and progression of the disease (Figs. 14.2 and 14.3). A major problem in these trials, however, is the breadth of driver mutations with small patient subset for each tumor subsite and key differences across intrahepatic cholangiocarcinoma (IHCC), extrahepatic cholangiocarcinoma (EHCC), and GBC [43]. Bridgewater et al. have shown that there is quite a distinct genetic

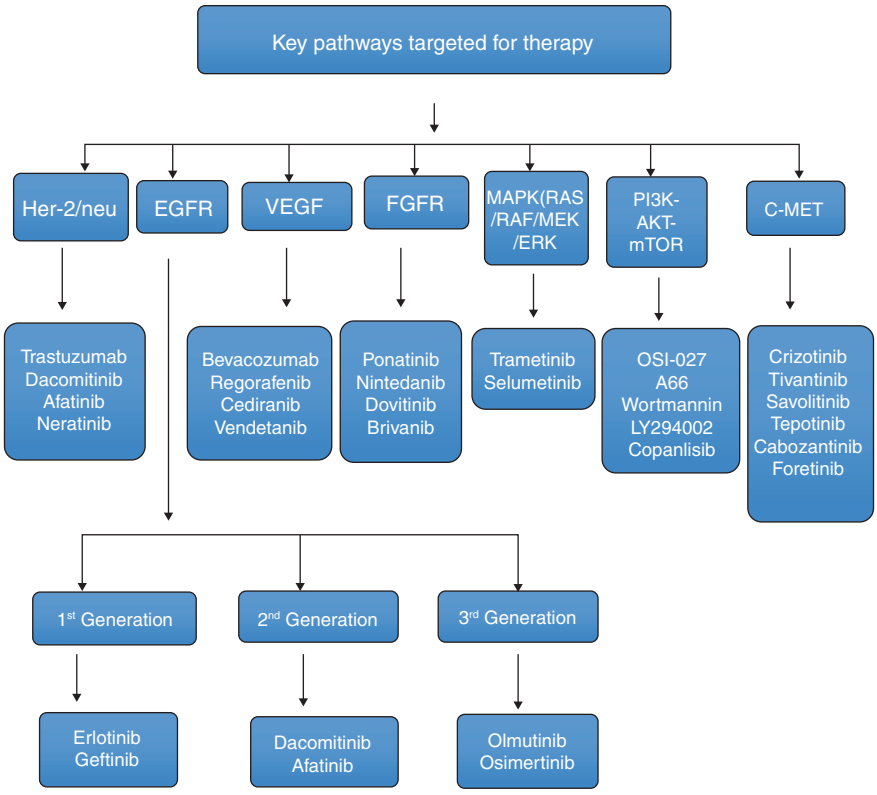

Fig. 14.2 Potential targeted agents being explored for use as therapeutic agents for gallbladder cancer

mutation landscape variation between IHCC, EHCC, and GBC based on anatomical subsite [44]. Mutation profiling has highlighted the genomic differences between the intra, extrahepatic cholangiocarcinoma, and GBC [45]. These trials have been done across the spectrum of all BTCs, very few studies have been done on GBC alone. Few exceptions being a recent large case-control genome-wide association study (GWAS) on more than thousand Indian subjects of GBC from Mumbai. This study observed genome-wide significant associations for several markers in the chromosomal region 7q21.12 harboring both the ABCB1 and ABCB4 hepatobiliary phospholipid transporter genes and common single nucleotide polymorphism (SNP) variants being associated with substantial variation in risk of GBC [46].

The lack of studies combined with the rarity of GBC in the western world creates challenges with testing novel therapies. Most trials of molecular targeted therapy for BTCs have GBC as only a small subset and the clinical outcomes are not very encouraging.

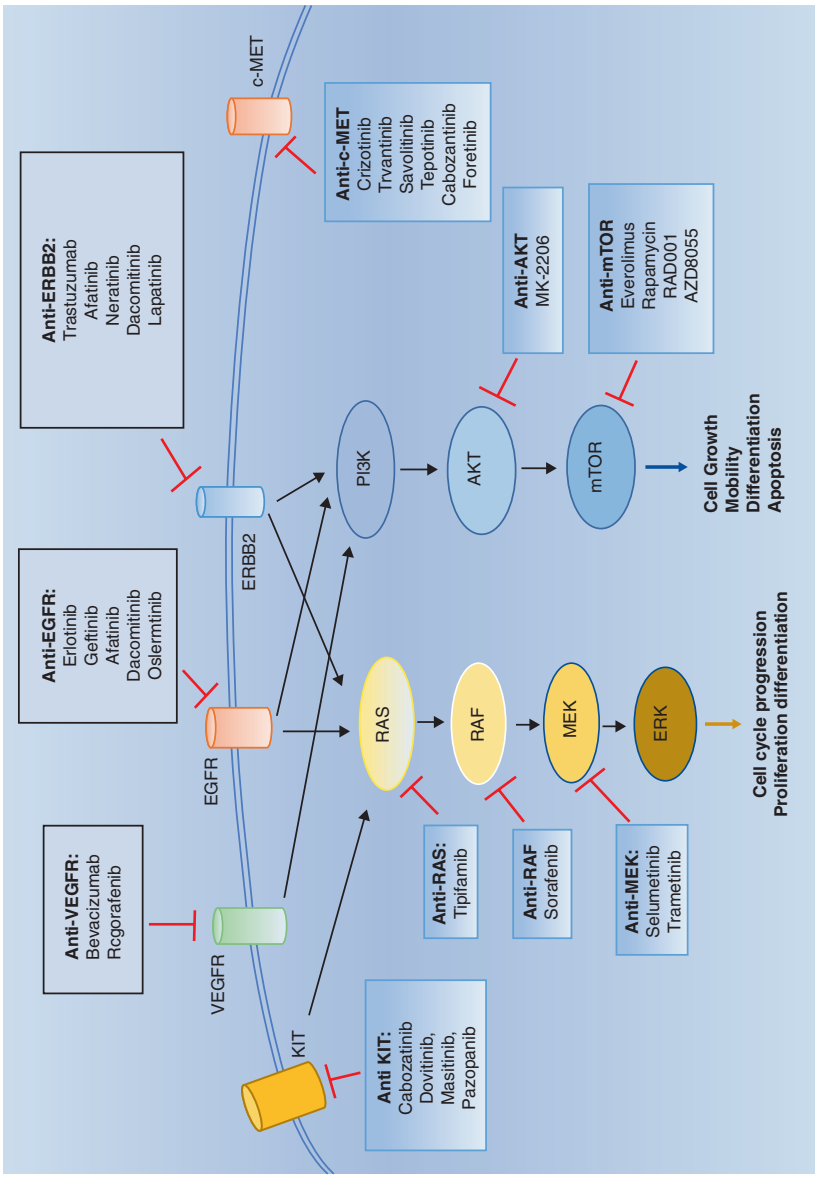

Fig. 14.3 Diagrammatic representation of potential genetic pathways involved in GBC pathogenesis along with available targeted agents and their site of action

14.4.1 Key Pathways Targeted for Therapy

14.4.1.1 HER-2/Neu

In the study by Iyer et al., trastuzumab as a monotherapy and in combination with gemcitabine demonstrated a stronger antitumor effect and greater cell apoptosis than gemcitabine treatment alone in GBC patients [47]. Wang et al. showed that gemcitabine/5-fluorouracil increased the expressions of total and phosphorylated forms of HER-2 in GBC cells, thus enhancing the cytotoxicity of trastuzumab, suggesting that sequential therapy with gemcitabine/5-fluorouracil followed by trastuzumab may perhaps be a promising therapeutic strategy [48]. Inagaki et al. reported decrease in the size of tumor emboli and hepatic lesions in GBC case harboring HER-2 mutation on the primary and metastatic site, after two cycles of treatment with anti-HER-2 lapatinib and capecitabine [49]. Among the various anti-HER-2/neu targeted agents being evaluated, most common are trastuzumab, dacomitinib, afatinib, and neratinib.

14.4.1.2 Epidermal Growth Factor Receptor

Development of drugs against EGFR basically involve humanized monoclonal antibodies that target the EGFR extracellular domain and prevent EGFR activated dimerization, thus inhibiting the downstream signaling (cetuximab and panitumumab) and small molecules of TKIs (tyrosine kinase inhibitors) which bind to the ATP binding pockets on the intracellular catalytic kinase domain of RTKs, leading to the disruption of downstream signaling (gefitinib, erlotinib, and afatinib) [18]. Three generations of EGFR-TKI have been developed to combat against mutational activity of EGFR (Fig. 14.3). Erlotinib and gefitinib represent the first-generation having the capability to compete with ATP for ATP binding pockets in the intracellular tyrosine kinase domain of EGFR. Treatment with these agents in NSCLC is associated with point mutation (T790M) of EGFR resulting in emergence of drug resistance [50, 51]. The second-generation EGFR TKIs like dacomitinib and afatinib were developed to increase the potency of drugs by inhibiting ATP binding in irreversible fashion. Eventually, the third-generation TKIs, olmutinib and osimertinib, were developed which display strong inhibition of mutational activities of EGFR, even in patients showing T790M mutations [52].

There was no incremental benefit observed from the addition of cetuximab to GemOx (Gemcitabine-Oxaliplatin) in the randomized phase II BINGO study [53]; similar negative findings were observed with erlotinib or panitumumab with no clear correlation with EGFR overexpression or Kras mutation status [54]. Philip et al. reported a phase II study of erlotinib in 42 patients with advanced biliary cancer, in which 16 cases were GBCs. The overall confirmed response rate was 8% (3 patients; 95% CI 2–20) and the median TTP was 2.6 months (95% CI 2–4 months), while EGFR level was not associated significantly with clinical outcome [55]. In meta-analysis by Cai et al. to evaluate a combination therapy of EGFR-targeted drugs (erlotinib, cetuximab, or panitumumab) with GEMOX (gemcitabine and oxaliplatin) in 612 BTCs, combination of GEMOX and EGFR-targeted therapy demonstrated improved PFS (HR 0.80, 95% CI 0.66–0.94, $P = 0.03$) compared with GEMOX alone, although OS was not significantly different [56].

Contrasting results were shown by another meta-analysis of 12 studies, comprising 410 patients in combination chemotherapy arm and 514 patients using chemotherapy with anti-EGFR antibody (panitumumab), which did not reveal any significant survival advantage for the addition of anti-EGFR agents to combination chemotherapy, either in PFS (median PFS for the experimental arm of 7.6 months compared to 6.7 months for chemotherapy alone) or OS (12.6 months versus 11.6 months, respectively, between the two arms) [57].

14.4.1.3 Vascular Endothelial Growth Factor (VEGF)

VEGF expression has been correlated with increasing grade, hematogenous metastatic potential, and overall poor prognosis. A multicentric phase II study of VEGF antibody bevacizumab in combination with gemcitabine and oxaliplatin in advanced BTC with a single-arm trial demonstrated that response rate was 40% and median PFS was 7 months, and OS was 12.7 months [58]. A similar single-arm phase II trial of bevacizumab in combination with erlotinib but no traditional cytotoxic drugs in patients with unresectable BTC demonstrated a response rate of 18.4%, mOS of 9.9 months, and time to progression (TTP) of 4.4 months [59]. A phase II study suggested promising efficacy of regorafenib (VEGFR 1–3 inhibitor) in chemotherapy-refractory advanced/metastatic BTC, which demonstrated that mPFS was 15.6 weeks, mOS was 31.8 weeks, PR was 11%, and stable disease was 44% with a disease control rate of 56% [60].

However, prospective randomized phase II studies targeting VEGF have failed to show a benefit of adding sorafenib (an oral multi-tyrosine kinase inhibitor) to single-agent gemcitabine or addition of cediranib (an oral VEGFR-1, 2, and 3, PDGF, and c-Kit tyrosine kinase inhibitor) or multi-targeted kinase vandetanib (an oral inhibitor of VEGFR2, EGFR/HER1, and RET) to the cisplatin-gemcitabine combination [61–63]. The major stumbling block remains the absence of a reliable biomarker of efficacy for VEGF inhibitors.

14.4.1.4 Fibroblast Growth Factor Receptor

FGFR-targeted therapies undergoing clinical evaluation include multi-targeted tyrosine kinase inhibitors (TKIs) that also inhibit FGFR (such as ponatinib, nintedanib, dovitinib, and brivanib), as well as specific FGFR-directed small molecule TKI (e.g. BGJ398/infigratinib), FGFR antibodies, and FGFR trap molecules. The ongoing phase II trial with the oral FGFR inhibitor infigratinib (BGJ398) in advanced CC with FGFR aberrations (gene fusions, translocations, or other genetic alterations) showed an overall response rate of 14.8% (18.8% for FGFR2 fusions only) and the disease control rate was 95% with progression-free survival of 6 months [64].

14.4.1.5 MAPK (RAS/RAF/MEK/ERK) Pathway

RAS/RAF/MEK/ERK is the most common pathway in MAPK signaling by which a variety of cancerous cells promote cell proliferation, death, differential, cell cycle progression, apoptosis, survival, metastasis, metabolism, and angiogenesis [65]. Activated RAS starts a phosphorylation cascade, which involves RAF kinase, MEK1/2, and ERK1/2, and, ultimately, these affect cellular function. Inhibition of

the MEK/ERK signaling pathway lends itself as a therapeutic target for BTCs and other solid malignancies. A Japanese Phase IIa open-label trial investigated efficacy and safety of oral Trametinib 2 mg once daily dose (MEK inhibitor) in patients with advanced BTC refractory to gemcitabine-based therapy. Trametinib showed safer and more effective drug responses than single gemcitabine treatment. The reported results of Trametinib were 10.6 months of PFS (95% CI 4.6–12.1), 20% of 1-year OS, 65% of stable disease, and 35% of PD in 20 Japanese patients [66]. Selumetinib (AZD6244, ARRY-142886), a second generation of MEK1/2 drug, was developed for the selective and uncompetitive small-molecule inhibition of MEK1/2.A recent multi-institutional phase II study of selumetinib demonstrated acceptable drug tolerability in patients with metastatic BTC, with median PFS and median OS in selumetinib-treated cases being 3.7 months (95%CI 3.5–4.9 months) and 9.8 months (95% CI 5.97–not available), respectively [67].

14.4.1.6 PI3K-AKT-mTOR Pathway

Several inhibitors targeting the PI3K/AKT/mTOR pathway, including OSI-027, A66, Wortmannin, and LY294002, have been demonstrated to inhibit GBC cell proliferation, migration, and invasion both in vitro and in vivo [68]. OSI-027 blocked mTOR, enhancing the sensitivity of GBC cells to 5-fluorouracil [69]. MK-2206, a single-agent targeting AKT, exhibited acceptable tolerability in eight patients with advanced, refractory BC [70]. Copanlisib, a PI3K inhibitor, is under evaluation together with gemcitabine-cisplatin in advanced CCA (NCT02631590).

14.4.1.7 C-Mesenchymal Epithelial Transition Factor (MET)

MET is an oncogene encoding tyrosine kinase receptor of the hepatocyte growth factor (HGF). Once HGF binds to MET, the receptor undergoes dimerization and induces downstream signaling pathways, such as PI3K/AKT, RAS/RAF/MEK/ERK, and Wnt/ß-catenin signaling which regulate cell proliferation, metastasis, and drug resistance [71]. In GBC, MET overexpression ranged from 5% to 74% of patients, and was also associated with clinical poor outcome [72]. Three categories of MET inhibitors presently available are: small molecules targeting MET receptors (e.g., crizotinib, tivantinib, savolitinib, tepotinib, cabozantinib, and foretinib), monoclonal antibodies targeting MET receptor (e.g., onartuzumab), and antibodies against its ligand HGF (e.g., ficlatuzumab and rilotumumab) [73]. Studies are underway to evaluate the effectiveness of MET inhibitors in the treatment of patients with gallbladder cancer.

Overall, targeted therapies have failed to bring about significant improvement in survival outcomes in the treatment of unresectable and metastatic BTC in general and GBC in particular, both in first- and second-line settings, though future looks promising with many new targeted agents in development stage. Combination chemotherapy still remains the standard of care in advanced disease [74, 75].

14.5 Immunotherapy

BTC represents a potentially attractive subset for immune-based therapies because of its association with chronic inflammation. In a retrospective series of resected BTC, a low expression of cytotoxic T-lymphocyte antigen-4 (CTLA-4) in the peritumoral tissue was associated with a better survival compared to patients with a higher expression ($P = 0.02$). Therefore, CTLA-4 had a prognostic role, reflecting the capacity of the immune system to react against the tumor. BTCs with a high mutational load and with elevated expression of checkpoint molecules such as CTLA-4 and programed death ligand 1 (PD-L1) have been shown to have the worst prognosis [76].

Approaches to modulating the immune system include:

- Vaccination with putative tumor antigens either as peptides or loaded within dendritic cells to enhance recognition.
- Adoptive immunotherapy where patients' own T cells are expanded ex vivo and reinfused.
- Reversing tumor cell-induced immune suppression,

Vaccination against tumor-associated antigens is attractive and at least two tumor-related antigens have been identified with moderate to high expression in biliary cancers—Wilms tumor 1 (WT1) and mucin-1 (MUC-1) [77]. Trials of dendritic-based cell vaccine against both these antigens as well as a randomized trial of chemotherapy (Gemcitabine and Cisplatin) in combination with WT1 vaccine in patients with advanced biliary cancer have been described, with reportedly modest benefits [78]. ONYX-015 is a genetically modified adenovirus with a deletion of the E1B early gene and is therefore designed to replicate preferentially in p53-mutated cells. Makower et al. conducted a phase II clinical trial of intralesional administration of oncolytic adenovirus ONYX-015 (dl1520, CI 1042), which interrupts the p53 pathway, in 19 patients with advanced hepatobiliary tumors. Amongst these, 15 cases expressed p53 mutations, and 5 patients had GBCs. In this study, ten patients showed some response (prolonged disease stabilization) with no serious toxicities (>grade 2) observed [79].

Immune check point modulation is emerging as a promising therapeutic strategy to treat GBC. Upregulated expression of PD-L1 in ERBB2/3 mutant GBC cells leads to the inhibition of normal T-cell mediated cytotoxicity in vitro through the activation of PI3K/AKT signaling pathway [80]. ERBB2 and ERBB3 mutations have been reported in 16% and 12% cases of GBC respectively and their association with PDL1 expression may be a new targeted therapy in this subset of patients [81].

The subset of cancers with MMR system defects is very sensitive to programed cell death protein 1 (PD-1) blockade using checkpoint inhibitor agents like pembrolizumab. BTC patients with mutations in the DNA repair pathways can represent a subset where specific DNA repair inhibitors in addition to immunotherapy may be effective. PD-L1 expression in GBC (23%) was comparable to breast cancer (23%),

urothelial cancer (20%), and pulmonary squamous cell carcinoma (27%) [33]. PD-L1 in GBCs is a therapeutic marker for immune checkpoint blockade.

Interim results of the KEYNOTE-028 trial, a Phase Ib trial of pembrolizumab in advanced PD-L1-positive BTC progressing after a first-line therapy, showed a 34% ORR with 17% of partial response, 17% of stable disease, 52% of progressive disease, and a good profile of tolerability [82]. KEYNOTE-158 (NCT02628067; phase II) study also evaluated efficacy of pembrolizumab treatment in BTC patients and results showed that pembrolizumab provides durable antitumor activity, regardless of PD-L1 expression [83].

Results of another phase I study by Xie et al. evaluating tremelimumab [anti-CTLA-4 monoclonal antibody (mAb)] in refractory biliary cancer (NCT01853618) showed that PFS and OS were 3.4 months (95% CI 2.5–5.2) and 6.0 months (95% CI 3.8–8.8) demonstrating the potential usefulness of tremelimumab for patients with advanced BTC [84]. A phase I study (NCT01938612) evaluated durvalumab (D) (anti-PD-L1 mAb) with/without tremelimumab (T) in Asian GBC patients. Median OS was 8.1 (95% CI 5.6–10.1) months and 10.1 (95% CI 6.2–11.4) months for the single and dual treatment, respectively, suggesting that conjunction of anti-PD-L1/PD-1 with anti-CTLA-4 therapies may be effective for patients with GBC. Both D monotherapy and D + T combination therapy were tolerable for Asian patients with BTC, and no unexpected toxicities were observed with either regimen [85].

A phase I trial assessed safety and efficacy of ramucirumab (VEGFR2 antagonist) with pembrolizumab (PD-1 antagonist) in biomarker unselected patients of previously treated advanced or metastatic BTC. Ramucirumab–pembrolizumab combination showed limited clinical activity with infrequent grade 3–4 treatment-related adverse events (hypertension and neutropenia). The ORR was 4%, median PFS was 1.6 months, and median OS was 6.4 months [86]. The anti PD-1 nivolumab is currently being tested in a phase II, two-arm study with a randomization between the combination of nivolumab and gemcitabine-cisplatin versus dual immunotherapy with nivolumab and ipilimumab [87].

14.6 Future Perspectives

Tumor organoids are a novel three-dimensional (3D) cell culture technology that utilizes tumors grafts or single cells to culture into self-organized tissues. Therefore, it can maintain the histological features, expression profiles, and marker expression of the parental tissues. Tumor organoids can be used to study the tumor development, personalized treatment, drug screening, discovery of prognostic markers, and other aspects of tumors [88]. At present, there are few studies on the construction and application of gallbladder cancer organoids, which may be related to the low incidence of gallbladder cancer and obvious regional differences. Methods to address the low success rate of gallbladder cancer organoid construction (20%) compared with success rate of 76% for liver cancer, 80% for ovarian cancer, and 63% for pancreatic cancer will help overcoming the primary limitation to applying organoids to gallbladder cancer [89].

Recently, with the widespread use of this technique, organoid are being used in research to study the cellular metabolism, pathogenesis, chemotherapy resistance, and development of precision medicine therapy for GBC.

14.7 Conclusions

The ability to deliver successful optimal treatment for GBC is a major challenge, primarily because of delayed presentation in majority of patients. Though there has been significant progress in understanding the molecular pathways of carcinogenesis for various biliary tract cancers, including gallbladder carcinomas, development of effective targeted therapies to replace or add on to conventional chemotherapy is still a work in progress. It is highly recommended to exploit combination targeted therapies aiming at different key pathways underpinning cancer metastasis to yield synergistic efficacy with minimal toxicities.

However, clinical trials till date have not shown very favorable results of the new agents evaluated. There is hope that with improved knowledge and understanding of the molecular oncology of GBC, there is an unexploited potential to benefit patients suffering from this lethal disease.

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Targeted Therapy: Molecular Pathology and Targets of Gallbladder Cancer

15

Nilam Bhasker and Faraz Ahmad

15.1 Introduction

Gallbladder cancer is also renowned as orphan cancer that relates to infrequency in the western populations and has remarkable geographic dispersal; however, it is relatively uncommon in Northern America while it is more frequent malignancies in North India, Bangladesh, Pakistan, Japan, Chile, Korea, and Ecuador in contrast to other countries [1–3] which indicated that genetic variations promote to gallbladder cancer. Gallbladder cancer more frequently occurs in women in contrast to men. Despite gender and genetic variation, chronic inflammation condition is another risk factor for gallbladder cancer [4]. Globally it is the sixth most frequent cancer among general gastrointestinal tract cancer [5–7] and extremely devastating malignancy with inferior medical diagnosis. Incidence of gallbladder cancer is 2.5/100,000 individuals globally [8] and 5-year overall survivability is not more than 5% without treatment [9, 10]. Depressive outcomes have been reported with gallbladder cancer owing to the anatomic position, asymptomatic nature as well as obscurity. In spite of this, various other factors including age, genetic makeup, obesity, gallstone, gallbladder polyps, fatty liver disease, congenital biliary cysts, reproductive factors, primary sclerosis, cholangitis, *Helicobacter pylori* infection, typhoid, cigarette smoking, alcohol consumption, harmful diet, and environmental chemical exposure [10–12]. Hence, early prevention of carcinogenesis of gallbladder cancer has been imposed in the clinical applications because globally, it accounts for 1.2% of all neoplasm diagnosed [4]. Nowadays, radical resection is potential strategy for the management of gallbladder cancer. Unfortunately, only a small population falls into

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the operational course as most of the patients opt for non-surgical treatment. Non-surgical treatment mostly involved chemotherapy and radiotherapy. Cytotoxic chemotherapy remains the centerpiece treatment for gallbladder cancer while it has a moderate effect on longstanding survival [13]. Conversely, combined chemotherapy did not enhance the survival rate in contrast to single-agent chemotherapy [13]. However, over the last few decades several additional therapeutic modalities have been continuously developed. Rapid discoveries of the advanced technology, including RNA-sequencing (RNAseq), whole-exome sequencing (WES), next-generation sequencing (NGS), and single-cell isolation, as well as characterization opens the window to identify genetic and epigenetic hallmarks and specific molecules which may acts as potential therapeutic targets. Recently, one of the studies analyzed the epigenetic, genetic, and transcriptomic alterations in a large group of patients with gallbladder cancer [14]. Furthermore, this study suggested that targeting the immune microenvironment of tumor merely or cytotoxic drugs possibly may have better therapeutic efficacy for the management of gallbladder cancer, especially immunotherapy, vaccine therapy, biotherapy, specific molecular target treatment, and nanoparticles, which have been employed in the pre-clinical and clinical studies associated with gallbladder cancer. In this overview, we highlighted the targeted therapy treatments which received considerable attention in recent times to enhance the patient's quality of life, as well as overall survival rate of gallbladder cancer patients.

15.2 Histopathological Features of Gallbladder Cancer

Gallbladder wall is made up of three layers—mucosa, muscle layer, and serosa. There is no submucosal layer. Mucosa is made up of tall ciliated columnar layer and serosa is adherent to liver. Gallbladder carcinomas, epithelial in origin and it constitute 98% of all gallbladder malignancies while the remaining one is lymphomas, sarcomas, metastases, carcinoid, and other unusual malignancies. Approximately, 90% of gallbladder carcinomas are adenocarcinomas which are manifested by glands that are lined with columnar or cuboidal cells that may hold mucin. Depending on the level of gland formation, they may be poorly, moderately, or well differentiated. Multiple histologic variants of adenocarcinoma are explored: intestinal, papillary, signet-ring cell, clear cell, and mucinous [15]. Several tumors possess more than one variant. A meta-analysis study reported the frequency of most common histological types of gallbladder carcinoma (Table 15.1) [16]. The papillary adenocarcinoma contains branching fibrovascular stalks that are lined with columnar or atypical cuboidal cells. Papillary carcinomas serve to fill the gallbladder lumen prior to attacking the gallbladder wall. Hence, they are useful for better prognosis in contrast to other variants [17]. The portion of tumor involved in invading normally forms tubular structures instead of papillae. Metastatic deposits may contain both patterns. The intestinal-type adenocarcinoma mimics epithelium of the intestine and it is a well differentiated type of adenocarcinoma. The intestinal-type adenocarcinoma has two sub-types that are manifested by the presence of the intestinal glands: (a) one striped by the goblet cells and (b) mimicking the glands of colonic

Table 15.1 Different kinds of gallbladder cancer

Variant of tumor	Percentage (%)
Adenocarcinoma (NOS)	75.8
Carcinoma (NOS)	7.6
Papillary adenocarcinoma	5.8
Mucinous adenocarcinoma	4.8
Adenosquamous carcinoma	3.6
Squamous cell carcinoma	1.7
Small cell carcinoma (oat like)	0.5

NOS not otherwise specified; Henson et al. [17]

adenocarcinoma [18]. They may present in the same tumor and may also possess the foci of typical well-differentiated adenocarcinoma. Tumors which contain >50% extracellular mucin is known as mucinous adenocarcinomas [15] having two histologic variants: (1) contains huge ponds of extracellular mucin while having small clusters of malignant epithelial cells and (2) contains glands filled with mucin along with cystic dilatation. Foci of these two variants could be intermixing with traditional well-differentiated adenocarcinoma. Signet ring-cell carcinoma possesses cells with ample amounts of intra-cytoplasmic mucin which dislocates the nucleus toward the periphery. When the tumor is restricted to the invaginations or surface epithelium then it is considered as in situ carcinoma. When invasion of stromal takes place, the cells grow in nests, cords, and sheets and may construct the incomplete glandular structures inside mucoid stroma [18]. The main features of signet ring-cell tumors are infiltrative sub-mucosal growth that mimics the stomach *linitis plastica*. Clear cell adenocarcinoma associated with gallbladder is made up of sheets, cords, and trabeculae and nests with ample amount of clear cytoplasm [19]. Other epithelial cell variants existing in the gallbladder include squamous cell carcinoma, adenosquamous carcinoma, undifferentiated carcinoma, and small cell carcinoma. The adenosquamous carcinoma has a combination of squamous components and malignant glandular. The pure squamous cell carcinoma accounts for only 1% of all tumors of malignant gallbladder and comprises islands, sheets, and cords of malignant squamous cells set apart by fibrous stroma [18]. Although these tumors particularly occur in the areas of earlier squamous metaplasia, their histologic properties may differ from anaplastic to well-differentiated. Small cell carcinomas are an unfamiliar form of gallbladder carcinoma and extremely invasive tumors and histologically similar to small cell carcinomas of the gastrointestinal tract and lung. Gallbladder small cell carcinoma may have paraneoplastic syndromes [20]. Most of the gallbladder carcinomas (68%) possess diffusely infiltrating lesions while the rest one displays an intraluminal polypoid growth (32%) [21]. Most of the tumors (60%) associated with gallbladder originate from the fundus of gallbladder while 30% from the body, and 10% from the neck [18]. Submucosal expansion of infiltrating carcinomas emerges as diffuse or focal areas of nodularity, wall thickening, or induration in the wall of the gallbladder. In few cases in which direct invasion is involved, a thick neoplastic wall encloses the gallbladder when immediate widening to the liver has taken place [18]. The papillary adenocarcinomas display intraluminal polypoid growth and sessile in nature [18].

15.3 Molecular Pathogenesis of Gallbladder Cancer

Recent hypotheses suggested that gallbladder cancer emerges from pre-malignant epithelial dysplasia which continuously progress into non-atypical hyperplasia and carcinoma, and finally transforms into invasive carcinoma. Most of the gallbladder cancer cases originate in the setting of gallstone-related cholecystitis and cholelithiasis [22, 23]. However, specific molecular mechanisms behind this are still unknown; it is assumed that chronic cholelithiasis promotes the continual irritation resulting in neoplastic changes in the gallbladder mucosa [24, 25]. In the nineteenth century, Virchow described the association between irritation, inflammation, and cancer. Diverse active molecules including TNF (tumor necrosis factor), cytokines, and pro-angiogenic molecules are secreted by inflammatory cells that may stimulate the differentiation, proliferation, migration, and survival of cells in the neighboring tissues resulting in pre-disposition of the biliary mucosa to tumorigenesis [26]. Upregulation of EGF (epidermal growth factor) and mutation of *ras* (oncogenes) are observed during the transformation from pre-malignant tumor to conspicuous gallbladder cancer. Abnormal expression of *cyclin E* (cell cycle regulator), *p53* (tumor suppressor gene), and anti-apoptotic protein Bcl-2 are involved during emergence of invasive gallbladder cancer. Dysfunction of p53 gene plays an important and early role in the development of gallbladder cancer which is associated with chronic inflammation and gallstones [27–30]. Recent progress is emerging to explore the carcinogenic events which play a key role during the development of gallbladder cancer. Bile acids may be involved in stimulating inflammation and tumorigenesis in the gallbladder by inducing multiple kinase signaling pathways, especially by stimulating the EGFR (epidermal growth factor receptor). A recent study showed that bile acids activate the EGFR resulting in induction of COX-2 (cyclooxygenase-2) expression through MAPK cascade. Bile acids increase the expression of COX-2 in the cholangiocarcinoma cell lines, and both EGFR and Src-kinase inhibits the activity of molecules that are involved in deactivation of COX-2. Unraveling the association between EGFR/MAPK activation and COX-2 induction by bile acids may be helpful to treat gallbladder cancer [31–33]. TP53 and Kras are well known genes involved in gallbladder cancer. Carcinogenic pathways for gallbladder may include (1) inflammation due to gallstones that promotes p53 mutations and eventual carcinoma, (2) point mutation of Kras leading to hyperplasia, and (3) presence of neoplastic foci in gallbladder polyps secondary to Kras mutation [26]. Epigenetic also plays a well-defined role in gallbladder carcinogenesis. Pattern of methylation of the tumor suppressor genes like p16, APC (anaphase promoting complex), p73, MGMT (O6-alkylguanine DNA alkyl transferase), RARβ2 (retinoic acid receptor- β2), and hMLH1 (human MutL homolog 1) have been observed in 28% of chronic cholecystitis and 72% of gallbladder cancer which is not found in the healthy tissue [34, 35]. A significant difference has been observed in the methylation pattern of p73 (40% versus 14%) and APC (13% versus 42%) in the gallbladder patients of the USA and Chile which indicated toward a peculiar geographic-dependent biology. Methylation pattern associated with gallbladder cancer accumulates during the progression of chronic cholecystitis to metaplasia

[34]. The involvement of microsatellite instability (MSI) in the development of gallbladder cancer still remains poorly explained, the rate of MSI ranged between 0% and 40% of cases. One of the studies reported the 7.8% of incidence of MSI in case of gallbladder cancer. A robust correlation has been reported between global DNA methylation pattern and depletion of mismatch repair proteins which indicated that methylation promotes silencing of these genes [34]. MSI is more prevalent in patients with developing gallbladder cancer and it is also linked with Lynch syndrome while there is no significant difference has been observed in tumor stage, tumor grade, and overall survival in the presence or absence of MSI. Connection of loss of heterozygosity (LOH) has been observed in the multiple tumor suppressor genes in gallbladder cancer including chromosomes 3p (FHIT, RAR-β, VHL, and RASSF1A), 9p21 (p15, p16), 1p34–36 (p73), 16q24 (FRA16D and WWOX) 9q (DBCCR1), 13q14 (RB), 8p21–23 (FEZ1 and PRLTS), 5q21 (APC), and 17p13 (p53) [36]. Enhanced expression of ADAM-17 has been observed in patients of gallbladder cancer, especially in high-grade tumors [37, 38]. ADAM-17 gene has been found to be intricate in the modulation of cell migration and extracellular matrix (ECM) remodeling. ADAM17 cleaves TNF-α from its precursor and also releases the heparin-binding epidermal growth factor (HB-EGF), amphiregulin, and ligands of EGFR (epidermal growth factor receptor). HMGA2 (High mobility group protein-A2) is a non-histone protein that plays a key role in tumorigenesis and metastasis of tumors. One of the previous studies reported the high expression of HMGA2 in patients of gallbladder cancer in contrast to healthy tissue, chronic cholecystitis, and polyps. This study also reported the reduced expression of CD9 in cancers in contrast to benign tissues [39]. Mobility related protein-1 (MRP1 also known as CD9) is a glycoprotein and included in the trans-membrane-IV super family and it is involved in the tumor progression. Consequently, the pathogenesis of gallbladder cancer is still not well defined. Unraveling the mechanisms associated with immune signaling system may be helpful in the treatment of gallbladder cancer.

15.4 Molecular Intended Therapy of Gallbladder Cancer

It is a class of cancer treatment which utilizes drugs or other molecules to recognize and attack the specific kinds of cancer cells. It can be used merely or in combination with other treatments like radiotherapy, chemotherapy or surgery. Targeted therapy for tumor treatment originated in 1988 on the basis of specific chemicals which were capable of eradicating some microorganisms [40]. Subsequently, the potency of targeted therapy has been massively explored in different kinds of cancers to block the activity of specific molecular targets which are closely associated with proliferation, differentiation, migration, vascular angiogenesis, cancer stemness of tumor cell, and antitumor immune responses [41, 42]. Numerous drugs have been developed for targeted therapy which consists primarily of immunized antibodies and small molecules growth (Table 15.2). The small molecules with <900 Da molecular weight can be easily entered into the cells to block activity of specific proteins or enzymes resulting in inhibition of tumor cell growth [43] while

Table 15.2 Molecular targets for gallbladder cancer

Drug	Molecular target	Variants of cancer	Clinical phase
Sorafenib	Multi-targeted TKI	Gallbladder cancer, biliary tract cancer	II
Erlotinib	EGFR	Solid tumors	I
Bevacizumab	EGFR, VEGFR	Upper gastrointestinal cancers, biliary tract cancer	II
Apatinib	EGFR, HER2	Gallbladder cancer	II
Afatinib	EGFR, HER2	Gallbladder cancer	II
Lapatinib	HER2	Biliary tract cancer	II
Trastuzumab	HER2	Gallbladder cancer/solid tumors	II/I
Ramucirumab	VEGFR2	Biliary tract cancer	II
Cediranib	VEGFR	Biliary tract cancer	II
Merestinib	c-MET	Biliary tract cancer	II
Panitumumab	Kras, BRAF	Biliary tract cancer	II
Trametinib	MEK	Gallbladder cancer or biliary tract cancer	II
Selumetinib	MEK	Biliary tract cancer	II
Everolimus	mTOR	Solid tumors	I
Selumetinib	AKT	Biliary tract cancer	II
Merestinib	MET	Solid tumors	I
Ceralasertib	PARP	Solid tumors	II
Intrafusp alfa	TGF-β	Biliary tract cancer	II and III

antibodies precisely bind to receptors of cell membrane or their ligands to control apoptosis or cell proliferation [44]. Few drugs were designed to target extracellular molecules which are involved in angiogenesis or immune reaction in the microenvironment of tumor culminating with suppression of metastasis, angiogenesis, and tumor [45, 46]. The signaling cascade molecules and receptor are targeted as therapeutic approach to manage the gallbladder cancer (Fig. 15.1):

15.4.1 Herceptin (HER)-2

It is a promising target to cure the gallbladder cancer and mutation in the HER2 was observed in multiple cancers. Mutation in HER2 protein is the commonest mutation observed in gallbladder cancer with 0.9%–4.7% frequency [4]. Overexpression of HER2 gene due to mutations has been observed in multiple cancers including such as stomach cancer, colon cancer, lung cancer, breast cancer, and bile tract cancer [4, 47, 48]. A study performed on transgenic mice demonstrated that upregulation of HER2 in the basal cell layer of biliary tract leads to the establishment of gallbladder adenocarcinoma [49]. A study led by Ah-Rong showed that SNU-2773 and HER2+ SNU-2670 gallbladder cancer cell lines are more sensitive to afatinib, dacomitinib, and trastuzumab in contrast to HER2 biliary tract cancer cell lines [50]. This study also demonstrated that trastuzumab and its combination with gemcitabine elicit strong antitumor effect and increased cell apoptosis in contrast to gemcitabine

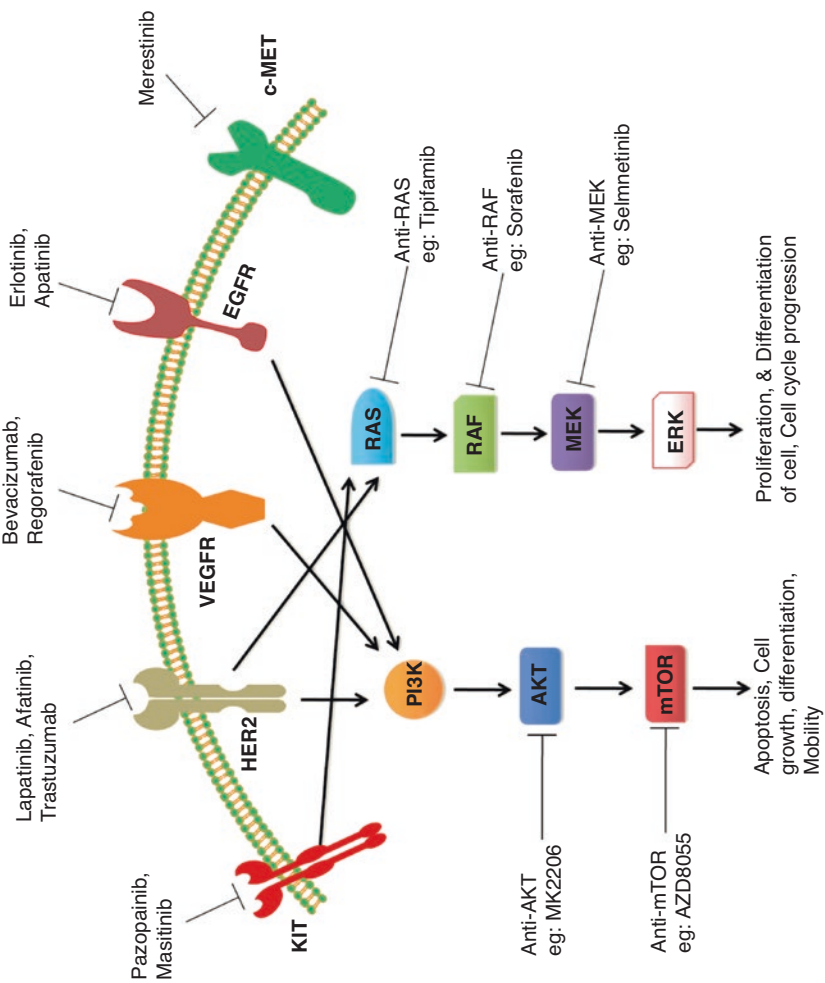

Fig. 15.1 Molecular targets of gallbladder cancer

treatment [50]. A recent study showed that EGFR-specific shRNA, ErbB2-specific short hairpin RNA (shRNA), or afatinib are able to inhibit the invasiveness of gallbladder cancer cells [51]. Interestingly, a study led by Wang et al. [52] demonstrated that gemcitabine/5-fluorouracil enhanced the expressions of phosphorylated forms of HER2 in gallbladder cells, hence increasing the cytotoxicity of trastuzumab. Another study also reported the HER2 targeted drugs (lapatinib and capecitabin) to cure the gallbladder cancer [53]. The above clinical data pinpointing that targeted HER2 therapeutic approach may acts as a promising treatment strategy for advanced gallbladder cancer with somatic mutations of HER2.

15.4.2 Vascular Endothelial Growth Factor (VEGF)/VEGF Receptor-(R)

VEGF/VEGFR, another molecular target for therapy against gallbladder cancer [4]. A growing body of evidence disclosed that the VEGF-VEGFR axis linked molecules is involved in the improvement of gallbladder cancer. Increased serum level of VEGF-A was observed in the patients of gallbladder cancer [54] and stimulates the angiogenesis, invasion, and cell proliferation while it inhibits the apoptosis in gallbladder cancer cells. A phase-II multi-centric study performed on a combination of VEGF-A antibody (bevacizumab) with oxaliplatin and gemcitabine in advanced biliary tract cancer showed that the response rate was increased up to 40% while overall survival was 12.7 months and mPFS was 7 months [55]. Similarly, a single-arm phase-II trial of bevacizumab with erlotinib conducted on un-resectable biliary tract cancer patients reported the 18.4% response rate, while mOS (median overall survival) was 9.9 months, and TTP (time to progression) was 4.4 months [56]. In addition to this, phase-II study performed at multicenter showed that combination of bevacizumab with gemcitabine and cisplatin in advanced biliary tract cancers enhanced the PR (partial response) up to 24% while median PFS (mPFS) was 8.1 months and mOS was 10.2 months [57]. Another phase-II study showed a promising benefit of regorafenib (VEGFR1–3 inhibitor) and reported that mOS was 31.8 weeks, PR was 11%, mPFS was 15.6 weeks, and stable disease was 44% with respect to diseased controls (56%) in chemotherapy-refractory metastatic or advanced biliary tract cancer. A few studies also reported the failure events like Sorafenib (a multi-kinase inhibitor of VEGFR2/3) manifest only minimal level of efficacy in biliary tract cancer in a non-randomized phase-II clinical study with an overall response rate (ORR) (2%), PFS of 2.3 months (range from 0 to 12 months), rate of stable disease at 12 weeks (32.6%), and a mOS of 4.4 months (range: 0–22 months) [58]. Similarly, phase-II study carried out at multicenter in different countries suggested that sunitinib (various RTKs inhibitors including VEGFR) monotherapy exhibited marginal efficacy in patients with metastatic biliary tract cancer. This study reported the median TTP which was 1.7 months, the rate of disease control was 50.0% and the ORR was 8.9% [59]. Vandetanib (antagonist of VEGFR-2) monotherapy or in combinations with chemotherapy did not provide

better results in PFS in biliary tract cancer especially in a phase-II clinical trial [60]. Additionally, a phase-I clinical trial of ramucirumab (a humanized monoclonal VEGFR2 IgG antibody) demonstrated that ORR was 4%, and mPFS was 1.6 months and mOS was 6.4 months in advanced biliary tract cancer [61]. Discrepancies among these individual clinical trials on angiogenic blockade indicated that mechanism behind this must be decoded. Recently the combination system with angiogenic-targeted antibodies and additional chemo-therapeutic agents provide the suitable treatment against gallbladder cancer.

15.4.3 EGFR (Epidermal Growth Factor Receptor)

Scientific community also targeted the EGFR as molecular target in case of gallbladder cancer and involved in the development of cancer [4]. Developments of drugs against EGFR basically involve humanized monoclonal antibodies that target the EGFR extracellular domain and small molecules of TKIs (tyrosine kinase inhibitors). Conventional antibodies are panitumumab and cetuximab which are capable of inhibiting the dimerization of EGFR resulting in deactivation of downstream signaling [62]. TKIs comprise erlotinib, gefitinib, and afatinib which have capability to interact with the ATP binding pockets reside in the intracellular catalytic kinase domain of RTKs which leads to inhibition of downstream signaling [63]. Up to the recent times, three generations drugs associated with EGFR-TKI have been developed to combat against mutational activity of EGFR [64, 65] in which Erlotinib and gefitinib represents the first-generation having the capability to compete with ATP for ATP binding pockets in the intracellular tyrosine kinase domain of EGFR. Emergence of point mutation (T790M) was observed during the treatment of non-small cell lung cancer patients with the first-generation EGFR TKIs which enhance the drug resistance capability of cancer cells [66, 67]. The second-generation EGFR TKIs like dacomitinib and afatinib were developed to increase the potency of drugs, which inhibit the ATP binding in irreversible fashion [68]. In some of the trials, afatinib failed to inhibit activity of T790M mutant associated with EGFR [69]. Eventually, the third-generation TKI solmutinib and osimertinib were developed to increase the effectiveness of the drug [65]. The third-generation drugs display strong inhibition of mutational activities of EGFR which were attained in 50%–60% of patients having T790M mutations [70] and approved as the second line treatment drugs inpatients who withstand the first-generation drugs. Overactivation of EGFR in gallbladder cancer was found between 44% and 77% of patients in several independent clinical studies [71, 72]. Enhanced activation of EGFR in gallbladder cancer tissues was firmly correlated with inferior diagnosis of the patients [73]. A recent study also reported that frequency of somatic mutations in EGFR gene were found to be low (range: 2.5–3.9%) in case of gallbladder cancer [4]. Several therapeutic trials that target the EGFR in patients with gallbladder cancer have been achieved, but final results were differing. A study performed by Mody et al. [74] reported the emergence of metastatic gallbladder cancer case particularly

in patients who received treatment of gemcitabine (1000 mg/m^2) on day 1 and 8 and in every 21 days in combination with erlotinib (100 mg daily). The disease persisted for 18 months without progression post 12 cycles of combination therapy followed by treatment with erlotinib for 6 months for maintenance which suggested the strong therapeutic response of EGFR-TKI on gallbladder cancer. A phase-II study conducted by Philip et al. [75] in 42 patients with advanced biliary tract cancer reported the development of gallbladder cancer in 16 patients after the treatment with erlotinib. This study reported the ORR was 8% and the mTTP was 2.6 months while level EGFR was not found to significantly associate with clinical outcome. A phase-II study carried out at multicenter published the outcomes by testing the combination of erlotinib with bevacizumab in 53 patients with un-resectable biliary tract cancer. 12% of patients had a partial response while TTP was 4.4 months and mOS was 9.9 months. One of the studies reported the negative outcomes in the phase-II Southwest Oncology Group (SWOG) study on erlotinib and sorafenib and erlotinib in 14 advanced gallbladder cancer patients and 20 cholangiocarcinoma patients [76]. A meta-analysis study conducted by Cai et al. [77] to measure a combination therapy of EGFR-targeted drugs (cetuximab erlotinib, panitumumab) with GEMOX (oxaliplatin and gemcitabine) in 612 biliary tract cancers. Improved PFS has been reported after treatment with a combination of EGFR-targeted drugs and GEMOX in contrast to GEMOX alone. Numerous clinical settings with combined procedures targeting EGFR are vital to provide a better treatment for clinical practice.

15.4.4 MAPK/ERK (Also Called as RAS/RAF/MEK/ERK) Pathway

It is involved in various cellular activities and deactivation of this signaling has been reported in various cancers including gallbladder cancer, hence it is also considered as molecular target for gallbladder cancer therapeutic approach [4]. Altered MAPK pathway has been reported in 40% of all cancers in humans including alteration in the gene of RAS (~30%) and BRAF (~10%) [78]. In case of gallbladder cancer, point mutations in KRAS were found between 0% and 41% while amplification of the BRAF gene was observed in 5% patients [79]. Polymorphism in codon 25 of KRAS gene (Gln25His) was found to be associated with the pathogenesis of gallbladder cancer [80]. This polymorphism was intimately found to be associated with risk and diagnosis of cancers in 541 gallbladder cancer patients and 307 normal healthy controls in Indians [81]. Trametinib (MEK1/2 inhibitor) hampers gallbladder cancer cells migration, proliferation, and invasion in a time and dose dependent fashion and induces the apoptosis in gallbladder cancer in animal and cellular model [82]. A study led by Horiuchi et al. [83] showed that U0126 (MEK inhibitor) prevents the invasion of liver tumors and enhances survival of nude mice that possess human gallbladder cancer cells [84]. In addition to this, few traditional Chinese medicines like pachymic acid, bufalin, and artemisinin had the capability to deactivate the proliferation of gallbladder cancer cells and prevent the invasion by impairing the MEK/ERK signaling [85, 86]. Progression of gallbladder cancer was also impaired by MiR-663a which halt MAPK/ERK pathway through impaired

regulation of EMP3 [87]. By contrast, miR-101, SLC25A22, and lncRNA MALAT1 activate the proliferation of tumor cells of gallbladder by initiating the MAPK/ERK pathway resulting in metastasis [88, 89]. A study carried out by Giannini et al. [90] demonstrated that patients who received inhibitors of BRAF/MEK for secondary gallbladder cancer yielded stronger therapeutic potency with 6 months PFS in two cases. Another study performed by Yu et al. [91] reported that combination therapy with MEK and BRAF inhibitors after surgical excision shows improvement in advanced gallbladder cancer and evidence of metastasis has been not observed with OS for 26 months and PFS for 14 months post 8 months of treatment. One of the phase-II study of GEMOX and its combination with cetuximab (EGFR inhibitor) showed that mutation in KRAS did not influence the difference in PFS and ORR between GEMOX and GEMOX with EGFR inhibitor in gallbladder cancer [92] that suggest that the addition of cetuximab to gemcitabine, as well as oxaliplatin did not appear to increase the activity of chemotherapy in gallbladder patients. A phase-II study base on trametinib (GSK1120212, JTP-74057) which is the first-generation inhibitor of MEK1/2 that was validated by FDA (Food and Drug Administration) [93] and suggested that it is safer and more promising drug than treatment with single gemcitabine. In phase-II study of trametinib, PFS was 10.6 months, OS was 20.0% with 1 year, 65% of stable disease, and PD was 35% in 20 Japanese patients having advanced biliary tract cancer refractory to therapy based on gemcitabine (NCT01943864) [94]. Similarly, a SWOG S1310 study which admitted 44 patients (32% patients with gallbladder cancer) for trametinib treatment. This study reported the ORR which was 10% vs. 8% observed in fluoropyrimidine therapy while the mPFS was 3.3 months in trametinib therapy in comparison to fluoropyrimidine therapy in which mPFS was 1.4 months [95]. AZD6244 (ARRY-142886) is a second-generation drug that selectively inhibits the MEK1/2 [96]. A phase-II study carried out multi-institute with selumetinib (NCT00553332) showed that the drug response is very effective in metastatic biliary tract cancer patients and mOS and mPFS were 9.8 months and 3.7 months, respectively [97].

15.4.5 PI3K/AKT/mTOR Pathway

It is involved in multiple physiological cellular processes including mobility, cell growth, metabolic activity, differentiation, and programmed cell death and may be potential molecular target for gallbladder cancer [4]. Several lines of oncogenic evidence proved that PI3K/AKT/mTOR pathway is one of the important signaling pathways which are notably upregulated in gallbladder cancer [4]. PI3K/AKT/mTOR pathway primarily triggers the initiation as well as progression of gallbladder cancer. A study carried out by Lunardi et al. [98] observed that 90% of heterozygous mice (+/−) of PTEN mice developed gallbladder cancer due to higher level of phosphorylated AKT suggesting that PI3K/AKT signaling involve in the conversion of gallbladder epithelial cells into metastatic form. Gallbladder cancer with PI3KCA mutations were distinctly reported in individual studies carried out globally [4]. PIK3CA with E545K mutation was observed in ~5.9% of gallbladder

cancer patients, which exhibited a dreadful prognosis [4, 8]. Epigenetic alteration of PTEN also promotes the development of gallbladder cancer as approximately 30% of gallbladder cancer patients display hypermethylated PTEN promoter [99]. A study conducted by Jin et al. [100] showed that miR-143-3p impedes the PI3K/AKT pathway by targeting ITGA6 resulting in suppression of angiogenesis and growth of gallbladder cancer. Numerous inhibitors that target the PI3K/AKT/mTOR including LY294002, Wortmannin, and A66 have been reported which is involved in inhibition of invasion, migration, and proliferation of gallbladder cancer cells in both animal and cellular model [101]. In addition to this, AZD8055, RAD001, and rapamycin were also able to deactivate the mTOR resulting in inhibition of migration and growth of gallbladder cancer cells in vitro condition [102]. A study performed on a transgenic mouse model showed that rapamycin impedes the incidence of gallbladder cancer [103]. OSI-027 occludes the mTOR resulting in increased sensitivity of gallbladder cancer cells to 5- fluorouracil [104]. Some traditional Chinese drugs such as dioscin, liensinine, and bufalin are able to hamper proliferation of gallbladder cancer cells and stimulate cell apoptosis by targeting the PI3K/AKT pathway [4]. A phase-I trial study (NCT00949949) was carried out to decide the MTD (maximum tolerated dose) of everolimus (mTOR inhibitor-5 mg) together with either gemcitabine plus cisplatin (12.5 mg/m^2, Cohort II) or gemcitabine (800 mg/m^2, Cohort I) in cancers. Other clinical settings that include ten patients with gallbladder cancer or cholangiocarcinoma were recruited for the Cohort III study treated with three drugs. They reported that six patients were in stable condition while four patients emerged with progressive stage demonstrating that the combination of three drugs may provide better comfort to gallbladder cancer [105]. Furthermore, numerous mechanistic studies are needed to prove the potency of these drugs for the treatment of gallbladder cancer.

15.4.6 DNA Damage Repair (DDR) Pathway

It can repair or eliminate the damaged cells to defend the host against carcinogenesis [4]. There are four principal DDR pathways including nucleotide excision repair (NER), base excision repair, mismatch repair (MMR), and double strand break repair [4]. A study performed by Javle et al. [106] reported the 7.8% ATM or BRCA2 mutations in 623 patients with advanced gallbladder cancer. A study carried out by BaekYeolRyoo et al. [107] showed that mutation of DDR gene was detected in 62.5% patients of biliary tract cancer (including 20.2% gallbladder cancer patients) and mutation of DDR gene has been found to associated with greater OS (21.0 vs. 13.3 months) and mPFS (6.9 vs. 5.7 months) in biliary tract cancer that were managed with first line chemotherapy based on platinum for metastatic or unresectable. The results of above clinical trials suggest that this may act as predictive markers for the response against chemotherapy based on platinum biliary tract cancer patients. Still, there is no confirming clinical trial evidence for the potency of inhibitors of DDR in case of gallbladder cancer patients. In recent times, a clinical trial (NCT03878095) focused on targeting the PARP is still ongoing.

15.4.7 TP53

It is a crucial tumor suppressor gene and mutations associated with gallbladder are usually observed in 50% of all cancers. It is well known to be involved in the cellular process like programmed cell death, DNA damage response, and induction of cell cycle arrest [4]. Apoptosis was diminished by pre-treatment with pifithrin-a (inhibitor of p53) [108]. The mutations of TP53 were found to lower in Greek patients with gallbladder cancer in contrast to Chile and Japanese gallbladder cancer patients [109]. About 1/third population of North India have mutations in exons 5–8 of TP53 [110]. However, numerous TP53 mutations in case of gallbladder cancer were elucidated in Hungary, Chile, and Japan [4]. Higher mutation frequency and overactivation of p53 protein that has tumor-promoting signature were found to correlate with an inferior survival in gallbladder cancer [111] and hence it can act as a cancer biomarker. A recent study based on mutagenesis of gallbladder cancer reported that TP53 was the most common among ablated genes which are responsible for development of the gallbladder cancer [4, 8]. Additionally, a study conducted by Singh et al. [112] showed that p53 can acts as an independent prognostic factor for the imperfect prognosis of gallbladder cancer. Further studies are needed for the assessment of p53 for predictive prognosis biomarker in case of gallbladder cancer.

15.4.8 C-Mesenchymal Epithelial Transition Factor (MET)

MET, an oncogene encoding receptor of tyrosine kinase of the HGF (hepatocyte growth factor). Dimerization of receptor occurs when HGF interacts with MET resulting in induction of downstream signaling pathways like Wnt/β-catenin signaling, RAS/RAF/MEK/ERK, and PI3K/AKT culminating with regulation of metastasis, proliferation of cell, and drug resistance [4]. Enhanced level of MET was found to link with inferior prognosis in cervical cancer, breast cancer, hepatic cancer, gastric cancer, pancreatic cancer, colorectal cancer, and NSCLC [4]. In case of gallbladder cancer, overexpression of MET has been found from 5% to 74% of patients as well as having poor clinical outcome [113, 114]. In addition to this, NK4 (HGF inhibitor) impeded the growth of tumor growth as well as invasion of gallbladder cancer in the study that are performed on animal models [115, 116]. Another study led by Kim et al. [117] showed the unfamiliar results which had no correlation between MET expression and inferior prognosis. In recent time, only three categories of MET inhibitors are available for clinical practice: antibodies for ligand HGF (erilotumumab and ficlatuzumab), small molecules that targets MET receptors (foretinib, cabozantinib, savolitinib, tivantinib, tepotinib, and crizotinib), mAbs for MET receptor (onartuzumab) [118, 119]. A clinical study (NCT03027284) is trying to assess the potency of MET inhibitors for the management of gallbladder cancer.

15.4.9 Cyclin Dependent Kinase Inhibitor (CDKN)-2A/B

It impedes CDK6 and CDK4 as well as prevents the phosphorylation of pRB (retinoblastoma) resulting in cell cycle arrest particularly at the junction of G1/S phase [120, 121]. A genomic profiling with NGS showed that CDKN2A/B gene is the most common mutated gene which is frequently observed in 107 US and 108 Chinese patients with gallbladder cancer [4]. The mutation rate was 26% in Chinese patients which is similar to 25% observed in US populations [122]. Stronger correlation between ERBB2 genetic mutations with CDKN2A/B variations has been reported in US patients (odds ratio 10.8, $P = 0.0001$) compared with in Chinese cohort (odds ratio 5.4, $P = 0.0014$), suggesting that somatic genetic alterations in CDKN2A/B were significantly found to associate with other distant metastases [123]. One of the studies reported that the mutation rate of CDKN2A/B was ~5.9% in case of gallbladder cancer [8]. Several other reports also support this notion that mutation of CDKN2A/B induces the gallbladder cancer pathogenesis [4, 124]. All the above clinical data suggest that it can act as a potential target for gallbladder cancer therapy. Another study led by Leiting et al. [125] observed that CDKN2A was not found to be associated with the survival of biliary tract cancer. However, there are no potential CDKN2A/B drugs candidates for the treatment of cancer.

15.4.10 KIT (A Protoncogene)

It belongs to type-III transmembrane receptor tyrosine kinase expressed in numerous human cells [126]. The descending signaling of KIT primarily involves JAK/STAT, PI3K/AKT, and MAPK, pathways and it is involved in the modulation of survival, growth, migration, differentiation, and cell proliferation [127, 128]. One of the studies showed that expression of KIT gallstones patients was found to lower in gallbladder tissue in contrast to healthy subjects [129]. Moreover, KIT expression was found to enhance in case of gallbladder cancer [130]. Approximately 16 inhibitors of KIT are available to block its enhanced activity in numerous cancers including prostate cancer, leukemia, renal carcinoma, and gastrointestinal stromal tumor [131, 132]. Prototypical blocking agents are used to target the KIT including dovitinib, cabozantinib, pazopanib, and masitinib [133, 134]. Only two clinical trials (NCT01153750, NCT02115542) have been underway in recent times.

15.5 Future Perspective

Gallbladder cancer is an uncommon cancer belonging to a high-grade malignant tumor. Numerous efforts have been made for the better development of novel therapeutic agents including vaccines and targeted drugs. Advanced technologies such as next-generation sequencing, genomic profiling, proteomics, and transcriptomics are paving the way for development of specific molecules that selectively deactivate the abnormal signaling cascade in gallbladder cancer. These technologies are also

helpful in the assessment of drug action mechanisms, genetic signature of tumor cells and therapeutic response of targeted molecules. Combination therapies are employed to increase the efficacy of drugs against tumor cells. Numerous unpredicted hurdles occur during the treatment like non-target toxicity of drugs. Unraveling the molecular mechanism associated with tumor microenvironment may open the window for identification of specific molecules for the development of antitumor drugs against gallbladder cancer. Furthermore, multiple drug therapies targeting specific alteration in gallbladder cancer cells together with tumor microenvironment will be emerging targeted therapy to treat the gallbladder cancer.

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Targeted Therapies in Gallbladder Cancer: Current Status and Future Perspectives

16

Manjusha Dixit, Vinay J, and Soham Choudhury

16.1 Introduction

Gallbladder cancer is the common cancer of the biliary tract and ranks sixth in global gastrointestinal cancer burden [1]. The incidence of gallbladder cancer is specific to certain geographical regions and ethnicity. High-risk regions include Chile, India, Pakistan, China, and Japan [1]. The incidence of gallbladder cancer among American Indians, Alaskan native people, Eastern European people, and North Indians is relatively higher compared to other races [2].

Due to the poor prognosis and late clinical intervention, the overall survival rate in gallbladder cancer is very low [3] which compels the research community to look for new therapeutic targets to combat gallbladder cancer. Current therapies such as complete removal of gallbladder (cholecystectomy), radical gallbladder resection, palliative surgeries, radiation, and chemotherapies are available, but the risk of its recurrence and remission remains high [4]. To overcome the drug resistance, toxic side effects and to improve treatment efficacy of conventional therapeutic agents, there is a need for the development of specific inhibitors and employing combination therapies. Also, some microRNA, long noncoding RNA (lncRNAs) have already been reported as a therapeutic target in gallbladder cancer. In order to improve the efficiency of current chemotherapeutic agents, researchers are focusing on small molecule inhibitors against key signaling molecules (TP53, KRAS, VEGF, Hif1α, etc.) and pathways (PI3K/AKT/MTOR, MAPK/ERK, HER2/EGFR, and Hedgehog) that are responsible for gallbladder cancer progression.

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In this chapter, we are particularly focusing on advancement in the research that led to the discovery of potential targets which might be bedrock for the development of novel therapeutic strategies with reduced side effects and improved efficacy in the treatment of gallbladder cancer. The chapter has two sections in which we have discussed major dysregulated signaling pathways and molecules, respectively. For each pathway/molecule we have summarized the findings of studies done so far in gallbladder cancer, along with the ongoing and completed clinical trials.

16.2 Potential Targets for Anticancer Therapy

Cancer is caused by aberrant expression of oncogenes or tumor suppressor genes which leads to the activation or inhibition of key signaling pathways. These pathways can be categorized as pro-survival or anti-apoptotic. Abnormal signal transduction in these pathways due to the loss of control not only contributes to the initiation but also the progression of cancer. Sections below describe the signaling pathways and the molecules which have frequently been reported to show aberrant activation or expression in cancers.

16.2.1 Major Signaling Pathways in Gallbladder Cancer

The most frequently altered signaling pathways in gallbladder cancer consist of pro-survival; PI3K/AKT, mTOR, MAPK/ERK, TP38 pathways, and anti-apoptotic; AKT/MDM2/TP53 pathways. This section we have discussed recent updates on research advancement in major signaling pathways and related clinical studies.

16.2.1.1 PI3K/AKT/mTOR Signaling Pathway

The PI3K/AKT/mTOR is a well reported pathway having functions in growth, metabolism, differentiation, and apoptosis [5]. The PI3K/AKT/mTOR pathway is significantly upregulated in major cancer types including breast cancer [6], pancreatic cancer [7], gastric cancer [8], non-small-cell lung carcinoma [9], colorectal cancer [10] as well as, biliary tract cancers, viz. cholangiocarcinoma [11] and gallbladder cancer [12]. The membrane receptor kinases such as G protein coupled receptors (GPCR), epidermal growth factor receptor (EGFR), and vascular endothelial growth factor receptor (VEGFR) induce signaling cascade by interacting with cell surface ligands. This activates the PI3K pathway by phosphorylating PIP2 (phosphatidylinositol bisphosphate) to PIP3 (phosphatidylinositol triphosphate). Tumor suppressor, phosphatase, and tensin homolog (PTEN), that dephosphorylates PIP3 to PIP2, thereby affects the downstream activation of AKT and PDK1 (Fig. 16.1) [13, 14]. mTOR (mechanistic target of rapamycin) is either directly activated by phosphorylated AKT or indirectly via inactivation of mTOR inhibitor TSC1/2 (tuberous sclerosis complex 1/2) complex. There are two mTOR complexes, mTORC1 (mTOR, mLST8, PRAS40, and raptor) and mTORC2 (mTOR, Sin1 mLST8, and rictor). Wherein, mTORC1 activates p70SK6 (S6 kinase 1),

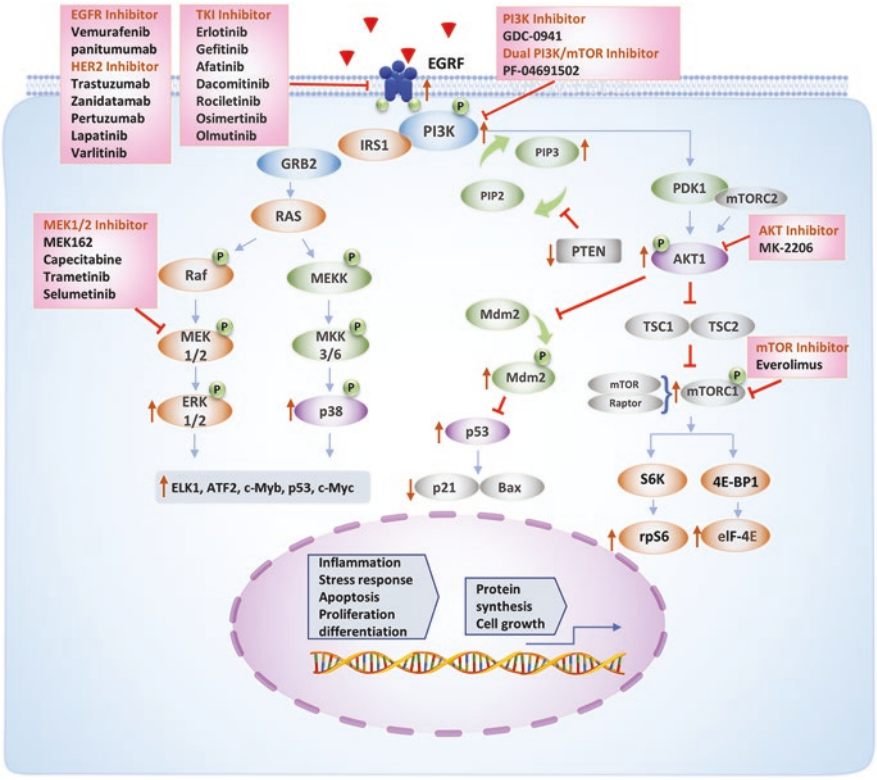

Fig. 16.1 The overview of molecular inhibitors and clinical targets in PI3K/AKT/mTOR and MAPK/ERK pathways selected for preclinical and clinical drug development in gallbladder cancer. The small molecule inhibitors are named in a rectangular box indicating respective signaling targets. The red colored bold upward and downward arrows represent the increased and decreased level of expression. Red colored bold T shaped arrows indicate inhibition of target molecules

which facilitates dissociation of inactive bound form of eIF4E (4E-BP1) to give active eIF4E and promotes cell growth and proliferation [15–17].

Studies in the recent past have shown the role of this pathway in gallbladder cancer growth and progression, and the role of their potential inhibitors as antitumor agents [18, 19]. The mTOR substrate p70S6K is phosphorylated in 66.7% dysplasia, 84.6% early gallbladder cancer, and 88.3% advanced gallbladder cancer patients. Leal et al. also found expression of phosphorylation of mTOR in 64.1% of gallbladder cancer patients and 24% of chronic cholelithiasis patients. The phospho-mTOR was a good prognosis predictor of advanced gallbladder cancer [19, 20]. The mutation of the PI3K pathway has been reported in 22% of early stage and 14.6% in advanced stage gallbladder cancer [21]. In PIK3CA, activating mutations were discovered in 12.5% gallbladder cancer patients [22]. The epigenetic alteration, mutation and aberrant activation of the PI3K/mTOR pathway is common in gallbladder cancer [23].

Along with mutation and epigenetic regulation of key signaling molecules such as PIK3CA, mTOR, and PTEN, there are many other molecules which modulate activation of PI3K/AKT/mTOR pathway in gallbladder cancer, and can be used as therapeutic targets [12]. Forkhead box k1 (FOXK1) transcription factor promotes cell cycle and increase the expression of epithelial to mesenchymal transition (EMT) markers such as vimentin, E cadherin, and N cadherin via PI3K/AKT/mTOR pathway in gallbladder cancer [12]. Fibronectin, a heterodimeric glycoprotein, shown to activate PI3K/AKT/4E-BP1 pathway promotes proliferation and metastasis of gallbladder cells [24]. The same pathway was activated by SPOCK1 (testican-1) to promote proliferation and metastasis, and to block apoptosis in gallbladder cells as well as in tumor xenograft mice model [25]. Similarly, serine threonine tyrosine kinase 1 (STYK1) [26], nectin-4 [27], and topoisomerase II alpha (TOP2A) [28] activate PI3K/AKT pathway and promote tumor growth, proliferation, and invasion of gallbladder cells.

Our improved understanding of important key signaling molecules in the PI3K/AKT/mTOR pathway has enabled us to target this pathway therapeutically in gallbladder cancer. Many studies have reported its inhibitors which can be classified into dual PI3K-mTOR inhibitors, pan-PI3K inhibitors, and isoform specific inhibitors [29]. The treatment with PI3K inhibitors GDC-0941 and PF-04691502 in gallbladder cancer patient-derived cell lines reduced their growth and proliferation [30]. Flavonoid compound isorhamnetin with anti-inflammatory and antitumor properties, inhibited gallbladder cancer cell proliferation, metastasis, and induced cell cycle arrest and apoptosis via inhibition of PI3K/AKT signaling [31]. lncRNAs and microRNA also regulate the PI3K/AKT pathway. The lncRNA PVT1 promotes gallbladder cancer cell proliferation via miR-143/HK2 axis [32]. However, miR-143-3p targeting, ITGA6 (Integrin Subunit Alpha 6) reduces gallbladder cells growth and angiogenesis via inhibition of PI3K/AKT pathway in gallbladder cancer [33]. lncRNA-HGBC promotes gallbladder cancer metastasis via stabilization of HuR (Hu Antigen R) following activation of the miR-502-3p-SET-AKT signaling cascade [34]. LncRNA CRNDE acts as a scaffold molecule to facilitate SP1 mediated expression of LINC00152. The LINC00152 further promotes migration and invasion of gallbladder cancer cells via activation of the PI3K-AKT pathway [35, 36].

A number of clinical trials are underway in biliary tract cancers targeting PI3K/AKT/mTOR pathway (Table 16.1). Phase I clinical trial (NCT00949949) of combination drug therapy using everolimus (mTOR inhibitor), gemcitabine, and cisplatin in two and three drug combinations to evaluate maximally tolerated dose (MTD), adverse event, and toxicity profile in gallbladder cancer [37]. Similarly, combination chemotherapy was also carried out with gemcitabine, cisplatin, and S-1 to study recommended dose (RD), dose limiting toxicities (DLTs), and MTD in advanced biliary tract cancer patients [38–41]. The combination therapies with specific molecular inhibitors will improve current treatment efficacy and reduce unwanted side effects in the treatment of gallbladder cancer.

Table 16.1 Ongoing and completed clinical trials targeting signaling molecules involved in biliary tract cancer (https://clinicaltrials.gov, accessed on 31-05-2021)

Drug treatment	Molecular target	Tumor type	Phase	Clinical trial ID	Location	Status
Everolimus, gemcitabine, cisplatin	mTOR	Cholangiocarcinoma, gallbladder cancer, solid tumor	I	NCT00949949	USA	Completed
Ado-trastuzumab emtansine	HER2	Solid tumor	II	NCT02675829	USA	Recruiting
Trastuzumab	HER2	Cholangiocarcinoma, biliary tract cancer, cholangiocarcinoma	II	NCT03613168	South Korea	Completed
Trastuzumab, pertuzumab, erlotinib, vemurafenib, cobimetinib, vismodegib, alectinib, atezolizumab	HER2,TKI, EGFR, BRAF	Solid tumors, biliary cancer, salivary cancer, bladder cancer	II	NCT02091141	61 cities USA	Recruiting
Zanidatamab	HER2	Biliary tract cancers	II	NCT04466891	USA, Canada, UK, Chile, China, France, Italy, South Korea, Spain,	Recruiting
Lapatinib ditosylate	HER2	Gallbladder cancer, extrahepatic bile duct cancer, primary liver cancer	II	NCT00107536	USA	Completed
Varlitinib, capecitabine	HER2	Biliary tract cancers	II	NCT03231176	China	Completed
FORFIRINOX, gemcitabine, oxaliplatin, cetuximab, trastuzumab, gefitinib, lapatinib, everolimus, sorafenib, crizotinib	TKI, EGFR, HER2, mTOR, ROS1, ALK	Gallbladder cancer, extrahepatic bile duct cancer	II	NCT03768375	China	Recruiting
Selumetinib, cisplatin, gemcitabine	MEK1/2	Biliary tract cancers, cholangiocarcinoma, gallbladder cancer	I	NCT01242605	UK	Completed
Trametinib, capecitabine, fluorouracil, leucovorin calcium	MEK1/2	Cholangiocarcinoma, gallbladder cancer, hilar cholangiocarcinoma,	II	NCT02042443	295 cities USA	Completed

(continued)

Table 16.1 (continued)

Drug treatment	Molecular target	Tumor type	Phase	Clinical trial ID	Location	Status
Trametinib	MEK1/2	Biliary tract cancers	II	NCT01943864	Japan	Completed
MEK162	MEK1/2	Biliary cancer, colorectal cancer, solid tumors	I	NCT00959127	USA	Completed
MEK162, capecitabine	MEK1/2	Biliary tract cancer	I and II	NCT02773459	South Korea	Completed
MEK162 (Binimetinib), gemcitabine, cisplatin	MEK1/2	Biliary tract cancers	I and II	NCT01828034	USA	Completed
MK-2206	AKT	Gallbladder carcinoma, extrahepatic bile duct carcinoma, distal bile duct cancer, liver carcinoma	II	NCT01425879	USA	Completed
Gemcitabine oxaliplatin and panitumumab	KRAS-BRAF	Metastatic biliary tract and gallbladder carcinoma	II	NCT01308840	USA	Completed
GEMOX with afatinib	Open label	Resectable gallbladder carcinoma patients	II	NCT04183712	China	Recruiting
Selumetinib, cisplatin, gemcitabine	MEK1/2	Biliary cancer	II	NCT02151084	Canada	Recruiting
Sorafenib tosylate and erlotinib hydrochloride	VEGFR	Extrahepatic bile duct adenocarcinoma, gallbladder adenocarcinoma, hilar cholangiocarcinoma	II	NCT01093222	USA	Completed
Gemcitabine, cisplatin, placebo, cediranib	VEGFR2	Biliary tract neoplasms	II/III	NCT00939848	London	Completed
Cabozantinib	c-MET, VEGFR2	Bile duct cancer, intrahepatic cholangiocarcinoma	II	NCT01954745	USA	Completed
Regorafenib	Open label	Cancer of the bile duct	II	NCT02115542	USA	Recruiting
Ramucirumab	VEGFR2	Cholangiocarcinoma, liver and intrahepatic bile duct carcinoma, gallbladder carcinoma	II	NCT02520141	USA	Active

16.2.1.2 MAPK/ERK Signaling Pathway

MAPK (mitogen-activated protein kinase) signaling conveys growth and stress signals from extracellular space to intracellular machinery to carry out various cellular functions such as proliferation, migration, and apoptosis. MAPK signaling cascade consists of three major kinases namely, MAPKKK, MAPKK, and MAPK. MAPKKK (Raf, ASK1, MLK, and MEKK1/4) gets activated by binding of specific ligands to membrane receptor kinases/Ras complex. MAPKKK phosphorylates MAPKK (MEK1/2, MKK4/7, and MKK3/6) which further phosphorylates MAPK (ERK1/2, JNK, and p38) leading to transcription of their targets (Fig. 16.1) [42, 43]. The regulation of MAPK/ERK pathway is crucial in maintaining balance between proliferation and apoptosis. In most cancers, this pathway is constitutively active due to frequent mutation in the RAS or RAF. In a study, KRAS gene was mutated in 31% and BRAF gene was mutated in 11% of bile duct cancer patients [44].

ERK/MAPK pathway showed frequent activation in gallbladder cancer and chronic cholecystitis patients in a high-risk Chilean population [45, 46]. Solute carrier family 25 member 22 (SLC25A22), a mitochondrial glutamate transporter, promotes gallbladder cancer growth and metastasis via activation of MAPK/ERK pathway [47]. Prohibitin (PHB), a scaffold protein, was found to be a good prognostic marker for gallbladder cancer, which promotes cell proliferation and invasion through activation of ERK pathway [48]. Likewise, CCR7 (CC-chemokine receptor 7) mediated expression of tumor necrosis factor (TNF)-α promotes lymph node metastasis via activation of ERK1/2/AP-1 and JNK/AP-1 pathways in gallbladder cancer [49, 50]. A systemic analysis of frequent mutation and activation of specific pathways revealed mutation and aberrant expression in MAP kinase, Wnt/β-catenin, and NF-κB. Out of three pathways MAPK pathway was carrying higher mutation burden with 50% of mutation in key signaling molecules such as ADAM12, MAP 2 K1/MEK1, MAPKBP1, NF1, and PDGFR [51, 52].

lncRNAs and microRNA also regulate ERK/MAPK pathway and are promising therapeutic targets [53]. The lncRNA MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) promotes gallbladder cancer proliferation and metastasis via activation of ERK/MAPK pathway [54]. MALAT1 has also been shown to be a good indicator of recurrence and prognosis of gallbladder cancer [55–57]. The miR-101 microRNA is a direct target of ZFX (zinc finger protein X-linked) which reduces TGF-β-mediated EMT in gallbladder cancer via inhibition of MAPK/ERK/SMAD signaling [58]. Similarly, microRNA-29c-5p, a direct target for CPEB4 (Cytoplasmic polyadenylation element binding protein 4), reduced tumorigenic properties of cells via inhibition of MAPK pathway [59].

Increased evidence of cisplatin resistance and severe cytotoxicity in various cancers has prompted the use of combination therapy [60]. Dual-specificity phosphatase 1 (DUSP1) was reported to be involved in p38 MAPK mediated Cisplatin resistance in gallbladder cancer cells [61]. DiGeorge syndrome critical region gene (DGCR5) was shown to inhibit oncogenic properties of gallbladder cancer, via regulating MEK/ERK1/2 and JNK/p38 MAPK pathways in gallbladder cancer [62]. Combination of cisplatin with p38 MAPK inhibitor [63] or with metformin enhanced combination therapy induced cell cycle arrest and apoptosis in gallbladder cancer

cells via PI3K/AKT and ERK pathway [64]. Many clinical trials are currently underway specially targeting MEK along with traditional chemotherapeutic drugs (Table 16.1) [65–67]. The GSK1120212 (JTP-74057), which is a selective MEK1/2 inhibitor, blocked Raf mediated phosphorylation of MEK [68]. Similarly, U0126 (MEK inhibitor) reduced liver metastasis and prolonged survival, in mice model with orthotopic KRAS mutation [69, 70]. Trametinib (MEK inhibitor) has shown therapeutic potential in both in vitro and in vivo gallbladder cancer models [51]. In a phase II randomized clinical trial, its efficacy has also been studied in combination with traditional 5-fluorouracil or capecitabine in refractory advanced biliary tract cancer patients [65]. These studies support that combination therapy along with selective molecular inhibitors, have potential to improve the current treatment regime against common drug resistance and system toxicity.

16.2.1.3 EGFR Pathway

The ErbB receptor, also known as the EGFR (ERBB1/HER1), is a transmembrane glycoprotein which belongs to the family of receptor tyrosine kinases (RTK). Other than ERBB1, this family consists of ERBB2 (HER2), ERBB3 (HER3), and ERBB4 (HER4) [71]. Among these RTKs, ERBB1 is most frequently mutated and aberrantly expressed in multiple types of cancers. Activation of these receptors leads to initiation of downstream signaling cascades such as ERK/MAPK, PI3K/AKT, and JAK/STAT pathways, which are involved in proliferation and differentiation [72, 73]. Frequent mutation and aberrant expression of ERBB1 is considered to be a good prognostic marker and considered as an important therapeutic target in the treatment of cancer [74, 75].

Currently available EGFR targeting drugs are of two types, humanized monoclonal antibodies against EGFR extracellular receptor domain and small synthetic molecules called tyrosine kinase inhibitors (TKIs) [76]. Cetuximab is a chimeric human-murine monoclonal antibody that binds to the extracellular EGFR receptor domain, which prevents dimerization and subsequent internalization of the antibody-receptor complex [77]. Three generations of TKIs are currently available; primarily they bind to the catalytic pocket of ATP binding intracellular kinase domain, leading to inhibition of downstream catalytic activation [78]. Known TKIs include first generation of reversible inhibitors such as Erlotinib and Gefitinib [79, 80], second generation of irreversible inhibitors Afatinib and Dacomitinib which show increased efficacy to mutated EGFR (Leu858Arg and Thr790Met) [81, 82] and the third generation of inhibitors such as Rociletinib, Osimertinib, and Olmutinib which were devised to counter the acquired resistance of first generation and second generation TKIs [80, 83, 84].

Although the frequency of mutation in ERBB1 was reported to be in the range of 3.9% to 4% gallbladder cancer [85, 86] yet over-expression of EGFR has been reported in 44–74% gallbladder cancer tissues [86–88], which suggests the possibility of other mechanisms. Recently, Shen et al. reported PLEK2 (Pleckstrin 2), an oncogene known to interact with the kinase domain of EGFR, leads to subsequent activation of EGFR signaling and promotes invasion and metastasis of gallbladder cancer [89].

Recent clinical trials have specially focused on targeting ERBB1 along with traditional anticancer therapy of gallbladder cancer. Elevated response in the inhibition of EGFR signaling was observed by employing a dual inhibition approach including anti-EGFR antibody and tyrosine kinase inhibitor. Extracellular receptor domain and intracellular kinase domain were inhibited by the combination of Cetuximab monoclonal antibodies with either gefitinib or erlotinib TKIs. Combination of antibodies and TKIs showed more significant inhibition in EGFR phosphorylation along with downstream MAPK and AKT pathways [90, 91]. Hezel et al. carried out a multicenter phase II trial with combination of GEMOX (Gemcitabine and Oxaliplatin) and Panitumumab in 31 gallbladder cancer patients with unresectable KRAS wild-type tumors. This combination therapy showed improved efficacy with progression free survival (PFS) of 10.6 months (95% CI 5–24 months) [92]. In another clinical trial (NCT00779454) Jensen et al. showed a similar outcome using the same treatment regimen with median PFS of 8.3 months (95% CI 6.7–8.7 months) [93]. Conversely, clinical trial (NCT01389414) by Leone et al. reported only marginal improvement in efficacy with PFS of 5.3 months (95% CI 3.3–7.2 months) by anti-EGFR (Panitumumab) therapy in cholangiocarcinoma patients with wild-type KRAS [94]. It is important to note that even though both are bile tract cancers, only the gallbladder cancer group showed improved efficacy.

16.2.1.4 Hedgehog Signaling Pathway

Hedgehog (Hh) signaling is named after its ligand. It was first identified in fruit flies (*Drosophila* sp.). In mammals, there are three homologs of Hh; Sonic Hedgehog (SHH), Desert Hedgehog (DHH), and Indian Hedgehog (IHH). This signaling plays an important role in the development by transmitting information to the embryonic cell and helping them differentiate. In vertebrates, SHH is the most studied ligand and mainly involved in autocrine signaling. In target cells SHH binds to the Patched-1 (PTCH1) receptor. In its absence PTCH1 blocks the downstream target molecule Smoothened (SMO). In the presence of SHH, SMO gets activated, which further activates the transcription factor- GLI. GLI goes to the nucleus and in turn controls the Hh gene transcription.

Different cancers have been associated with the defects in this pathway which include brain cancer, basal cell carcinoma [95], lung cancer [96], breast cancer [97], prostate cancer [98], and skin cancer [99]. Very little has been discovered about the role of hedgehog in gallbladder cancer. High expression of GLI1, SHH, and SMO have been found in gallbladder tumor cells. Examination of 10 normal mucosa, 32 gallbladder cancer, and 95 cholecystitis samples revealed increased expression of patched and GLI1, suggesting an aberrant activation of sonic hedgehog signaling in gallbladder cancer and chronic cholecystitis patients [100]. SMO and GLI1 positive patients could be a target for the Hedgehog inhibitor treatment. Smoothened suppression, in fact, decreased the invasiveness of the tumor by the inhibition of MMP2 and MMP9 expression [101]. Interestingly, another study made a different observation where, out of all GLI isoforms (GLI1, GLI2, and GLI3), only GLI2 was found to affect the proliferative capacity of gallbladder cancer cell lines. The Gemcitabine treatment (a drug that blocks G1-S transition) suppressed GLI2 expression and

increased the survival of gallbladder cancer cells. Gallbladder cancer patients with higher GLI2 expression have a smaller number of CD3+ and CD8+ cells and have increased PDL1 expression [102]. It is possible that GLI1 and GLI2 both play a role in gallbladder cancer, which depends upon the tumor microenvironment. Combined treatment with Rapamycin and Vismodegib (SMO antagonist) reduced cell proliferation and viability in biliary tract cancer cell lines. It also reduced hedgehog signaling in mouse xenografts of cholangiocarcinoma. This combination of drugs reduced phospho-p70S6K, phospho-GLI1 as well as phospho-mTOR and phospho-AKT in biliary tract cancer patients [103]. SMO suppression by Cyclopamine (binds to SMO) can be a potential approach in treating gallbladder cancer. si-RNA based reduction of SMO was also found to be effective against gallbladder cancer (Fig. 16.2) [101]. Overall, although existing studies show the therapeutic potential of targeting hedgehog signaling yet it has scope for further exploration.

16.2.2 Major Signaling Molecules in Gallbladder Cancer

In the previous section we discussed multiple pathways that are altered in cancer progression. Studies so far suggest multiple molecules in each pathway which can be therapeutically targeted and have potential in prognosis and prevention of gallbladder cancer. We have dedicated this section to major signaling molecules which have either shown very high mutation frequency in cancers or predominantly

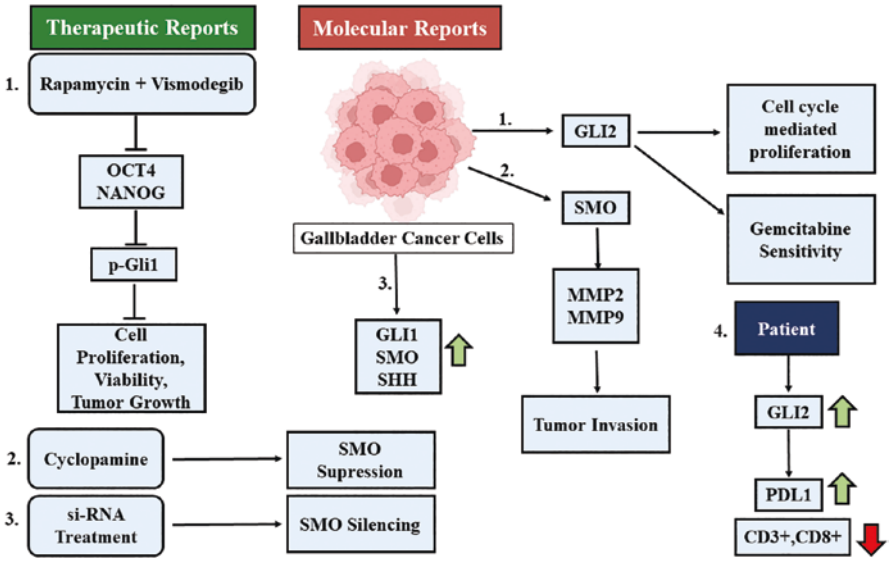

Fig. 16.2 An overview of the available molecular studies and therapeutic reports of Hedgehog signaling in gallbladder cancer. The numbers represent different studies. Upward green arrow represents upregulation and downward red arrow represents downregulation

control the specific pathway. We have described the evidence of the role in different cancers including gallbladder cancer and therapeutic potential of TP53, KRAS, HER2. HIF1A, and VEGF.

16.2.2.1 TP53

TP53 is the major tumor suppressor gene that plays a central role in key cellular processes such as guarding against DNA damage, cell cycle arrest, and apoptosis. TP53 is the most frequently mutated gene in almost all human cancers such as lung, colorectal, breast, hepatobiliary, and bile duct cancer [104, 105]. Murine double minute 2 (MDM2), an E3 ubiquitin-protein ligase balances p53 protein level through constant proteasomal degradation [106].

Initially TP53 was misdiagnosed as an oncoprotein, due to discovery of TP53 with oncoprotein simian virus 40 large T antigen (SV40LT) SV40 and partly due to cloning of a mutant TP53 cDNA from cancer cells which was overexpressed in cancers [107]. The ectopic expression of mutant TP53 promoted tumor properties in TP53 knockout mice [108]. Most human cancers show accumulation of TP53 mutation leading to expression of mutant TP53 [109–111]. In an immunohistochemical study, TP53 expression was found in 92% of gallbladder patients compared to 66% of extrahepatic bile duct/ampullary carcinomas [112]. Overexpression of p53 protein was often observed in moderately to poorly differentiated compared to initial stage of gallbladder cancer [113]. Increased TP53 expression and frequent mutation in gallbladder cancer led to expression of tumor promoting factors and failure to initiate apoptotic signaling. Accumulation of mutated p53 protein level was associated with poor survival in gallbladder cancer [114]. The MDM2 overexpression, along with KRAS and TP53 mutation are often reported in cholangiocarcinoma [115]. Whole-exome and targeted sequencing analysis found TP53 to be frequently mutated genes in gallbladder cancer patients [85, 114]. Interestingly, the most frequent missense point mutation was observed in the early dysplastic stage of cancer and mutations accumulated mostly in exon 5 and exon 7 regions of TP53 gene [116]. Next-generation sequencing of biliary tract cancer patients from both Japan and Indian population revealed frequent germline and somatic mutations in TP53/SMAD4/RAS/MAPK signaling molecules [114, 117].

Even after TP53 frequent mutations and associated poor prognosis, very limited clinical studies have been undertaken to evaluate mutant p53 as molecular targeted in gallbladder cancer. Elongator Acetyltransferase Complex Subunit 5 (ELP5) mediated TP53 expression overcame Gemcitabine resistance in advanced and metastatic gallbladder cancer by facilitating TP53 mediated apoptosis [118]. Phase II clinical trial of ONYX-015 (dl1520) has been done in gallbladder cancer, ONYX-015 (dl1520) an adenovirus mutant that lacks the E1B gene so as to replicate preferentially in p53-mutated cancer cells. Intra-lesional treatment of ONYX-015 in patients with frequent TP53 mutation show evidence of antitumor properties [119, 120]. Even though ONYX-015 is known to replicate in TP53 mutant cells, a clinical trial by Wadler et al. found expression of ONYX-015 viral gene hexon in adjacent normal stroma along with gallbladder tumor cells [121]. The frequent accumulation of mutant p53 protein and gain of tumor promoting function suggest mutant p53 protein as potential therapeutic target of interest in gallbladder cancer.

16.2.2.2 KRAS

Similar to TP53, KRAS mutation frequency is also very high in cancers. There are three different types of RAS—KRAS, NRAS, HRAS. Proto-oncogene KRAS is named after the Kirsten Ras virus (KRAS). KRAS mutation frequency shows variation among cancer types. According to the cBioPortal database KRAS was mutated in 89% pancreatic cancer, 45% rectal cancer, 39% colorectal cancer, 38% colon cancer, 32% lung cancer, 5.7% cholangiocarcinoma, and 7.8% gallbladder cancer, respectively (https://www.cbioportal.org/, access date-31/05/2021). Mutations in the KRAS activate the MAPK pathway, giving cancerous properties to cells. In normal cells KRAS acts as a switch (on and off signal) to regulate cellular growth, it gets activated when it binds to the GTP, when GTP is converted to GDP, KRAS is inactivated. When mutated it gets stuck to the on signal, resulting in continuous activation of the MAPK pathway leading to uncontrolled cellular growth and proliferation. The detailed signaling and targets of the MAPK/ERK pathway are described in the previous section.

These observations advocate KRAS as the potential candidate for therapeutic strategy. For a long time, despite many attempts, the KRAS remained difficult to target via drugs due to its relatively small targeting pocket. Often, the downstream effectors of KRAS, such as PI3K and MAPK signaling cascades were targeted. Eventually with the advancement of science, scientists were able to devise a therapeutic approach by directly targeting the mutant KRAS. In colorectal cancer, direct inhibition of interaction between GTP bound KRAS and RAF results in antitumor properties in KRAS-G12V mutated mouse xenograft model [122]. In lung metastatic cancer, a high affinity Pyrazolopyrimidine based compound disrupts KRAS and RAF interaction by allosteric inhibition [123]. A very recent study on blood cancer revealed that blocking SOS1-KRAS complex by BI-3406 (a drug) can be a potential approach in KRAS mutated cancers [124].

KRAS is also well studied in biliary tract cancers. Computational analysis of gallbladder cancer databases containing samples from Japan, USA, Chile, and China, showed that KRAS was one of the 14 most altered genes [125]. In Chile gallbladder cancer patients KRAS mutation was present in 30% of the samples [126]. In the North Indian population, codon 12 KRAS gene mutation was present in 48% gallbladder cancer patients [127]. Another study in the North Indian population found that KRAS exon 1 and 2 mutations were present in 23.5% of the gallbladder cancer patients, and were also associated with the advanced stage [128].

KRAS and the associated signaling molecules have also been targeted therapeutically in biliary tract cancers. Selumetinib, which is a MEK1/2 inhibitor, was found to be effective in treating biliary tract cancer. It showed acceptable tolerance and efficacy in patients with metastatic biliary cancers [129]. Afatinib (anti-RTK) treatment in the mouse model showed significant reduction in the tumor size in G13D KRAS mutated group but not in G12V KRAS mutation (Fig. 16.3) [130]. More research would further benefit targeting gallbladder cancer patients through KRAS, as it not only has a role in progression but also could be the causative.

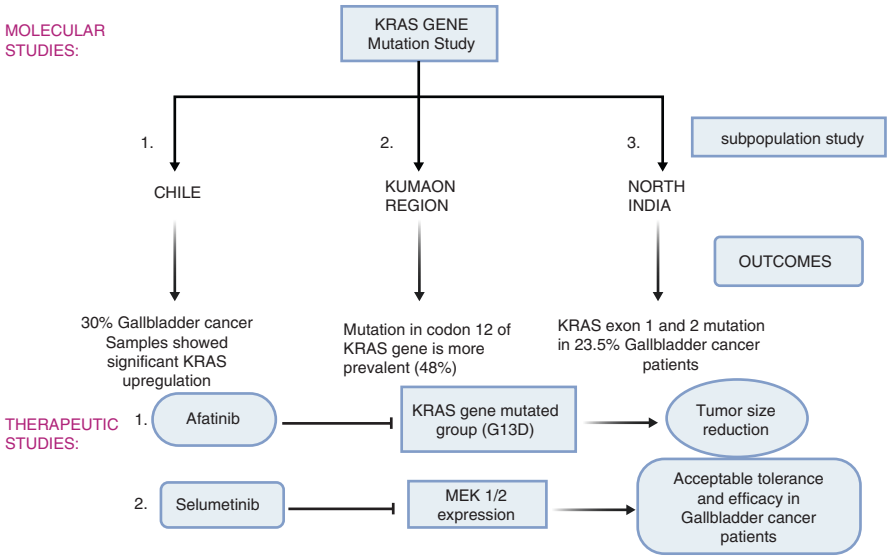

Fig. 16.3 Studies in gallbladder cancer showing role and therapeutic potential of KRAS. Upper panel summarizes reports about the molecular mechanism and the lower panel shows studies targeting KRAS directly or its downstream molecule

16.2.2.3 HER2

The human epidermal growth factor receptor 2 (HER2) receptor is a transmembrane glycoprotein belonging to the HER receptor family [131]. Like EGFR, HER2 is also a transmembrane receptor tyrosine kinase consisting of extracellular ligand binding domain, transmembrane domain, and intracellular tyrosine kinase domain. Ligand interaction with HER2 extracellular domain leads to its homodimerization or heterodimerization, which results in phosphorylation of intracellular tyrosine domain and the activation of downstream signaling molecules [132, 133]. The commonly activated downstream signaling pathways include PI3K/AKT and MAPK/ERK. These two pathways play a crucial role in cancer biology by regulating various cellular functions (detailed description is given in the above section).

Recently, Wei et al. reported that elevated levels of Kinesin Family Member 11 (KIF11) mediate cell growth and proliferation via activation of HER2/PI3K/AKT signaling pathway in gallbladder cancer [134]. Also, the epigenetic profiling from high-risk Chile population shows gain in HER2 copy number in 14% gallbladder cancer patients [135]. HER2 overexpression is a good prognostic indicator for advanced radically resected biliary tract cancer [136]. The meta-analysis by Galdy et al. also reported presence of HER2 overexpression in 57.6% in biliary tract malignancies and proposed it as a good prognostic marker [137]. Similarly, meta-analysis from China, Japan, Chile, and the USA population revealed frequent mutation and copy number alteration in HER2 [125].

Considering that HER2 frequently is mutated and shows copy number variation, significant amount of work on preclinical and clinical therapeutic trials have been

done in biliary tract cancer. Patient-derived HER positive gallbladder cancer cell lines SNU-2670 and SNU-2773 show sensitivity to Trastuzumab, Dacomitinib, and Afatinib treatment. Trastuzumab monotherapy in SNU-2670 mouse xenograft model showed reduced tumor property and in combination therapy with gemcitabine increased apoptosis of gallbladder cancer [138]. Pertuzumab reduced activation of downstream molecules in a dose-dependent manner in biliary tract cell line overexpressing HER2 and HER3 [139]. Phase 2 trial (NCT02675829) of Ado-Trastuzumab Emtansine in patients with aberrant expression and mutation (S310) in HER2 showed partial response [140]. ERBB2 targeting with specific shRNA or Afatinib reduced tumorigenic properties and the activation of downstream ERK [130]. However, another phase II trial using lapatinib (dual inhibitor of EGFR and HER2) did not show beneficial effect in biliary tract cancer [141, 142]. These controversial observations suggest that still we have incomplete knowledge about the complex signaling networks which warrants further research.

16.2.2.4 HIF1A

In cancer tissue, cells have high metabolic activity thus relatively high oxygen demand. Increase in tumor size leads to hypoxia in the tumor core, which induces angiogenesis via HIF1. The HIF1 is a heterodimer consisting of α and β subunits. Under normoxic conditions the protein level of β subunit is constantly maintained where HIF1A (α subunit) is constitutively expressed and eventually degraded. In hypoxic conditions HIF1A is stabilized and its protein level increases significantly [143]. HIF1 induces transcription of multiple genes by binding to hypoxia response element (HRE). HIF1A can regulate multiple pathways such as pKB/AKT, MAPK, eNOS signaling pathways via activation of VEGF and increases angiogenesis and vascular permeability [144]. HIF1A can control the tumor cell's adaptive response by controlling the transcription machinery of more than 100 downstream genes responsible for tumor growth and survival [145]. It can suppress cellular senescence by regulating the expression of p53 and CDKN1A [146]. Counteracting with Myc it induces cell cycle arrest (PMID: 15071503). Therefore, targeting this specific molecule could help us to regulate multiple signaling molecules.

Role of HIF1A has been reported in multiple cancer types. To cite a few, in pancreatic cancer cells, increased HIF1A significantly increases the anti-apoptotic property [147]. Induction of cell death in ovarian cancer cells is initiated by down-regulation of HIF1A [148]. HIF1A overexpression results in increased cell invasion in lung cancer [149]. Based on these reports, we can say that HIF1A has diverse functions and can be a potential target in gallbladder cancer.

HIF1A being a direct regulator of tumor angiogenesis is the major determinant of tumor microenvironment. So far most of the studies in gallbladder cancer have focused on its role in tumor neovascularization, which controls two aspects in tumorigenesis, the growth, and the metastasis. A clinicopathological study in gallbladder cancer in Japan showed that 70% of patients were positive for HIF1A and its expression correlated with the tumor stage. Moreover, survival rate was lower in HIF1A positive patients [150]. Drug based studies have also supported the role of HIF1A in tumor growth. Hispidulin (isolated from Chinese herb *Salvia involucrata*) has been

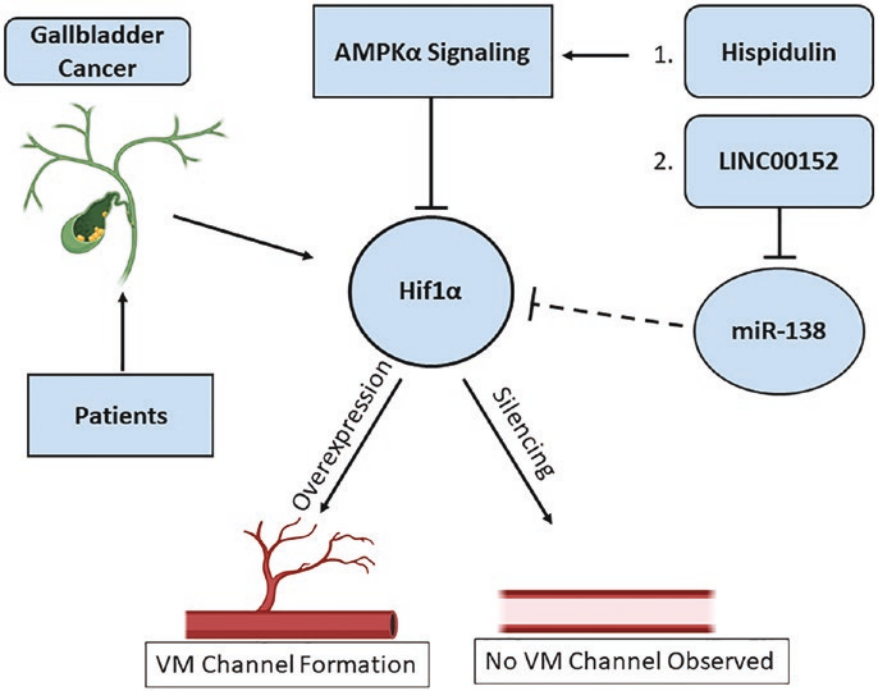

Fig. 16.4 Brief overview of the role of HIF1A in gallbladder cancer. Numbers show the therapy-based studies targeting HIF1A

known to induce apoptosis and affects AMPKα signaling. Hispidulin blocks the HIF1A signaling and acts as a potent tumor suppressor in gallbladder cancer. It reduces HIF1A protein level without affecting its mRNA expression [151]. A long noncoding RNA, LINC00152 has been reported to promote gallbladder cancer metastasis and EMT progression, acting as a miRNA sponge by abrogating the endogenous effect of miR-138, which is reported to suppresses the expression of HIF1A. Targeting the LINC00152/miR-138/HIF-1α signaling pathway regulatory network might be a novel therapeutic target for gallbladder cancer (Fig. 16.4) [152].

The ability of cancer cells to organize itself into vascular structure in order to obtain nutrients and oxygen independent of normal blood vessels is known as vasculogenic mimicry (VM). VM channels were present in human gallbladder cancer tissues. HIF1A expression perturbation changed VM network formation in both hypoxic and normoxic conditions [153]. Although the above-mentioned studies suggest the therapeutic potential of HIF1A in gallbladder cancer, yet more research is needed to establish its exact role in pathogenesis and in evaluation of its candidacy as therapeutic target.

16.2.2.5 VEGF

VEGF protein family members facilitate angiogenesis and lymphangiogenesis. These members include VEGF-A, B, C, D, and placental growth factor (PGF). Out

of these members VEGF-A is the most potent inducer of angiogenesis. However, other VEGF isoforms also have roles in angiogenesis in metastatic tumors. Growth in tumor size creates hypoxic conditions inside it leading to HIF1A mediated induction of VEGF-A which binds to its receptor VEGFR-2 (major receptor) leading to receptor dimerization and autophosphorylation. This in turn phosphorylates downstream proteins, protein kinase C, PI3K and phospholipase c-ɣ. PI3K further activates AKT and RAC. VEGF-A plays a role in normal angiogenesis as well as in disease conditions, including cancer. Formation of new blood vessels is essential in providing nutrients to the tumor tissue, hence VEGF-A directly or indirectly plays a very crucial role in facilitating the tumor cell proliferation. It is a key provider of survival and mitogenic stimuli to endothelial cells and cancer cells [154]. VEGF-A also induces vessel leakiness [155]. One VEGF-A isoform (VEGF189) was associated with papillary renal cell carcinoma and could serve as a prognostic marker [156]. TP53 gene mutation and high VEGF-A levels predict a poor prognosis in advanced breast cancer patients treated with tamoxifen [157]. VEGF-A induced, Sox2 mediated, cancer stem cell self-renewal, and metastasis [158]. Goel and Mercurio have extensively reviewed the role of VEGF-A in angiogenesis and tumorigenesis [159].

Other VEGFs also affect tumorigenesis, especially VEGF-B has garnered researchers' attention due to recent discoveries as a potent antioxidant [160]. VEGF-B promotes tumor metastasis independent of VEGF-A [161]. VEGF-C is important for the growth of lymphatic vessels. VEGF-C has also been shown to stimulate angiogenesis in mouse cornea [162]. VEGF-C mRNA was upregulated in human pancreatic cancer cells [163]. VEGF-D can regulate angiogenesis and lymphangiogenesis both (PMID: 11175849). In mouse skin keratinocytes and tumors, upon VEGF-D overexpression, it stimulated lymphangiogenesis and angiogenesis [164].

The presence of VEGFR on the tumor cells opens up an important therapeutic target, as blocking these receptors will stop downstream signaling. In pancreatic tumor cells the expression of both VEGF and VEGFRs are upregulated with respect to the normal pancreatic tissue [165]. Similarly, in malignant pleural mesothelioma, cells express VEGFR1 and VEGFR2 [166]. In different breast cancer cell lines such as MCF-7, MDA-MB-231, MDA-MB-453, and T47D; VEGFR1 and VEGFR2 are reported to have expressed [167]. This evidence supports the idea of targeting VEGF signaling in cancers, which can not only stop the tumor cell growth but also the tumor angiogenesis.

VEGF has been extensively studied in gallbladder cancer also, pertaining to various aspects of tumorigenesis. In gallbladder carcinoma expression of VEGF-A was increased in 80% of the samples and correlated with poor prognosis [168]. Cyclooxygenase 2 (COX-2) is responsible for the formation of prostaglandin from arachidonic acid. High COX2 level correlated with high VEGF-A and high micro vessel count (MVCs) [169]. In gallbladder cancer, VEGF-C and D have been reported to be involved in lymphangiogenesis and angiogenesis [170]. TNF-α levels correlated with VEGF-D in bile samples of gallbladder cancer patients. TNF-α promoted lymphangiogenesis and was involved in gallbladder cancer progression

through the ERK-1/2-AP1/VEGF-D signaling pathway [50]. VEGF-D knockdown decreased cell proliferation and invasiveness in gallbladder cancer cell lines and in tumor xenograft mouse models [171]. The expression of VEGF-C in gallbladder cancer patients positively correlated with lymphatic vessel density. In the gallbladder cancer cell line TNF-α activates VEGF-C expression via NF-κB binding to VEGF-C promoter [172].

Multiple clinical trials are already underway for biliary tract cancers including gallbladder cancer. Clinically, Erlotinib and Sorafenib combination treatment was used to target VEGFR and passed the phase II trial (NCT01093222). Cediranib targets VEGFR and has completed phase II/III trials in gallbladder cancer (NCT00939848). Similarly, Cabozantinib (NCT01954745), Regorafenib (NCT02115542) and Ramucirumab (NCT02520141) target VEGFR2 and have completed phase II clinical trials in metastatic gallbladder carcinoma (Table 16.1). These reports clearly suggest that VEGF and VEGFR have huge therapeutic potential which has scope for further exploration. Other VEGFs viz. VEGF-C and VEGF-D can also be explored for targeted therapy.

16.3 Conclusion

Gallbladder cancer being aggressive and highly metastatic is often identified at unresectable advanced stages. Alternative non-surgical therapeutic strategies can provide a treatment option in gallbladder cancer. Although, currently a few clinical trials are underway which are intended to improve efficacy of conventional chemotherapies as well as targeted therapy, yet there is scope for the identification and validation of more. Targeted therapy, based on patients' genetic, proteomic, and transcriptomic profiling, can be helpful in avoiding off-target effects, lack of drug response or drug resistance due to individuals' genetic differences. By employing multitarget combination therapy along with conventional chemotherapy treatment efficacy can be improved with minimum side effects.

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Integrative Omics: The Roadmap for Gallbladder Biomarkers Identification

17

Kirti Gondkar, J. R. Parvathi, and Prashant Kumar

17.1 Introduction

17.1.1 Gallbladder Cancer

Gallbladder cancer (GBC) is, highly aggressive, yet the commonest malignancy among biliary tract cancers [1]. It arises due to the abnormal growth in the epithelial lining of the gallbladder (GB); however, the underlying causative factors are still unknown [2]. The current statistics estimate the 5-year prevalence rate to be 0.1 million; with higher occurrence in women than men [3]. Worldwide, GBC ranks at the 21st position in the number of deaths and 24th in the number of new cases [3]. The incidence rates for GBC are specific to certain ethnic types located in Asia, Eastern Europe, Chile, and

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Latin America owing to multiple endogenic and exogenic factors [3]. Cholecystitis, cholelithiasis, biliary cysts, and abnormal pancreaticobiliary duct junction are potential risk factors associated with GBCs [4]. The majority of GBC patients (75–90%) have gallstones; however, the incidence of GBC in cases of gallstones is only 0.5–3% [5]. The incidence could be coincidental or indicative of GBCs. Nonetheless, the presence of larger stone has a significant association with GBC [5]. Histologically, GBCs are categorized into adenocarcinomas (most prominent, 80–95%), squamous cell carcinomas, neuroendocrine carcinoma, and undifferentiated carcinomas [6]. Despite being an aggressive cancer, GBCs remain undetected until the last stage. Their deep anatomical positioning and asymptomatic conditions, individually and cumulatively makes the complication go unnoticed during routine tests. This "missing out" leads to limited prognosis and fatality when diagnosed [7]. The severity and extent of GBC metastasis decide the action measures- "Cut, poison, burn"—for treatment. Surgical resection ("cut") is considered during extreme conditions, while chemotherapy ("poison") and radiations ("burn") as treatment options for initial stage GBCs [8]. The likelihood of recurrence in surgically resected cases remains very high [7]. Considering the few therapeutic options available for GBC patients, there is a dire need to explore new diagnostic and therapeutic approaches while minimizing the toxic side effects of chemotherapy and radiation thus improving disease management. Understanding molecular mechanisms of gallbladder carcinogenesis from DNA variations to altered signaling pathways lays the roadmap for novel targeted therapies for GBCs.

17.1.2 Overview of Current Therapies in GBC

The current treatment for GBC largely relies on the type and stage of the tumor. If diagnosed in the preliminary stage, GBC patients have much better survival chances. The treatment modality includes surgery, chemotherapy, and radiation therapy individually or in combination for early-stage GBCs. The extent of the surgical resection may involve either the removal of the gallbladder (cholecystectomy) or removal of multiple tumor-infested organs: liver, bile duct, lymph nodes, and pancreas [8].

Systemic chemotherapy involves cytotoxic drugs that inhibit the proliferating tumor cells by blocking their DNA synthesis, avoiding resection of the gallbladder. Gemcitabine and fluoropyrimidine are used in monotherapy or combined with cisplatin, oxaliplatin, and capecitabine as the first-line therapy for treating unresectable GBC. The combined therapy regimens of 5-fluorouracil (5-FU) and oxaliplatin (FOLFOX), capecitabine and oxaliplatin (CAPOX), gemcitabine and cisplatin (GC), and gemcitabine and oxaliplatin (GEMOX) are the current mainstream chemotherapy programs employed in clinical trials [9].

17.1.3 Multi-Omics Approach to Identify the Potential Targets in GBC

The term omics refers to comprehensive and integrative profiling of biomolecules to identify markers and variants for diagnostic and prognosis studies (Fig. 17.1).

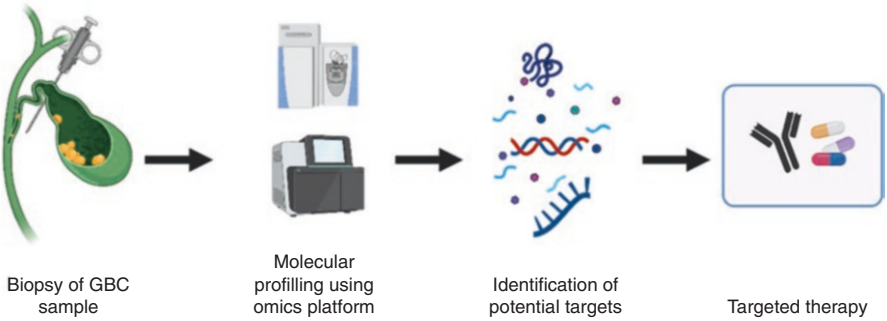

Fig. 17.1 Flowchart depicting utilization of omics technology to identify potential targets in gallbladder cancer. (Created with biorender.com)

Table 17.1 Table depicting different applications of omics techniques

Omics	Type	Data analysis	Application
Genomics	Whole-genome sequencing, whole-exome sequencing	Point mutations, InDels, copy number variations, structural variations	Genomic alterations, functional effect of mutations, Driver mutations
Transcriptomics	RNA sequencing	Gene fusions, differential expression, alternative splicing, RNA editing	Differential gene expression analysis, network analysis, pathway analysis
Epigenomics	Bisulphite sequencing, ChIP sequencing	Transcription factor binding, histone modification, methylation	Genome-wide methylation pattern, assess promoter methylation status, identification of tumor suppressor genes, upstream regulation of gene expression
Proteomics	Quantitative and qualitative mass spectrometry analysis	Pathway analysis, protein–protein interaction, kinase-substrate enrichment analysis	Differential protein expression, localization, modification, pathway analysis
Metabolomics	Deep metabolomics	Metabolite abundance, localization	Metabolite profiles, metabolite intermediates, hormones and signaling molecules

Genomics, transcriptomics, proteomics, metabolomics, and epigenomics—individual omic study—each represents systematic approach targeted at unique molecular stages and mechanisms (Table 17.1) to differentiate normal from abnormal. Molecular subtypes, differential gene expressions, isoforms, functional changes in proteins, altered biological pathways that may or may not be epigenetic influenced are potential diagnostic and prognostic biomarkers to differentiate diseased genotype from normal.

Multi-omics is the analytic approach that integrates respective "omes" studies and relies on comparisons, co-mapping, and correlations analysis of datasets [10].

The inter-relation of data from individual omics study helps decipher the variant and common genetic and phenotypic patterns.

The Cancer Genome Atlas Program (TCGA) [11] and Clinical Proteomic Tumor Analysis Consortium (CPTAC) [12] facilitate a multidimensional view on various cancer types through integrative omics. The output data help infer disease pathogenesis, molecular subtyping, prognosis prediction, and drug target identification. TRACERx Consortium utilizes multi-omics technologies and evolutionary analytical tools to trace the clonality in the tumor at different sites providing an evolutionary perspective to design novel therapeutic strategies [13]. The "Pan-cancer initiative" of TCGA—is a streamlined analytical and comparative approach to identify molecular signatures—from the 12 tumor data sets derived from TCGA. The data interpretation assists in developing targeted therapies among cancer types with similar genomic profiles [11].

In the past few years, multiple omic studies across cancers have yielded a better understanding and comprehensible knowledge of the disease. However, these studies are very limited in GBC. In addition, the molecular and genetic mechanisms associated with characterizing and tracing pathogenesis in GBC are restricted, thus limiting targeted therapy [14]. Recently, few studies have explored pathways associated with advanced GBC-a potential target region for inhibitors [9]. This targeted therapy approach could substantially reduce the side effects of chemotherapy, further improving the response in resistant tumor cells.

17.1.4 Molecular Signatures and Therapeutic Perspective

Molecular signatures in cancer are distinct variants that define or "mark" specific instabilities underlying tumors. Widely, molecular signatures enumerate differential expression patterns of macromolecules such as genes, proteins, metabolites, and microRNAs. These signatures are helpful in the discovery of novel molecules specific to the disease, thus unearthing targets for therapy. Furthermore, molecular signatures can be extrapolated to risk assessment, physiological toxicity, and drug responses to cancerous conditions.

It is well established that various biological processes are interconnected. Genes, proteins, metabolites participate in more than one signaling pathway for cell functionality. The upregulated pattern or differential expression is a "hallmark" of a specific pathological process. A deeper approach with multidimensional comparison and cross-relative analysis is necessary to understand the molecular similarities and complexities. Omics platform has been widely utilized to identify drug targets and biomarkers for efficient management of a disease. High-throughput data has tremendously expanded our knowledge in identifying cancer-specific mutations, alterations at epigenetic levels, molecular subtyping of cancer—each leading to possible biomarkers.

The different data types arising from the individual omic study can be integrated into network-based approaches, thus simplifying the disease interpretation. However, reproducibility, validation, and accuracy remain to be crucial challenges

faced by scientists. An overview of similarities and variations deciphered from individual omic studies can connect the thread of genetic information from gene selection to protein expression. Therefore, it is necessary to develop an integrative yet accurate approach to identify disease-specific molecular signatures.

17.2 Potential Biomarkers in GBC

17.2.1 With Genomics Studies

Next-generation sequencing (NGS) helps to "read" the order of bases that make a DNA segment and "scan" possible alterations from normal. With technological advances and upgraded sequencing platforms, NGS has tremendously improved the analysis of cancer genomes. One of the main factors leading to carcinogenesis is the change in DNA, making it essential to study the genome comprehensively. Sequence reads helps to draw a road map of possible genetic variations: mutational perturbations, copy number alterations, genetic fusions, etc. that signals the stage and progression of the disease. To date, many cancer types have been sequenced using whole-genome (WGS) and whole-exome sequencing (WES) approach. The high-throughput approaches in GBC are discussed below.

A comprehensive integrative genomic analysis across 167 GBC tumors, 7 GBC cell lines, 23 cholecystitis cases, 14 gallstones, and 2 gallbladder polyps identified neoantigens that were capable of T-cell activation including *ELF3*, *ERBB2*, and *TP53* genes suggesting potential targets for cancer vaccine [15]. The study also identified significant mutations in Erythroblast Transformation Specific (ETS) domain genes: *ELF3* and *EHF*, *CTNNB1*, *APC*, *NSD1*, *KAT8*, *STK11*, and *NFE2L2*. Recurrent alterations in *KEAP1/NFE2L2* and WNT pathway genes were also reported in GBC [15]. Exome sequencing identified significant mutations in 25 genes *CTNNB1, ELF3, TP53, ERBB2, ARID2, ERBB3, STK11, CDKN2A, SMAD4, ARID1A, KRAS, EHF, PIK3CA, BRAF, ACVR2A, PSIP1, NFE2L2, CHRM3, ZNF107, SMARCA4, APC, NF1, KAT8, MAP 2 K4,* and *HIST1H2AG* [15]. A WES study involving nine GBC cases observed TP53 as the most significantly altered gene with somatic mutations in 62% of cases [16]. Somatic mutations were observed in genes involved in chromatin-remodeling like *PBRM1* (22% cases); *KMT2C* (22% cases); *PIK3CA* (11% cases); and *SMAD4* (11% cases), respectively. Mutations were not detected in *BAP1, ARID1A, IDH1/IDH2* genes, suggesting a potential role of chromatin-remodeling genes in GBC pathogenesis [16]. Another study involving 57 tumor-normal GBC pairs reported mutations in *TP53* (47.1%), *KRAS* (7.8%), and *ERBB3* (11.8%) genes [17]. Multiple ErbB signaling pathway genes such as *EGFR, ERBB2, ERBB3, ERBB4* were extensively mutated in 36.8% of the cases suggesting a potential role of the ErbB signaling pathway in GBC carcinogenesis [17]. Studies have also explored predominant mutations in *ERBB3* gene and promoter mutations in *PTEN, ARID2, MLL2, MLL3, TERT,* and *APOBEC* genes in gallbladder cancer [18, 19]. Mass spectroscopy-based profiling and NGS studies across 72 GBC cases revealed hotspot mutations in *TP53* and PI3 kinase pathway

genes: *STK11, RICTOR, TSC2*; amplification in *FGF10* gene and fusion in *FGF3-TACC* genes [20]. Study using multi-gene NGS involving 153 biliary tract cancers reported mutations in *KRAS* (19.2%), *ARID1A* (11.5%), *BAP1* (3.8%), *PBRM1* (7.7%), *SMARCB1* (7.7%), and also genes involved in mTOR pathway *PIK3CA* (7.7%), *PTEN* (3.8%), and *TP53* (46.2%) in GBC [21]. Another study involving 85 GBC cases and hybrid capture-based comprehensive genomic profiling have reported genomic alterations in *TP53* (59%), followed by *CDKN2A/B* (19%), *ERBB2* (16%), *PI3KCA* (14%), *ARID1A* (13%), *KRAS* (11%), *EGFR* (4%), *FGFR1–3* (3%), *BAP1* (2%), *BRAF* (1%), and *MET* (1%), respectively [22]. A recent study with Ion AmpliSeq Cancer Panel on 14 GBC tissues against 50 cancer-associated genes reported mutations in *TP53* (64.3%), *SMAD4* (14.3), *CDKN2A* (7.1%), *PI3KCA* (21.4%), *KRAS* (14.3%), *RB1* (7.1%), *ATM* (7.1%), and *VHL* (1%), respectively [23]. An investigative study on somatic and germ-line driver mutations in 66 GBC cases showed significant somatic mutations in *TP53, KRAS, SMAD4, NF1, ARID1A, PBRM1,* and *ATR* genes; germ-line mutations were observed in *BRCA1, BRCA2, RAD51D, MLH1, MSH2* genes in 11% of BTC patients [24].

17.2.2 With Transcriptomics Studies

RNAs bridge the informational relay from DNA to protein and are regulated by various external and internal factors. Transcriptomics refers to the study of all intracellular transcripts within cells. Abnormal splicing events lead to aberrant variations in proteins resulting in isoforms with gain or loss of function. These protein variants range from transcription factors to signaling molecules, thus interfering with the regular functioning of cells leading to cancer progression. RNA-sequencing technology helps in the detection of splicing abnormalities events in cancer. It is also valuable for detecting cancer-related alternative splicing, which potentially could act as markers in cancer and help in targeted therapy.

Analysis of RNA-sequencing data from 115 GBC cases and 5 GBC cell lines identified 23 potential gene fusion events in GBC cases, one being *PTPRK-RSPO* fusion leading to overexpression of RSPO3. Increased expression of mitochondrial genes and apoptosis-related genes such as *BAX, BAD, FASTK,* and *NOXA1*, along with *BRAF, KRAS,* and *CBL*, were also reported [15]. Employing RNA-sequencing on 50 GBC samples, a relationship study to understand the association of gallstone and GBC revealed gene enrichment (over-representation) in PI3K-Akt, mitogen-activated protein kinase (MAPK), Ras, and genes related to wnt signaling pathways. The expression of *ALPP* and *GPR87* was higher in GBC samples than in gallstone samples [25]. Another study designed to characterize GBC molecularly investigated the genetic variants in GBC tumors. The study reported overexpression of *SERPINB3* and *KLK1* genes in GBC. The Ingenuity pathway analysis indicated alteration in LXR/RXR and FXR/RXR pathways as a result of *LXR* and *FXR* downregulation. The study emphasized lipid metabolism pathway and gallbladder cell transport

systems to play a key role in GBC pathogenesis [26]. A global transcriptome profiling of two GBC cases identified 12 differentially expressed genes (DEG), further validated across 35 GBC cases [27]. The expression levels of *BIRC5, TK1, TNNT1,* and *MMP9* correlated to post-operative relapse. BIRC5 expression was correlated positively with the tumor-node-metastasis stage (TNM), and cases with elevated expression of *TK1* and *MMP9* had limited prognostic options [27].

17.2.3 With Epigenomic Studies

Silencing of genes takes place due to hypermethylation in the promoter region of genes. Aberrant hypermethylation is an early and cumulative phenomenon observed in GBC. Epigenetic alterations like modification in histones (acetylation, methylation, phosphorylation, SUMOylation), promoter DNA methylation, and regulation of gene expression by microRNAs (miRNA) are widely implicated with gallbladder carcinogenesis. Genes involved in cell differentiation, cell growth, molecular signaling, repair, and apoptosis like *p16, APC, PTEN, CDH1, RASSF1, MGMT, MASPIN, THBS1, RARβ2, SOCS1, TP53, FHIT, RB* are the most common aberrantly methylated genes identified in GBC [28]. Studies have also reported hypermethylation in the promoter region of *CDKN2A, DAPK1, DLC1, CDH13, TIMP3,* and *GSTP1* genes using a methylation-specific polymerase chain reaction approach in GBC [29, 30]. Tumor suppressor gene silencing by DNA methylation further contributes to the progression of this invasive carcinoma [31].

A genome-wide methylome study on 24 samples confirmed a correlation between hypermethylation events and GBCs [32]. Among the 33,443 genes analyzed, 72% (24188) sites showed hypermethylated in GBC. The aberrant methylation was found localized in the proximal promoter region of the coding or non-coding genes, usually the regulatory sites. A comparative analysis of methylome and the proteomics data identified seven genes as hypermethylated/downregulated and 61 as hypomethylated/upregulated. The genes were involved in the wnt signaling pathway, TGF-β signaling pathway, GPCR signaling pathway, EGFR signaling pathway, and apoptosis-related pathways [32]. High percentage of methylation is reported in the genes like *SHP1* (80%), *3OST2* (72%), *CDH13* (44%), *P15INK4B* (44%), *CDH1* (38%), *RUNX3* (32%), *APC* (30%), *RIZ1* (26%), *P16INK4A* (24%), and *HPP1* (20%) [33]. Advanced GBC cases have reported high methylation frequency in *CDH13* (69.6%), *DAPK1* (60.9%), *FHIT* (56.5%), and *RAR beta 2* (43.5%) genes. Methylated *DLC1*, *APC*, and *FHIT* are associated with poor prognosis and *MGMT* methylation with better survival [27, 31]. Epigenetic inactivation in 3p chromosomal location is a frequent observation in GBC patients which affects tumor suppressive genes like *SEMA3B* (3p21.3) and *FHIT* (3p14.2) [34]. Epigenetic silencing of these genes has been reported in different human tumors, such as lung, breast, brain, prostate, pancreas, and kidney cancers [35]. Methylation in *RASSF1A* at exon 1 was reported in 36.4% of gallbladder carcinoma samples, 25.0% in adenoma, and 8.0% in normal epithelium samples [36, 37].

17.2.4 With Proteomics Studies

In arguably, protein expression provides phenotypic characteristics and functionality to cells and tissues, playing active roles as messengers, effectors, regulators, and suppressors. Proteomics profile the proteome to detect the specific alterations relative to a pathologic condition, thus serving as a guiding tool for discovering potential biomarkers [38]. Mass spectrometry based proteomics has revolutionized personalized treatments in cancer. However, due to GBC's lack of diagnostic markers, the global proteomic analysis will provide a deeper understanding of the molecular alterations leading to this disease.

iTRAQ based high-resolution mass spec study in GBC identified 286 upregulated proteins, a few being: prosaposin, nuclear ubiquitous casein, cyclin-dependent kinases substrate, lysosomal proteins, high mobility group protein B2, and cathepsins; transgelin, S100-A8, and neurofilament were downregulated [39]. Immunohistochemical analysis identified 83% strong positivity for prosaposin presence and complete absence of transgelin in GBC tumor tissues [39].

To study tumor suppressor influenced downstream pathways, authors have overexpressed a tumor suppressor gene DKK3 [40] in six GBC cell lines and studied the altered pathways upon its overexpression. Proteomics and phosphor-proteomics studies on DKK3 overexpression led to identification of 14 altered kinases, hyperphosphorylation of 2 phosphorylases–unique to this study-giving differential expression patterns. These signal modulators were traced to Protein kinase A signaling, Sirtuin signaling, and Cell Cycle Control of Chromosomal Replication pathways. Upon DKK3 overexpression, several molecules such as P-TEFb, PPME, SET, CIP2A showed differential activation across three GBC cell lines, concluding the link of the invasive nature of the GBC cell lines and differential expressions [41].

In another study, authors have utilized a 2-D gel electrophoresis and mass spec-based approach to identify the dysregulated proteins in primary GBC, cholecystitis, and normal gallbladder tissues. The molecule annexin A4 showed significant elevation while heat shock protein 90-beta and dynein cytoplasmic1 heavy chain1 showed decreased expression in GBC tissues compared to the normal [42]. Similarly, in other study, authors have identified 17 proteins to be dysregulated out of which 9 were overexpressed and the other eight proteins were downregulated in tumors. Annexin A3 was significantly overexpressed in GBC compared to cholecystitis and correlated with lower histological grading, lymph node metastasis and shorter survival time post-operation. Hence the role of AnnexinA3 in the initiation and progression of GBC was proposed [43].

To identify the biomarkers in fluids, authors compared serum samples from three GBC cases and controls samples using 2D gel electrophoresis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Out of the 64 proteins that were differentially expressed in 2D gel electrophoresis, 24 were successfully identified- S100A10, haptoglobin, cystatin-B, profilin-1, and superoxide dismutase are a few. Furthermore, higher expression of S100A10 and haptoglobin were correlated to late-stage GBCs with poor clinical prognosis [44].

17.2.5 Altered Pathways Identified in GBC

Literature studies and authors' experiences confirm that alteration in few signaling pathways shows a strong link to tumorigenesis and cancer maintenance in GBC (Fig. 17.2). The aberrant activation of signaling pathways provides a way to study cancer progression and act as a baseline to design targeted cancer therapy.

The aberrant activation of mTOR contributed to the malignant transformation of GBC in early development stages [45]. The expression of phosphor-mTOR was high in 61.4% GBC and 24% of cholecystitis cases with poor survival rates in advanced stages [45]. The mTOR activation was confirmed in 88.3% of GBC cases showing phosphorylation levels at p70S6K position [46]. Upon treating the cells with mTOR inhibitors rapamycin, RAD001and AZD8055, a reduction in cell growth and migration was observed, suggesting mTOR inhibitors as a therapeutic strategy in GBC. Preclinical data in transgenic mice study confirm the potential use of mTOR inhibitor rapamycin to reduce GBC incidence [47].

Studies show aberrant activation of the Sonic hedgehog (Shh) pathway in GBC [48–51]. Molecules involved in Shh signaling like Shh, smoothened (Smo), Patched (Ptch), and Gli1 were found to be overexpressed in 46–97%, 65%, 75–97%, and 50–97%, of GBC cases, respectively, each with poor survival rates [48, 49]. Aberrant expressions of Ptch and Gli1 in chronic cholecystitis (CC) are suggestive of their involvement in the progression of CC to GBC [49]. Furthermore, studies have shown that inhibition of Smo decreases GBC cell lines' proliferation and invasiveness ability by inhibiting MMP2 and MMP9 expressions in GBC cells [50].

Extensive mutation in ErbB signaling pathway molecules like EGFR, ERBB2, ERBB3, ERBB4, etc. is observed in 36.8% of GBC samples. Activating mutations

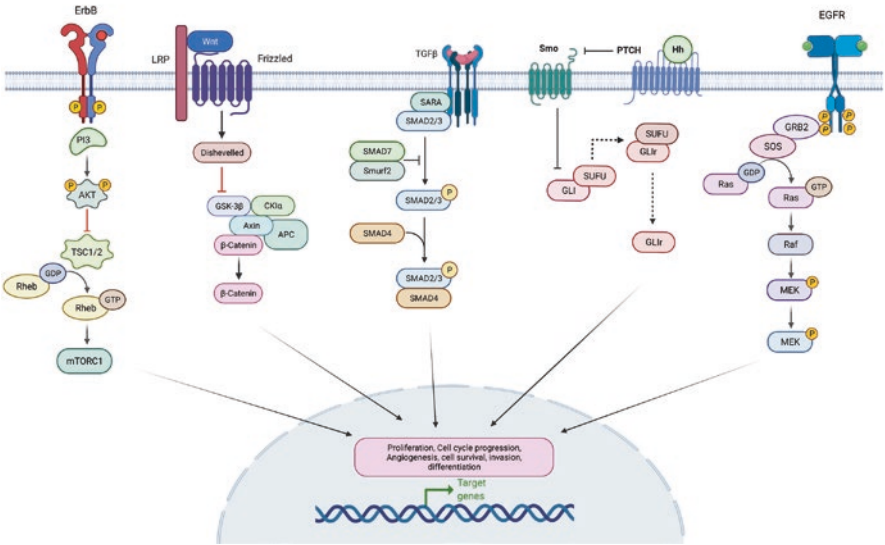

Fig. 17.2 Overview of altered pathways in GBC. (Created with biorender.com)

in ERBB2 and ERBB3 have shown oncogenic nature highlighting their role in GBC development and progression [17]. Studies have also reported EGFR as a promising potential target in gallbladder cancer therapy [52].

Dysregulation in MEK/ERK pathway due to mutations in kinases such as RAS, RAF, MEK is reported in biliary tract cancers [53]. Furthermore, the expression of phospho-ERK1/2 has been linked to PI3K/AKT pathway in cases of poor prognosis. In GBC, phospho-ERK1/2 and PI3K were positively stained in 58.3% and 50.9% cases, while 11.4% and 8.6% in cholecystitis cases [54]. Therefore, possible crosstalk between ERK1/2 and PI3K signaling offers the possibility of utilizing serine/threonine kinase inhibitors as therapeutic targets in GBC.

17.2.6 Liquid Biopsy for Molecular Diagnosis in GBC

17.2.6.1 Circulating Tumor Cells

Tumor metastasis is the primary cause of mortality in cancer. During the formation and growth of cancer, cells from primary tumors shed into the bloodstream and circulate to distant organs. These cells further invade surrounding tissue, eventually proliferating to the metastatic tumors, and are referred as circulating tumor cells (CTCs). Early detection and characterization of CTCs can potentially help identify therapeutic targets, detect drug resistance mechanism, early detection of cancer, evaluate metastatic risk, and study tumor evolution and its heterogeneity [55, 56]. Most of the GBC cases are diagnosed in advanced stages when survival is poor. The only treatment module for advanced cases is palliative; an early knowledge of treatment response would benefit cancer management. Till date, there are only countable studies, which have successfully isolated CTCs from GBC. CTCs were detected and enumerated using cell surface markers like EpCAM, cytokeratins 8, 18, and 19 using CellSearch® assays [57]. However, a large number of cases are needed to validate the prognostic significance of CTCs in biliary cancers. Authors have successfully detected CTCs using the EasySep™ Direct Human CTC Enrichment kit (Stemcell Technologies) in 25 of the 27 GBC cases [58]. This kit targets hematopoietic cells and platelets with antibodies targeted to recognize CD2, CD14, CD16, CD19, CD45, CD61, CD66b, and Glycophorin A markers. While the field of CTCs is expanding, the possibility of finding multiple cell surface markers during different transition states is a reality. The presence of CD61 marker on hybrid mesenchymal cells and CTCs limit the exclusively of CTCs based biomarker studies. However, with growing knowledge on cell surface marker expression, precision diagnostic with CTCs will become the next game changer [59].

17.2.6.2 Ct-DNA

The tumor-derived fraction of the cell-free DNA is known as circulating tumor DNA (ctDNA). ctDNA has gained much importance as a minimally invasive tumor biomarker for cancer patients. The majority of the studies rely on the potential use of ctDNA in the detection of specific mutations in plasma or serum of cancer patients. The mutations detected in ctDNA could be extrapolated in early cancer

detection, prognosis, monitoring response, and to assess drug resistance. Although studying ctDNA is an attractive diagnostic approach, obtaining sufficient cytologic material is difficult partly due to late diagnosis of GBC. In a recent study, the role of serum cfDNA was assessed in the diagnosis of GBC in 34 patients [59]. The authors observed significantly lower cfDNA in cholecystitis controls and healthy subjects compared to the GBC cases.

Additionally, cfDNA was significantly associated with conditions such as jaundice, metastatic lymph nodes, and GBC stage. Thus, quantitative analysis of cfDNA could serve as a noninvasive marker in GBC diagnosis [60]. Another study suggested the useability of bilect DNA for GBC diagnosis. In spite of 85% concordance in mutations between bile ctDNA and GBC tissue DNA, no driver mutations—sequential changes leading to cancer—were identified. Due to its deep anatomical position, problems arise in obtaining sufficient gallbladder biopsy samples, and in view of this, performing liquid biopsy of bile might be an ideal scenario [61].

17.3 Future Perspectives

Gallbladder cancer is a genetically heterogeneous disease. Multiple genetic and epigenetic factors have been associated with GBC. In the era of next-generation sequencing, molecular characterization of GBC using omics technologies has led to the identification of many novel mutations, dysregulated proteins, epigenetic alterations, and aberrant activation of pathways in GBC patients. All these approaches help in the management of GBC towards personalized therapy. There are very few studies exploring the utilization of liquid biopsies as an early noninvasive diagnosis of GBC. There is a need to advance technology and validate these techniques in larger cohort samples, especially targeted therapies that aimed at altered pathways leading to cancer metastasis.

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Anti-EGFR Therapy in Gallbladder Cancer

18

Lovenish Bains and Tanuj Chawla

18.1 Introduction

Epidermal growth factor receptor (EGFR) is a cell-surface receptor and it belongs to a family of ERbB receptors. This family is comprised of four homologous receptors: the EGFR (ErbB1/EGFr/HER1), ErbB2 (HER2/neu), ErbB3 (HER3), and ErbB4 (HER4). These receptors have an extracellular binding domain, a transmembrane lipophilic segment, and an intracellular protein tyrosine kinase domain with a regulatory carboxyl terminal segment [1].

EGFR activation stimulates multiple intracellular downstream signaling cascades, including RAS/RAF, ERK/MAPK, PI3K-AKT, SRC, PLC-γ1-PKC, JNK, and JAK-STAT pathways, which mediates cancer proliferation, angiogenesis, cell motility, adhesion, and metastasis [2].

The EGFR is normally expressed in many epithelial tissues, including the skin and hair follicle and is also detected in many human cancers including head and neck, colon, and rectum [3]. EGFR is aberrantly activated by various mechanisms such as receptor overexpression, mutation, ligand-dependent receptor dimerization, ligand-independent activation. Interaction of EGFR with its normal ligands (e.g., EGF, transforming growth factor-alpha) leads to phosphorylation and activation of a series of intracellular proteins, which regulate transcription of genes involved with cellular growth and survival, motility, and proliferation.

The EGFR expression in gallbladder cancer (GBC) is highly variable ranging from 11 to 100% in various studies [4, 5]. Most of the first line chemotherapy in biliary tract cancers (BTC) are Gemcitabine-based. In 2016, a meta-analysis by Chen

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et al. [6] showed that combining EGFR-targeted therapy with standard Gemcitabine and platinum-based chemotherapy is a safe option and can improve the progression free survival (PFS), and overall response rate (ORR) in patients with advanced BTC. However, the meta-analysis by Rizzo et al. in 2020 [7] found that the addition of EGFR-monoclonal antibodies to Gemcitabine in advanced BTC, including GBC, are more toxic and does not provide any statistically significant benefit in overall survival (OS), PFS, and ORR. Zang et al. [8] also showed that median survival time in high EGFR expression GBC group was almost half than the low expression group.

18.2 What Are EGFR Inhibitors?

EGFR, also known as ErbB-1 or HER-1, inhibitors are drugs that bind to certain parts of the EGFR and slow down or stop cell growth. EGFR is a protein that is found on the surface of some cells that causes cells to divide when epidermal growth factor binds to it. EGFR is found at abnormally high levels in cancer cells, and its activation appears to be important in tumor growth and progression. Some types of cancers show mutations in their EGFRs, which may cause unregulated cell division through continual or abnormal activation of the EGFR.

EGFR inhibitors can be classified as either:

- Tyrosine kinase inhibitors (TKI) (e.g., erlotinib, gefitinib): these bind to the tyrosine kinase domain in the epidermal growth factor receptor and stop the activity of the EGFR.
- Monoclonal antibodies (e.g., cetuximab, panitumumab): these bind to the extracellular component of the EGFR and prevent epidermal growth factor from binding to its own receptor, therefore preventing cell division.

EGFR mutation was an independent prognostic marker in BTCs in addition to tumor stage and differentiation. EGFR and KRAS mutations should be evaluated when tailoring molecular-targeted therapy to patients with BTCs. The various EGFR inhibitors used in biliary tract cancer are discussed below.

18.3 Anti-EGFR Tyrosine Kinase Inhibitor

18.3.1 First Generation

18.3.1.1 Erlotinib

Erlotinib is an EGFR antagonist and selective inhibitor of several tyrosine kinase (TK) receptors which are associated with tumor growth and angiogenesis. Erlotinib was approved for FDA for medical used to treat patients with NSCLC in 2004 [9–11]. It is on the World Health Organization's List of Essential Medicines. It is a reversible inhibitor of the EGFR tyrosine kinase, competitively inhibiting ATP binding at the active site of the kinase. Erlotinib has an IC50 of 2 nM for EGFR

kinase activity whereas protein kinases have range of 20 μM. It is 60% absorbed with bioavailability of 60% which is increased by food intake to almost 100%. Simultaneous use of proton pump inhibitors decreases the bioavailability of erlotinib by 50%. It peaks in plasma at 4 h. with half-life of 36 h. Erlotinib is metabolized in the liver through the cytochrome P450 system (largely CYP 3A4) and to a lesser extent by CYP1A2, and the extrahepatic isoform CYP1A1. It is excreted 83% in feces and 8% in urine [12]. Smoking accelerates clearance of erlotinib and may decrease its anti-tumor effects. Erlotinib is used in treatment of patients with advanced or metastatic Non-small cell lung cancer (NSCLC) after failure of platinum-based treatment and in combination therapies for advanced, unresectable, or metastatic pancreatic cancer and biliary cancer. Side effects include fatigue, rash, diarrhea, anorexia, skin discoloration, hand-foot syndrome, edema, muscle cramps, arthralgias, headache, abdominal discomfort, anemia, cough, and pruritus. Uncommon, but potentially severe side effects include heart failure, interstitial lung disease, gastrointestinal perforation, pancreatitis, hemolytic anemia, renal failure, and severe skin reactions [12, 13] (Fig. 18.1).

Erlotinib can cause liver injury or serum amino transferase elevation which varies in severity from minor, transient serum enzyme elevations to acute symptomatic hepatitis and rarely acute liver failure. The abnormalities are usually asymptomatic and self-limited but may require dose adjustment or discontinuation. These enzyme elevations above five times the upper limit of normal should lead to temporary

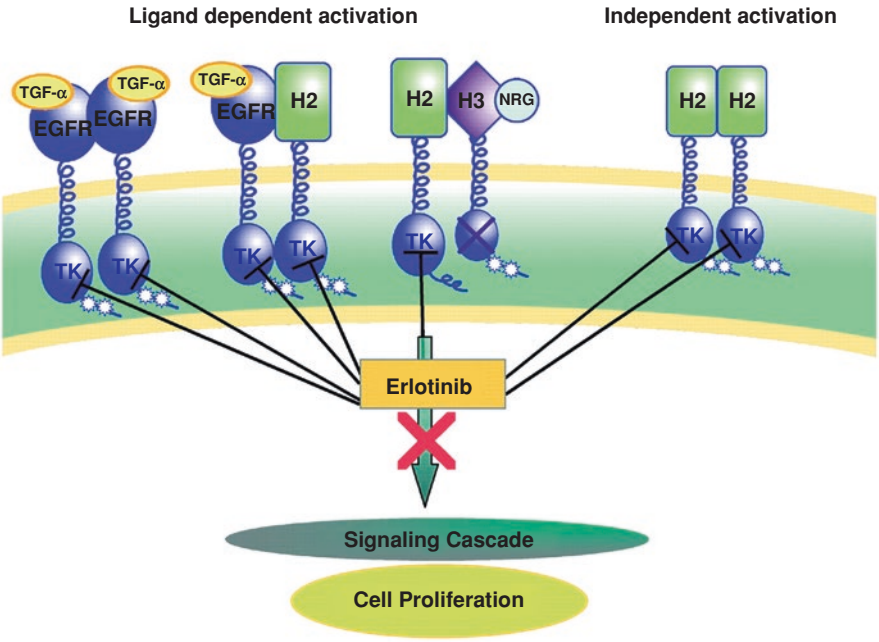

Fig. 18.1 Mechanism of action of Erlotinib (Reproduced from (Source- Schaefer G et al. [14]) with permission from American Association for Cancer Research © 2007)

discontinuation, and permanent if does not improve significantly or resolve within 3 weeks. Regular monitoring of liver function tests during therapy is recommended [15].

18.3.1.2 Gefitinib

Gefitinib is an anilinoquinazoline with antineoplastic activity. Gefitinib reversibly inhibits the tyrosine kinase activity associated with the EGFR, and thus blocks intracellular signal transduction pathways emanating from this receptor implicated in the proliferation and survival of cancer cells. It competes with the binding of ATP to the tyrosine kinase domain of EGFR, thereby inhibiting receptor autophosphorylation and resulting in inhibition of signal transduction, blocking EGFR-dependent proliferation, causing cell cycle arrest and inhibiting angiogenesis. Gefitinib has been used in Japan since 2002 and received approval for use in the USA in 2009 for the treatment of advanced non-small cell lung cancer after failure of other therapies. The FDA approved gefitinib as a first line treatment for NSCLC in 2015 [12, 16, 17].

It is 60% absorbed and reaches peak plasma concentration in 3–6 h. Its half-life is 48 h. It is metabolized in the liver by cytochrome P450 enzymes, primarily by CYP3A4 and to a lesser extent by CYP3A5 and CYP2D6. It is excreted 86% in feces and 4% in urine. The absorption of gefitinib is not significantly altered by food but is reduced by drugs that cause elevations in gastric pH [12].

The cause of the liver injury due to gefitinib may be due to accumulation of a toxic or immunogenic intermediate. Side effects are common and include diarrhea, skin reactions, nausea, vomiting, anorexia, mouth ulcers, increases serum transaminases, conjunctivitis, dry eyes, rash, pruritus, and fatigue. Uncommon serious side effects include interstitial lung disease, keratitis, corneal erosions, and nail disorders. Patients using warfarin should be monitored for poorer extrinsic coagulation (elevated INR) while taking gefitinib [18].

Second-generation EGFR TKIs were developed in an attempt to overcome some of the issues related to acquired resistance to the first-generation agents. Two key features of second-generation EGFR TKIs, which distinguish them from first generation agents, are:

1. their irreversible mode of binding,
2. their broader activity against human epidermal growth factor receptor (HER) family members.

18.3.2 Second Generation

18.3.2.1 Afatinib

Afatinib is an oral, selective inhibitor of the receptor tyrosine kinases of the ErbB family. It irreversibly binds to cysteine 797 of the EGFR and the corresponding cysteines 805 and 803 in HER2 and HER4, respectively, leading to reduced auto- and transphosphorylation within the ErbB dimer and inhibition of the consecutive pathways [19, 20]. It covalently binds to the kinase domains of EGFR (ErbB1),

HER2 (ErbB2), and HER4 (ErbB4) and irreversibly inhibits tyrosine kinase autophosphorylation, resulting in downregulation of ErbB signaling. Afatinib induces apoptosis and reduces tumor cell growth in vitro and in vivo [21]. It was approved by the FDA in 2013 for the treatment of advanced non-small-cell lung cancer.

It is 60% absorbed with bioavailability of 92%. It peaks in plasma at 2–5 h. with half-life of 37 h. It is excreted 95% in feces and 4% in urine. Food intake reduces the systemic exposure to afatinib significantly therefore it is better to take it empty stomach. Side effects include diarrhea that results in dehydration with or without renal impairment. Patients with severe renal impairment will need dose reduction. It may increase risk for sunburn/phototoxicity and may worsen rash or acne or cause skin reactions. Dry skin, hypokalemia, decreased appetite, pruritus, nausea, epistaxis, decreased weight, stomatitis are among common side effects. Hepatotoxicity, keratitis, Interstitial lung disease (ILD) or ILD-like adverse reactions are uncommonly reported. The patients with gastrointestinal ulceration, underlying diverticular disease or bowel metastases may be at increased risk of perforation [12, 22, 23].

18.3.2.2 Dacomitinib

Dacomitinib (Vizimpro®) is an orally administered, small-molecule irreversible kinase inhibitor of the human EGFR family (EGFR/HER1, HER2, and HER4) and certain EGFR-activating mutations (exon 19 deletion or the exon 21 L858R substitution mutation) that was developed by Pfizer Inc. for the treatment of solid tumors. Dacomitinib exerts its effect on HER-family tyrosine kinases by irreversibly (covalently) binding at a site within the ATP binding pocket of the kinases leading to dose-dependent inhibition of EGFR and HER2 autophosphorylation and tumor growth [24]. Dacomitinib was approved in 2018 by USA for first line treatment of patients with metastatic NSCLC with EGFR exon 19 deletion or exon 21 L858R substitution mutations. The anti-tumor activity of dacomitinib has also been demonstrated in NSCLC, Squamous Cell Carcinoma (SCC), gastric cancer, bladder cancer, and glioblastoma and other wide range of cancers in clinical studies [25, 26]. It has bioavailability of 80% which is not affected by food intake. It peaks in plasma at 6 h. with half-life of 70 h. Hepatic metabolism is main route of clearance, with oxidation and glutathione conjugation as the major pathways. It is excreted 79% in feces (20% unchanged) and 3% in urine (<1% unchanged) [27]. Dacomitinib absorption is reduced if taken along with a proton pump inhibitor, therefore it is recommended that locally-acting antacids or an H2-receptor antagonist be used as an alternative to PPIs [28]. Adverse effects include diarrhea, nausea, skin rash, paronychia, stomatitis, anemia, hypocalcemia, decreased appetite, dry skin, hypokalemia, hypomagnesemia, stomatitis, increased liver enzymes, pruritis, cough, conjunctivitis, and palmar-plantar erythrodysesthesia syndrome [12].

18.3.2.3 Lapatinib

Lapatinib (Tykerb; GlaxoSmithKline) is one of the first dual inhibitors of epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) tyrosine kinases. It is a synthetic, orally-active quinazoline with potential antineoplastic properties which was approved by the US Food and Drug

Administration (FDA) in 2007. It binds to the ATP binding site of protein kinases and competes with the ATP substrate, thus blocking receptor phosphorylation and activation, preventing subsequent downstream signaling events. It reversibly blocks epidermal growth factor receptor (EGFR), ErbB2, also the Erk-1 and-2, AKT kinases and inhibits cyclin D protein [29, 30]. It is indicated for use in combination along with capecitabine for the treatment of patients with advanced breast cancer or metastatic breast cancer whereas it's efficacy in other malignancies that overexpress EGFR and/or HER2 is under evaluation. It is metabolized by the cytochrome P450 3A4 isozyme, with one metabolite remaining active against EGFR but not HER2. The single-dose terminal half-life is 14.2 h whereas due to drug accumulation, the half-life of lapatinib is 24 h with continuous dosing [30]. The most common side effects are diarrhea, hand-foot syndrome, nausea, fatigue, and rash. QT prolongation has been observed with the use of lapatinib ditosylate and reversible decreased left ventricular function are found when used in combination with capecitabine [30, 31].

18.4 Anti-EGFR Antibody

18.4.1 Cetuximab

It is a recombinant chimeric IgG1 monoclonal antibody which binds to the extracellular domain III of EGFR and prevents ligand-dependent signaling and receptor dimerization, thereby blocking cell growth and survival signals. It binds with nearly tenfold higher affinity to EGFR than normal ligands and prevents both homodimerization and heterodimerization of the EGFR, which leads to inhibition of autophosphorylation and inhibition of EGFR signaling [12, 32]. The half-life of cetuximab is about 5 days. Adverse effects are pruritus, dry skin, acne form skin rash, fatigue, peripheral sensory neuropathy, insomnia, hypomagnesemia, diarrhea, asthenia, dyspnea, cough, generalized malaise, and paronychial inflammation with swelling of the nails. The infusion-related symptoms include fever, chills, flushing, urticaria, headache, bronchospasm, fatigue, dyspnea, angioedema, and hypotension. Pulmonary toxicity in the form of ILD is uncommon [12]. It has been approved by US-FDA for RAS wild type metastatic colorectal cancers (CRCs) and is a current standard first line therapy for this disease.

18.4.2 Panitumumab

Panitumumab is the first fully human monoclonal antibody (recombinant) to be found effective for treatment of solid-tumor cancers. Panitumumab works by binding to the extracellular domain of the EGFR preventing its activation. It binds with nearly 40-fold higher affinity to EGFR than normal ligands, which results in

inhibition of EGFR. It prevents both homodimerization and heterodimerization of the EGFR, which leads to inhibition of autophosphorylation and inhibition of EGFR signaling, resulting in inhibition of critical mitogenic and anti-apoptotic signals involved in proliferation, growth, invasion/metastasis, and angiogenesis.

It was approved in 2006 by the U.S. Food and Drug Administration (FDA) for the treatment of RAS wild type metastatic CRC with disease progression despite prior treatment. Its half-life is about 6–7 days. The incidence of infusion reactions is lower when compared with cetuximab, as panitumumab is a fully human antibody. Adverse effects include pruritus, dry skin with mainly a pustular, acneiform skin rash, Infusion-related symptoms with fever, chills, urticaria, flushing, and headache, hypomagnesemia, diarrhea, asthenia, and generalized malaise. Ocular toxicity or keratitis, pulmonary fibrosis, and interstitial lung disease were observed in clinical trials [12, 33, 34].

18.4.3 Vandetanib

It is a tyrosine kinase inhibitor (TKI) with selective activity against RET, VEGFR-2, and EGFR. The inhibition of these receptor tyrosine kinases results in inhibition of critical signaling pathways involved in proliferation, growth, invasion/metastasis, and angiogenesis. Vandetanib was approved by the FDA in 2011, for treatment of late-stage thyroid cancer. It achieves peak plasma concentration in 6 h. with elimination half-life of >100 h and is unaffected by food. It is metabolized in the liver, essentially by CYP3A4. It is excreted in feces (45%) and in urine (25%) [35]. Adverse effects are skin reactions with rash, acne, dry skin, dermatitis, pruritis, photosensitivity reactions, and palmar-plantar erythrodysesthesia syndrome. Diarrhea, nausea/vomiting, fatigue, hypertension, bleeding complications, QT prolongation and Torsades de Pointes, cystitis, low thyroid hormone levels, hypokalemia, hypercalcemia, hyperglycemia, balance disorders, changes in sense of taste, visual impairment are also found. ILD or pneumonitis, seizures, headache, visual disturbances, confusion, or altered mental function are uncommon ones [36].

18.4.4 Varlitinib

Varlitinib is a selective and potent ErbB1 (EGFR) and ErbB2(HER2) inhibitor with IC50 of 7 nM and 2 nM, respectively. It is an orally bio available, reversible ATP-competitive inhibitor of the epidermal growth factor receptor family with potential antineoplastic activity resulting in inhibition of the associated signal transduction pathways, inhibition of cellular proliferation and cell death [37] In 2011, the drug was used for treatment of solid tumors and in 2015, it was used in U.S. for the treatment of cholangiocarcinoma.

18.4.5 Neratinib

Neratinib is an orally available dual inhibitor of the epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (Her2) and kinases. It inhibits them by covalently binding with a cysteine side chain in those proteins. It was approved by US FDA in Feb 2020 in combination with capecitabine for adult patients with advanced or metastatic HER2-positive breast cancer [38]. The most common side effect is diarrhea which affects nearly all patients. Other common side effects include nausea, vomiting, decreased appetite, tiredness, back pain, rash, renal impairment, stomatitis, and muscle spasms.

18.5 Role of Anti- EGFR Therapy in Gallbladder Cancer

Erlotinib has been tried in both GBCs and non-gallbladder BTCs as first as well as second line palliative chemotherapy. One case report showed a complete and durable response as first line along with Gemcitabine [39] without any EGFR mutation while another report showed good response to Erlotinib with chemotherapy with single EGFR mutation [40]. In a phase II study published in 2006, most of the BTC had EGFR expression and showed response to Erlotinib as second line therapy [41] with an acceptable toxicity. A Phase III trial published by Lee et al. in 2012, showed anti-tumor response in CCA but failed to improve ORR in patients with GBC [42]. Although, the study did not reach in primary endpoint, i.e., PFS, but patients with cholangiocarcinoma improved PFS to almost double (5.9 vs. 3 months). Another phase II study in hepatobiliary cancers suggested that tumor's molecular and genetic profiling is important in planning studies in these cancers [43]. The toxicity profile of Erlotinib either alone or with combination chemotherapy was, although manageable but without much clinical benefit and better molecular characteristics need to be defined for it to come to clinics. Due to above reasons the drug has not been well accepted as a therapeutic option for both first and subsequent lines of therapy in advanced/metastatic BTCs.

Unlike Erlotinib, Gefitinib has not been represented much in clinical studies done on BTCs. A preclinical study in 2012 [44] showed that combination of Gefitinib and Gemcitabine may have anti-tumoral effect on some cell lines (HuCCT1) of cholangiocarcinoma but studies on gallbladder cancer are scarce. Gefitinib has not been well represented in the management of advanced BTC/GBCs. Preclinical studies on Dacomitinib (PF00299804) have shown some promise on various BTC cell lines both as monotherapy as well as in combination with Gemcitabine [45]. We are hoping that it shows safety and efficacy in clinical trials as well.

Cetuximab has also been tried in various phase II trials [46–48] as an adjunct to chemotherapy but it has failed to make a mark in all except one small study which gave some promise [46]. A single arm phase II trial [46], published in 2013, recruited

34 patients (10 GBCs) where patients received Cetuximab along with Gemcitabine and Capecitabine. In this trial Cetuximab was well tolerated and showed promising results. A year later, another phase II trial [47] with comparative arms was published which enrolled total 150 patients (22 patients had GBC) to compare cetuximab plus chemotherapy (Gemcitabine with Oxaliplatin) with chemotherapy alone as first line therapy in BTCs. Addition of cetuximab did not improve the tumor response, PFS and OS. EGFR overexpression, KRAS or BRAF mutations seemed to have no correlation with response to therapy. Subsequently, a study [48] with patients stratified by KRAS status was done with similar design as mentioned above. Here, again, the addition of cetuximab did not impact the ORR and PFS. Although, this phase II trial showed that patients with KRAS wild type had slightly better response and survival than those with KRAS mutated ones, regardless of treatment group.

Cetuximab is not used currently used for advanced BTCs since the results of the studies are not very encouraging. Panitumumab has also been tested in BTC and has not shown any improvement in PFS or OS. A single arm phase II study [49], published in 2013, with primary objective of evaluating 5-month PFS combined Panitumumab with Gemcitabine and Irinotecan. Out of total 35 patients, two patients had complete response (CR) and nine had partial response (PR) which showed that this combination is active. In about half of the tumors, no EGFR or BRAF mutations were found while seven had KRAS mutations but there was no difference in OS by KRAS status. Since this study was single arm with no statistical difference in PFS and OS more studies were warranted to prove efficacy of Panitumumab. Another two single arm studies, one on Australian patients [50] and another by Hezel et al. [51], with KRAS WT tumors showed some efficacy.

The Vecti-Bil [52] study was testing Panitumumab along with the first line chemotherapy in patients with BTC (where ~31% were GBCs) having KRAS wild type (exon 2). In a subgroup analysis, IHCC showed some likely improvement in OS, however, it was not backed by statistics. Although Panitumumab was well tolerated but there was no significant benefit in PFS or OS.

Recently published phase II [53] trial also tried to show activity of Panitumumab with combination chemotherapy in KRAS WT tumors. The primary endpoint of PFS was not met because of which it did not qualify for the phase III trial. Although, Panitumumab showed some activity and was well tolerated in the above-mentioned phase II trials irrespective of KRAS status. Lapatinib is a dual inhibitor of EGFR and HER2/neu. Since both EGFR and HER2/Neu has been implicated in the carcinogenesis of BTCs, this drug seemed a perfect candidate to be tried against it. Two phase II studies [54, 55] were done but were closed prematurely due to poor accrual. Study by Peck et al. with the primary endpoint as ORR was done with only nine patients [54]. It did show any activity of lapatinib as a single agent. The mPFS and mOS were only 2.6 months and 5.1 months, respectively. Both studies, although closed prematurely, failed to show any clinically meaningful benefit ion BTCs.

Vandetanib (inhibits RET, VEGFR-2, and EGFR blocking angiogenesis and cellular proliferation) as first line therapy in BTCs was published in 2015 [56]. There

were three arms in the study, i.e. Vandetanib (V) alone vs. V plus Gemcitabine (G) vs. G plus Placebo (P). Although there was no statistical difference in the primary endpoint, i.e. PFS but ORR was significantly better in the V plus G group. OS was slightly longer in the placebo containing arm but it lacked statistical significance. The study did not confirm the activity of V in BTCs (including GBCs) to progress toward Phase III trial. Like Vandetanib, Afatinib is also an oral agent, which irreversibly inhibits panErbB family (EGFR, HER2neu, HER4) receptors. This drug is being used for the treatment of non-small-cell lung cancer (NSCLC). Afatinib was tried in a phase II study [57] in various EGFR naïve cancers (including BTC/GBC) but was terminated early due to poor recruitment. This low patient number made interpretation of the findings difficult. Since additive anti-tumor activity of Afatinib with Gemcitabine was already established, a phase I trial of Afatinib in combination with Gemcitabine and Cisplatin was done in patients with Cholangiocarcinoma (CCA) to determine dose limiting toxicities [58]. Here, Afatinib was safely administered, but failed to show any survival advantage in advanced CCA in all nine patients enrolled. There was overexpression of EGFR in almost all tumor tissues, and none of them expressed mutations in Exons 18, 19, and 21. There was a high variation of VEGF-C, -D, leptin, and sEGFR in sera of non-responders. It was then hypothesized that these pathways may be explored as drug target in this disease.

Varlitinib is another small molecule which has been tried in advanced BTCs. Recently, a phase II trial in second line settings was done with primary endpoints of ORR and PFS [59]. In this study, Varlitinib was combined with another oral agent, i.e. Capecitabine. The comparator arm was Capecitabine with placebo. Although the combination with Varlitinib was well tolerated but did not improve ORR, PFS or OS. Patients with GBC and female sex had better median PFS with Varlitinib in the exploratory analysis. Neratinib is another irreversible panHER TKI which is being tried in a Phase II basket (multi-histology) trial (ClinicalTrials.gov NCT01953926). Patients were given neratinib (240 mg oral daily) who had activating somatic HER2 mutations, including GBCs [60]. Disease control was observed in both CCA and GBC with HER2 mutations. Although, the responses in specific EGFR expressing GBCs were not reported but this multi-HER TKI gave some hope to improve survival in this aggressive disease. Analysis of other oncogenic mutations is in progress with a hope to improve outcomes in this setting.

A phase I study on BTCs, showed safety and activity of CAR-T cell therapy in EGFR positive cancer [61] after conditioning with nab-paclitaxel and cyclophosphamide. Out of total 19 patients in the study 5 had GBCs, among which 2 had SD and other 3 progressed on the therapy. The CART-EGFR cell immunotherapy showed response in EGFR positive advanced BTCs. This trial opened another paradigm in this disease group and we will wait to see further studies being developed to explore this potential and hoping to achieve better outcomes in GBCs (Tables 18.1 and 18.2).

Table 18.1 Clinical trials of anti-EGFR in GBC conducted in past

Author (Publication year)	Drug investigated	Combination chemo	Trial phase	Line of therapy	GBCs-total *N* (%)	No. of participants: experimental vs. Control arm	Endpoints/outcomes-whether met or not
Philip et al. (2006) [41]	Erlotinib	NA	II	First/ second	16 (38)	42	PFS, ORR, OS, AE profile- Met
Lee et al. (2012) [42]	Erlotinib	Gemcitabine, oxaliplatin	III	First	82 (30.5)	135 vs. 133	PFS-not met
Malka et al. (2014) [47]	Cetuximab	Gemcitabine, oxaliplatin	II	First	22 (14.6)	76 vs. 74	PFS, OS, ORR- Not met
Chen et al. (2015) [48]	Cetuximab	Gemcitabine, oxaliplatin	II	First	14 (11.4)	62 vs. 60	ORR, PFS, OS-not met
Rubovszky et al. (2013) [46]	Cetuximab	Gemcitabine, capecitabine	II	First	10 (29.4)	34 (single arm)	ORR, CBR-Met
Sohal et al. (2013) [49]	Panitumumab	Gemcitabine, irinotecan	II	First	6 (17)	35 (single arm)	PFS, OS, ORR- Not met
Hezel et al. (2014) [51]	Panitumumab	Gemcitabine, oxaliplatin	II	First	3 (9.7)	31 (single arm)	ORR, toxicity, PFS, OS- Met
Leone et al. (2016) [52]	Panitumumab	Gemcitabine, oxaliplatin	II	First	28 (31.4)	45 vs. 44	PFS-not met
Ferraro et al. (2016) [50]	Panitumumab	Gemcitabine, cisplatin	II	First	17 (34)	48 (single arm)	CBR- Met
Amin et al. (2021) [53]	Panitumumab	Gemcitabine, oxaliplatin	II	First	NK	45 vs. 43 (Arm with bevacizumab)	PFS, OS, ORR- Not met
Santoro et al. (2014) [56]	Vandetanib	Gemcitabine	II	First	31 (18)	59 vs. 58 vs. 56	PFS, DCR, OS, DOR-not met. ORR slightly better with Vandetanib + Gem combo

(continued)

Table 18.1 (continued)

Author (Publication year)	Drug investigated	Combination chemo	Trial phase	Line of therapy	GBCs-total *N* (%)	No. of participants: experimental vs. Control arm	Endpoints/outcomes-whether met or not
Javle et al. (2020) [59]	Varlitinib	Capecitabine	II	Second	NK	64 vs. 63	ORR, PFS, OS-Not met
Harding et al. (2021) [60]	Neratinib	NA	II	Second	10 (40)	25	ORR, DoR, CBR, PFS-Partially met
Peck et al. (2012) [54]	Lapatinib	NIL	II	First/ second	NK	9	ORR- Not met

PFS progression free survival, *ORR* overall response rate, *OS* overall survival, *CBR* clinical benefit rate, *DoR* duration of response, *AE* adverse effect

Table 18.2 Ongoing clinical trials of anti-EGFR in BTC/gallbladder cancer

Trial ID	Drug investigated	Trial phase	No. of proposed participants	Combination chemo	Primary endpoint/ outcome	Secondary endpoint/ outcome
*NCT02442414	KBP-5209	I	30	NIL	Safety and tolerability	Dose-dependency of toxicity
NCT04183712	Afatinib	II	54	Gemcitabine, oxaliplatin	3 years DFS	3 years OS
NCT02836847	Cetuximab	II	152	Gemcitabine, oxaliplatin	PFS	OS, ORR, DCR, CBR
*NCT03231176	Varlitinib	II	62	Capecitabine	ORR	PFS, DoR, DCR, OS, safety
NCT04976218	TGFβR-KO CAR-EGFR T cells	I	30	NIL	TRAE	ORR, PFS
NCT04838964	MRG003	II	80	NIL	ORR	DoR, TTR, DCR, PFS, OS, AEs, PK
# NCT04209465	BDTX-189	I/II	200	NIL	DLTs, ORR	
NCT04584008	Multi drug including EGFR-TKI	NA	400	Conventional therapies	ORR	PFS, OS, TRAE

Data taken from www.clinicaltrials.gov, last accessed on 18 Nov 2022
*Study completed
#Study terminated. *OS* overall survival, *ORR* overall response rate, *DCR* disease control rate, *CBR* clinical benefit response, *DoR* duration of response, *TTR* time to recurrence, *AEs* adverse events, *PK* pharmacokinetics, *TRAE* treatment related adverse event, *DLT* dose limiting toxicity

18.6 Resistance to EGFR TKIs and Third-Generation TKIs

Majority of the tumors with activating mutations in the EGFR initially respond to first- and second-generation EGFR TKIs, however, with progression of the disease, many (60%) of NSCLC acquire a second EGFR mutation (EGFR gatekeeper residue T790M) which prevents binding of these inhibitors to the kinase domain [12]. Other mechanisms of resistance include mutational activation of downstream signaling molecules such as KRAS or activation of parallel signaling pathways, for example, through MET amplification, EML4-ALK translocation, and small cell lung cancer transformation [62]. Third-generation TKIs (Osimertinib, Olmutinib) were developed to recognize and target the T790M-mutant EGFR.

Osimertinib was the first third-generation EGFR tyrosine kinase inhibitor (TKI) to receive FDA and EMA approval for metastatic EGFR-mutant non-small-cell lung cancer (NSCLC) patients that have acquired the EGFR T790M resistance mutation. Osimertinib is an orally taken kinase inhibitor of the EGFR which can form an irreversible covalent bond via the cysteine-797 residue and T790M or other EGFR mutations. Osimertinib selectively targets EGFR-sensitizing and T790M resistance mutations while still sparing wild-type EGFR tyrosine kinase at ~ninefold lower concentrations than wild type [63, 64]. It has bioavailability of 80% and peaks in plasma at 6 h with half-life of 48 h. It is metabolized by oxidation (predominantly CYP3A). It is excreted 68% in feces, 14% in urine, and ~2% (unchanged) (Fig. 18.2).

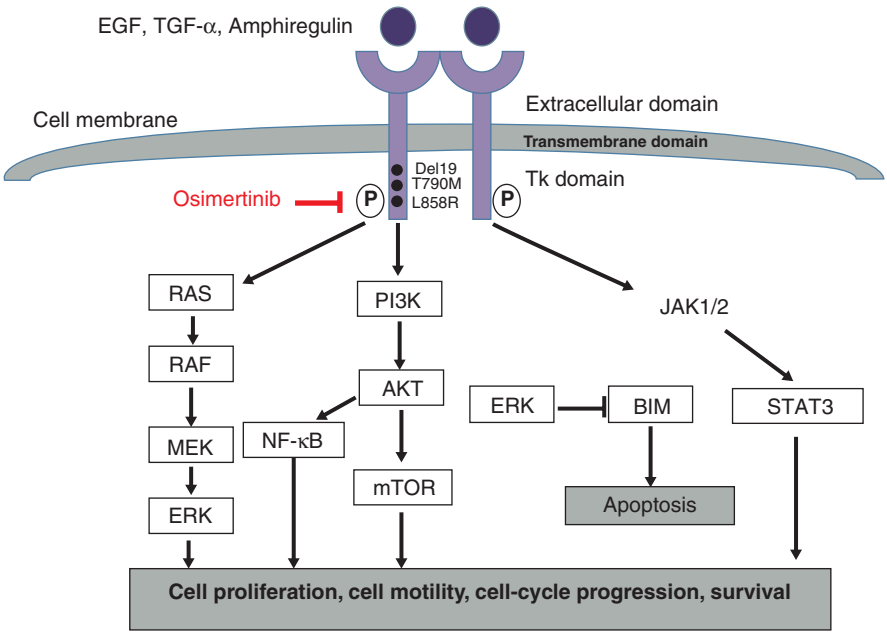

Fig. 18.2 Mechanism of action of Osimertinib. (Reproduced from (Santarpia et al. [65]) with permission from Dove Medical Press Limited © 2017)

It is emerging as the new standard of care for all EGFR positive patients as first line treatment.

Adverse effects include lymphopenia, thrombocytopenia, anemia, diarrhea, rash, neutropenia, hyponatremia, dry skin, nail changes, eye disorders, hypoglycemia, nausea, vomiting, stomatitis and pruritus. Keratitis, venous thromboembolism, QT prolongation, interstitial lung disease and cardiomyopathy are uncommon ones [66].

Olmutinib is an oral, third-generation EGFR TKI that is developed by Boehringer Ingelheim and Hanmi Pharmaceutical Co. Ltd. It selectively and irreversibly binds and inhibits epidermal growth factor receptors (EGFR) with the T790M activating mutation. Olmutinib covalently binds a cysteine residue near the kinase domain of mutant EGFRs to prevent phosphorylation of the receptor 7. This inhibits receptor signaling as phosphorylation is necessary for recruitment of signaling cascade proteins. US FDA approved Olmutinib in 2015 and in 2016, South Korea approved olmutinib. Its half-life is 8–11 h [67, 68].

Despite its efficacy, resistance to osimertinib inevitably develops and mechanisms of resistance can be grouped broadly in two categories: on-target EGFR-dependent and off-target EGFR-independent mechanisms. EGFR-dependent resistance is associated with additional EGFR mutations disrupting the osimertinib binding through changes in the binding site by allosteric/conformational transitions whereas EGFR-independent mechanisms are related mostly to alternate pathway activation or aberrant downstream signaling [69].

18.7 New Agents and Future Directions

Anti-EGFR therapies in GBCs keep evolving but we are yet to be convinced that it improves the survival in this cancer setting. Most of the trials have not gone beyond phase II and some of them stopped because of futility. Newer agents are being developed and tested in clinical trials. Two of the latest anti-EGFR agents are described below. The prospective studies with newer anti-EGFR drugs will layout the future roadmap in the management in advanced GBCs.

Amivantamab (Rybrevant™), a bispecific monoclonal antibody targeting epidermal growth factor receptor (EGFR) and mesenchymal epithelial transition factor (MET), which is being developed by Janssen Biotech. It received first approval in the USA, on 21 May 2021 for the treatment of adult patients with locally advanced or metastatic NSCLC harboring EGFR Exon 20 insertion mutations whose disease has progressed on or after platinum-based chemotherapy [70].

Lazertinib (LECLAZAR) is an oral, third-generation, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) being developed by Yuhan and Janssen Biotech for the treatment of non-small cell lung cancer (NSCLC). It is a brain penetrant, irreversible EGFR-TKI that targets the T790M mutation and activating EGFR mutations Ex19del and L858R, while sparing wild type-EGFR. Lazertinib received its first approval in January 2021, for the treatment of patients with EGFR T790M mutation-positive locally advanced or metastatic NSCLC who have previously received EGFR-TKI therapy. Its mean terminal half-life is 64.7 h and excreted largely in bile (60%) and feces (24%) [71].

18.8 Conclusion

The relevance of Anti-EGFR therapy in GBCs is yet to unfold. However, as science evolves, we keep striving to get signals from molecular studies to help guide treatment in this subgroup of BTCs. Targeting a single EGFR pathway may not be enough to control disease progression. More rational combinations of targeted therapies and conventional treatment which are backed by molecular and cellular studies are needed to gain success and improve survival in unresectable (locally advanced or metastatic) GBCs. With the new anti-EGFR drugs being developed we can hope to have their role in GBCs as well.

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