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Liquid biopsy in gallbladder carcinoma: Current evidence and future prospective

Sridhar Mishra [a,b], Swati Kumari [a,c], Nuzhat Husain [a,*]

[a] Department of Pathology, Dr Ram Manohar Lohia Institute of Medical Sciences, Lucknow, Uttar Pradesh, 226010, India
[b] Department of Plastic and Reconstructive Surgery, King George Medical University, Lucknow, Uttar 1pradesh, 226003, India
[c] Department of Pathology, King George Medical University, Lucknow, Uttar 1pradesh, 226003, India

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ABSTRACT

Although there have been significant advances in the early detection and treatment of gallbladder cancer (GBC), it is still considered a leading cause of morbidity and mortality. Molecular profiling of tumors is generally performed using samples obtained during surgery or biopsy. However, tissue genotyping has its limitations as it only provides a single snapshot and is susceptible to spatial selection bias due to the tumor heterogeneity. Over the past decade, there has been a remarkable transition from invasive diagnostic methods to non-invasive alternatives, including liquid biopsy, for cancer diagnosis and monitoring. Liquid biopsies have ushered in a new era in clinical oncology, enabling convenient tumor sampling, continuous monitoring through repeated analysis, development of personalized treatment regimens, and assessment of therapy resistance. While peripheral blood is the primary medium for these biopsies, other biological fluids, including urine, saliva, and bile, also serve as valuable sources of information. Currently, the focus of blood-based biopsy analyses is on four main sources of biomarkers for cancer detection and stratification: circulating tumor DNA (ctDNA) or circulating free DNA (cfDNA), circulating tumor cells (CTCs), and extracellular vesicle (EVs). There are over 300 clinical trials either ongoing or actively recruiting participants to investigate the diagnostic and prognostic applications of ctDNA/cfDNA in the context of cancer. This review outlines the current standard of care for individuals with GBC, anticipates future treatment developments, and evaluates the potential applications of liquid biopsies in various clinical contexts. The review addresses ctDNA/cfDNA, CTC, and circulating microRNA and highlights their prospective roles in management of GBC.

1. Introduction

Epidemiology and incidence of GBC: Gallbladder cancer (GBC) is the most common malignancy of the biliary tract, accounting for 80–95 % of all biliary tract cancers in many developed and developing countries, including India [1]. The highest incidence of GBC was reported in Chile and is the second most common cause of death from a malignant tumor in women compared to men [2]. Recent data from Indian Council of Medical Research, suggest India had highest incidence of GBC worldwide, with 8,00,000 new GBC cases and 5,50,000 deaths per year. The highest GBC incidence rates were reported in women from India (21.5/100,000), Chile (18.1/100,000), Pakistan (13.8/100,000), and Ecuador (12.9/100,000). Within India, western and northern cities as Uttar Pradesh, Bihar, Orissa, West Bengal and Assam have higher incidence of GBC [3]. It occurs two to six times more often in women than in men. Until 10 years ago, GBC was a rare form of tumor in India. But currently India records 7–9 cases per 1 lakh population [4]. In Indian cancer registries, age adjusted incidence of GBC is higher in females (2.3/100,000) as males (1.01/100,000). In North Indian female's incidence rate is 10–12 times higher as compared to South Indian (5.7/100,000 vs. 0–0.7/100,000 women). Delhi and Bhopal regions of India have highest incidence rate being 6.6 and 5.2 for women and 1.9 and 2.2 for men per 100,000 respectively [4,5]. It is uncommon in United States of America, Europe and Nigeria with incidence rate of 7.5 per 100,000, especially in American Indian females. In Japan, population-based registry programme incidence of GBC was 11.6/100,000 for men and 13.4/100,000 for females [6,7]. It affects females 2–6 times more frequently than in men; however, this varies in different geographical [7]. The significant differences in the incidence of GBC observed in different geographical locations suggest that, in addition to genetic,

* Corresponding author.
E-mail address: drnuzhathusain@hotmail.com (N. Husain).

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ethnic, racial, and social factors, nutritional and environmental factors are also likely to be involved in the etiology of the malignancy [8,9].

Current challenges in diagnosis of GBC using Blood-based Markers, Imaging and Histology: GBC is usually diagnosed in the late stages of the disease due to the lack of specific signs and symptoms. There are no precise and clearly defined criteria for diagnosing early-stage disease and curative surgery at time of diagnosis is possible in fewer than 20 % cases. However, in most cases the diagnosis is made in advance using ultrasound. Incidental GBC detected during cholecystectomy for benign disease varies between 0.14 % and 6.1 % depending on whether the area is low or high risk [2,10]. The tumor is usually undetectable at the time of diagnosis. Laboratory findings are also nonspecific and may include anemia, leukocytosis, and elevated bilirubin and alkaline phosphate levels. Tumor markers carcinoembryonic antigen (CEA) and Carbohydrate Antigen 19-9 (CA 19-9) may be elevated but do not play a role in the diagnosis as they have low sensitivity and specificity [11,12], (Fig. 1). Available imaging techniques include ultrasound (USG) and computed tomography (CT), magnetic resonance imaging (MRI) with magnetic resonance cholangiopancreatography (MRCP), magnetic resonance angiography (MRA), and endoscopic retrograde cholangiopancreatography (ERCP) have limited sensitivity and specificity, as shown in Fig. 2 [12–17]. These are considered essential for identifying structural changes that include bile bladder thickening, replacement of the gallbladder by a heterogeneous mass or tumor spread to the liver, extrahepatic spread, porta hepatis, portal vein, other adjacent structures or biliary stasis. In patients with jaundice, MRCP appears to be preferable to ERCP/PTC unless therapeutic intervention is planned [16]. The spread of GBC occurs through direct invasion of the tumor into the adjacent liver segments IV and V and other surrounding organs such as the duodenum, colon, anterior abdominal wall and common hepatic ducts. Early-stage carcinoma can be easily missed, with the diagnosis typically only made after microscopic analysis of paraffin-embedded samples. Impression cytology of the gallbladder mucosa represents a straightforward, rapid and effective technique for identifying GBC [18]. In addition, ultrasound-guided fine needle aspiration cytology is considered a safe diagnostic approach for GBC [19,20]. Endoscopic retrograde cholangiopancreatography of the bile duct and GBC is particularly specific and should be used to evaluate clinically suspicious lesions [21]. Cytology and histopathological examination have their own challenges and limitations [22,23]. Diagnosing GBC can be challenging because it may not be identifiable by radiographic imaging or gross pathology and often presents without a clear mass or characteristic symptoms. This can lead to underdiagnosis. In addition, some benign lesions can closely mimic GBC, making histopathological assessment difficult. Determination of CEA concentrations can provide valuable assistance in clarifying these diagnostic uncertainties.

Preoperative imaging studies play a crucial role in determining the appropriate surgical approach based on the T stage of GBC. However, T1 or T2 GBC pose significant challenges for accurate preoperative diagnosis. Pathologic confirmation is essential before initiating nonsurgical procedures. Although imaging technology has improved significantly, diagnosing GBC can be particularly difficult when it occurs in association with adenomyomatosis [24]. Therefore, a T1 or T2 GBC is often identified incidentally during routine cholecystectomy surgeries. In these cases, comprehensive pathological examinations of the entire tumor are performed to evaluate prognostic factors and determine the need for further comprehensive radical surgery. Intraoperative histologic studies are typically performed for lesions classified preoperatively as suspected GBC or potential T1 or T2 GBC. During surgery, the resected gallbladder sample, which may include en bloc liver resection (S4a+5 or liver bed), is sent for intraoperative histological analysis. Nevertheless, determining the depth of invasion by frozen section analysis of GBC remains a complex challenge and requires caution so as not to hinder the pathological assessment of formalin-fixed samples, especially when the tumor is small. While endoscopic imaging and tissue sampling can be beneficial, biopsy samples often prove inadequate for molecular profiling, and tissue sampling has been reported to have high specificity but low sensitivity in diagnostic accuracy. Therefore, there is an urgent need to develop innovative strategies aimed at facilitating the early diagnosis of GBC at the resectable stage and ensuring the procurement of sufficient material for genomic analysis.

Liquid Biopsy: The advent of liquid biopsy represents a significant advance in non-invasive biomarkers, enabling early detection of cancer, tracking its progression and assessing response to treatment. Liquid biopsy includes cfDNA, CTCs, circulating microRNAs, circulating proteins and EVs. CfDNA and CTC are the mainstays of liquid biopsy-based studies (Fig. 3). CfDNA & CTCs serve as non-invasive tests for molecular analysis in pre-cancer & cancer and quantification may help to optimized medical practise, personalized medicine and improve quality of life. Elevated levels of cfDNA have been reported in various malignancies when compared to healthy individuals such as in lung, ovarian, cervical, breast, colon, head and neck, gastric, bladder, prostate, testis, Pancreatic, Gliomas and hepatocellular cancer [25]. However, cfDNA levels have not been investigated in GBC so far. CTCs are extremely rare and occur in peripheral blood in a ratio of about 1 CTC per 10^6-10^8 leukocytes. Detection and quantification of CTCs may also help in identification of cancer patients at high risk of metastatic relapse and for more accurate tumor staging. In gastrointestinal malignancies CTC has been studied in colonic cancers, gastric carcinoma, Esophageal cancer, pancreatic carcinomas, and hepatocellular carcinoma [26]. CTC detection in blood serves as a liquid biopsy that is useful for numerous prognostic applications and would avoid the need for tumor tissue biopsies. The use of such a liquid biopsy offers the possibility of repeatedly

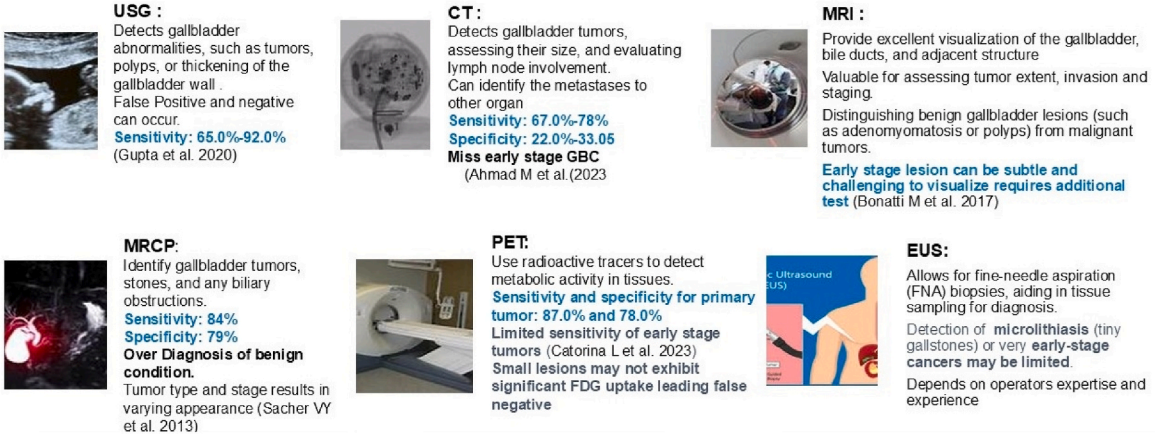

Fig. 1. Imaging methods used for early diagnosis of GBC with detection sensitivity.

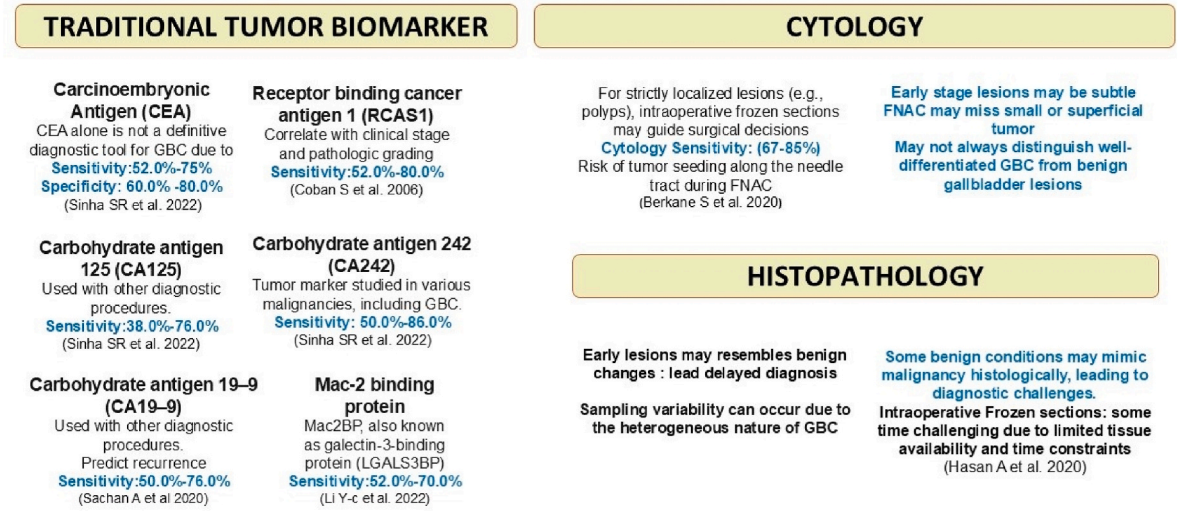

Fig. 2. Early detection: Diagnostic dilemma's using tumor markers, cytology and histopathology.

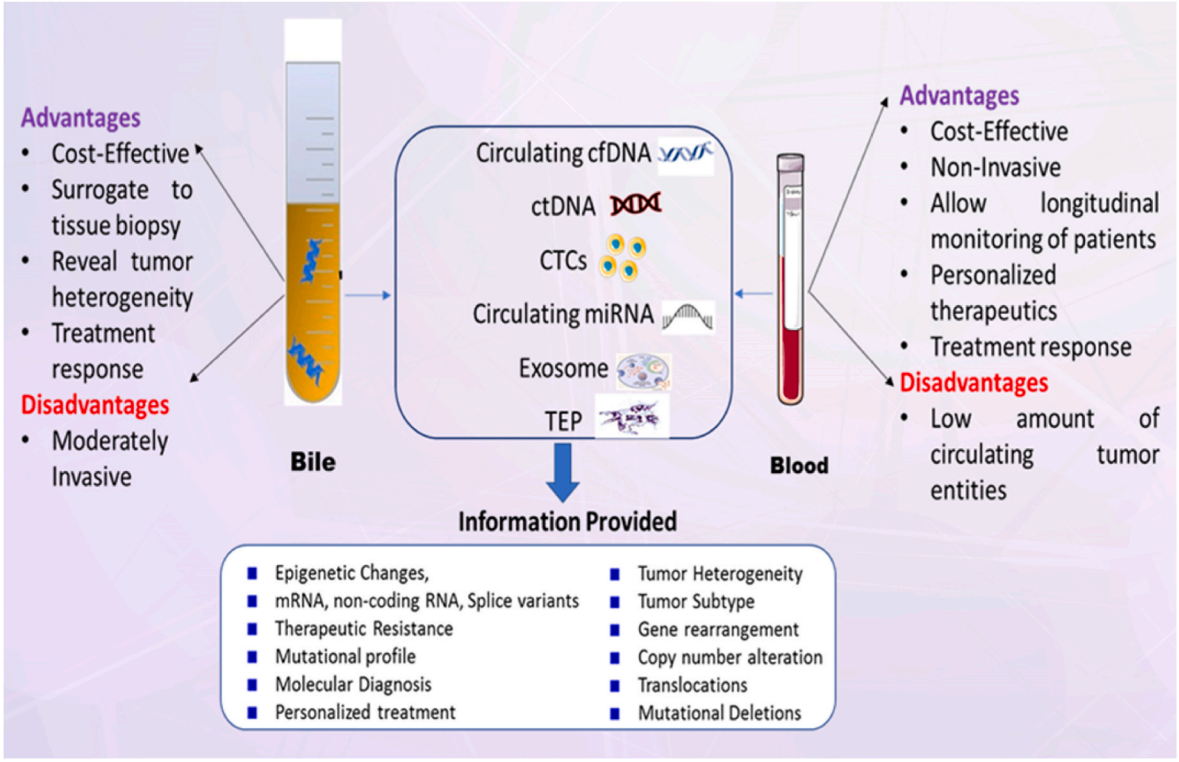

Fig. 3. Liquid biopsy component.

taking blood samples and thus tracking the changes in the CTC during the natural course of metastasis or during cancer treatment. CTC is an alternative to invasive biopsy for early detection of metastatic tumors and monitoring response to treatment as well as determining additional treatments [27]. Liquid biopsy measurement of CTC has emerged as a better alternative to invasive biopsy for predicting and monitoring treatment response in metastatic cancer. The presence of CTC in cancer patients with either metastatic or apparently localized disease is an important indicator of metastatic disease and poor prognosis. These cells could represent new targets for therapy to prevent their spread to distant locations. For clinical purposes, collecting blood samples for analysis of CTCs without surgical intervention is a more attractive alternative. CTCs have been characterized as epithelial tumor cells with a high nuclear-to-cytoplasmic ratio with irregular nuclear shape, large size and non-proliferative nature [28,29].

Noncoding RNAs, especially long noncoding RNAs (lncRNAs), are crucial in both physiological and pathological processes. Research has shown that lncRNAs influence key cancer-related signalling pathways in GBC, including *WNT/-catenin*, *PI3K/Akt, EGFR, NOTCH*, mTOR and *TP53*, and thus play a significant role in the full spectrum of GBC carcinogenesis and tumor progression [30]. Furthermore, the high stability of lncRNAs allows their detection in various body fluids, making them promising candidates for biomarkers in early diagnosis and prognosis. MicroRNAs (miRNAs), which are small non-coding RNAs, are also crucial in regulating gene expression through transcriptional interference and are involved in numerous biological processes,

including tumor initiation and progression [31].

1.1. Molecular Biomarkers of GBC

GBC is a globally prevalent biliary tract malignancy that often occurs in advanced stages and results in poor overall survival rates. The incidence of GBC shows significant regional variation, with geographical factors playing a crucial role in the pathogenesis of the disease. A comprehensive understanding of the molecular genetic alterations associated with GBC is crucial for elucidating the disease mechanisms and for developing future clinical trials targeting specific signalling pathways. Research on other types of cancer has shown that mutation patterns can have significant effects on disease progression and treatment outcomes. The genomic characteristics and molecular characteristics of bile duct cancer, including GBC, have been well documented in various studies [32–35]. In addition, specific gene mutations in GBC have been identified through whole exome sequencing of tumor samples [36,37] (Fig. 4).

In our previous research, we identified targetable somatic mutations in 90.0 % of GBC cases. This highlights the importance of early detection of somatic genetic mutations in GBC, which may facilitate the identification of more personalized therapeutic targets. Genomic profiling can improve our understanding of cancer-causing processes, aid in classification, and inform treatment strategies [38]. Identifying specific mutations during cancer progression could pave the way for novel treatment approaches for GBC. Barreto et al., have developed a genetic model for GBC based on specific gene changes observed during tumor development [39]. This helps predict the risk of GBC and can guide decision-making to perform gallbladder removal before disease manifestation. Furthermore, in a study by Simbolo et al., examined 56 cancer-related genes in 26 GBC cases, 70 intrahepatic cholangiocarcinoma (ICC), and 57 extrahepatic cholangiocarcinoma (ECC) and showed that 68 % of tumors had targetable pathway variations [40].

In GBC, tumor progression is characterized by a series of pathological changes, including dysplasia, in situ carcinoma, invasive adenocarcinoma, and ultimately metastasis. Each stage of this progression is associated with specific mutations that have been mapped. In particular, mutations in the ERBB2 gene have been identified as potential targets for therapeutic interventions. Current early-stage clinical trials are investigating targeted therapies for GBC that focus on various genetic alterations, including those in *SMAD4, HER2, EGFR, MET, RAS/RAF, and PIK3CA*. Research shows that alterations in the *ERBB2* gene, including both amplifications and mutations, occur in approximately 10–11 % of ECC and GBC cases. A study by Rao et al. found *HER2* overexpression in 13 % of GBC cases and correlated with worse survival outcomes [41]. Furthermore, research by Maurya SK et al., highlighted the importance of overexpression of *ERBB2 (HER-2/neu)* for early diagnosis and tumorigenesis of GBC [42]. Furthermore, findings from Javle et al., in 2015 suggested that *ERBB2* gene amplification is associated with better response to treatment compared to cases with *ERBB2* mutations [43].

A study by Szymaska et al. identified *TP53* as the most commonly mutated gene in various human cancers, including hepatocellular carcinoma and Esophageal carcinoma [44]. In a related study, Mishra et al. examined *TP53* mutations in patients with GBC and found that almost 90 % of these patients had *TP53* mutations [35]. Mutations in the *TP53* gene are associated with poor prognosis, increased metastasis and adverse effects on patient survival [45]. Furthermore, research by V DAfonseca et al., in 2020 identified *TP53* mutations as a marker indicating poor survival outcomes [46]. Despite these results, there is a notable lack of clinical trials targeting *TP53* mutations to evaluate their therapeutic potential.

The *PI3K/AKT/mTOR* signalling pathway emerges as the most commonly dysregulated signalling pathway within a range of malignancies, including breast cancer, non-small cell lung carcinoma, gastric cancer, hepatocellular carcinoma, colorectal cancer, pancreatic cancer, cholangiocarcinoma and GBC. The mutations in *PIK3CA* and *PTEN* may make tumors more susceptible to treatment with PI3K and mTOR inhibitors such as everolimus, temsorolimus and rapamycin, which are currently under clinical investigation. Phase II clinical data have demonstrated the efficacy of everolimus and rapamycin in the treatment of biliary tract cancer [47]. The prevalence of *PIK3CA* and *PTEN* mutations in GBC cases has been shown to be between 6.0-%13.0 % and 0%–7.5 %, respectively [48,49].

Research is currently focused on the genetic modifications of EGFR and its associated signalling pathways, particularly *KRAS, NRAS, and HRAS*. In studies concerning biliary tract cancer, EGFR overexpression and amplification have been observed in 20–30 % of cases [50]. Activating and resistant mutations within the *EGFR* gene are primarily

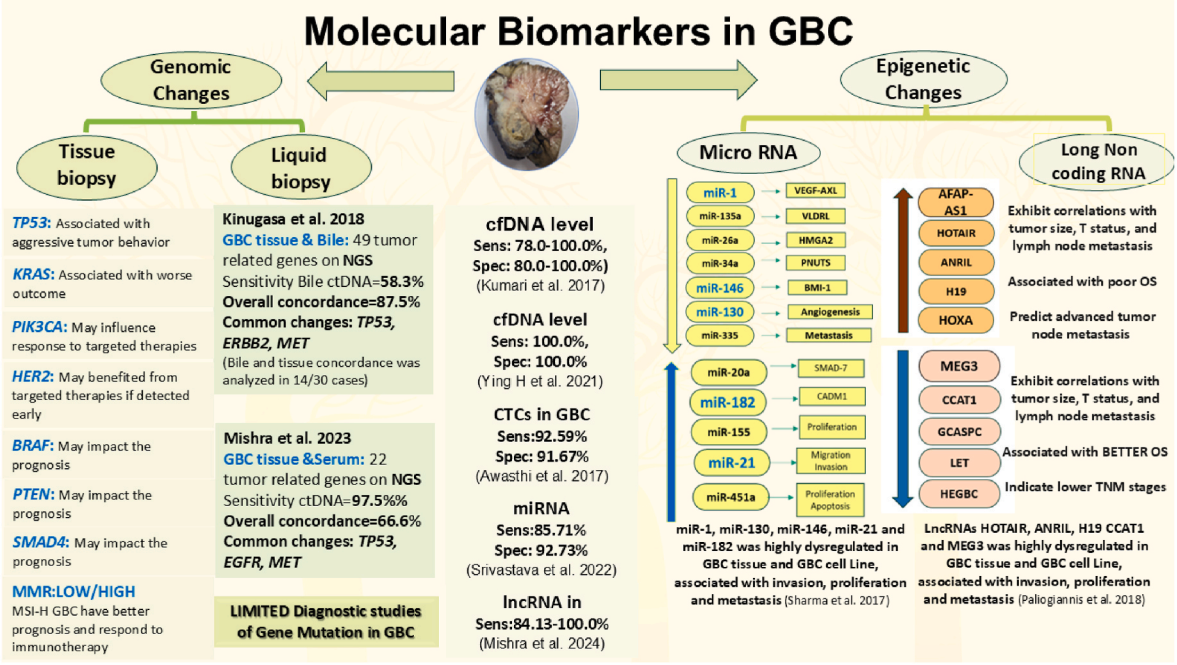

Fig. 4. Molecular biomarker of GBC

located in exons 18–21, which encode the adenosine triphosphate-binding pocket of the intracellular tyrosine kinase domain [51]. It has been reported that somatic mutations in the *EGFR* tyrosine kinase domain occur in approximately 15 % of GBC [52]. Earlier investigations into the therapeutic targeting of the *EGFR/HER2* pathways indicated that gemcitabine exhibits anti-proliferative effects in GBC, suggesting a correlation with poorer prognostic outcomes [53]. A study by Reid et al. established a link between *KRAS* mutations and the anomalous junction of the pancreaticobiliary duct (AJPBD), suggesting that *KRAS* may serve as a potential screening biomarker for GBC patients with AJPBD [54]. Additionally, Sharma et al. (2017) identified a significant association between KRAS mutations in exons 1 and 2 and advanced-stage GBC [55].

Very few studies have focused on the *BRAF* mutation in bile duct cancer. Results regarding BRAF mutation in GBC have been inconsistent. Some studies reported the absence of BRAF mutations in both bile duct cancer (BTC) and GBC [56,57]. In contrast, Saetta et al. (2004) identified a *BRAF* mutation in 33.0 % of GBC cases [58]. Furthermore, alterations in MET gene, including overexpression, amplification and mutation, have been shown to activate various signalling pathways such as *PI3K/AKT, MAPK*, Wnt/-catenin and STAT pathways [59]. Research has examined the oncogenic effects of *MET* in both intra- and extrahepatic cholangiocarcinoma and GBC, showing that *MET* overexpression in GBC ranges from 5 % to 74 % [60,61]. Furthermore, Xu et al. reported *MET* gene mutation in 4.1 % of BTC cases using NGS, with 2.6 % occurring in GBC [62].

The *SMAD4* gene has a high mutation frequency in GBC and encodes a protein that, through post-translational modifications, plays a crucial role in complex regulatory mechanisms and thereby influences various cellular processes [63]. As a tumor suppressor gene within the TGF signalling pathway, *SMAD4* is involved in several types of cancer. In particular, increased TGF levels are associated with increased tumor growth in the later stages of tumor progression. Nevertheless, there is little research specifically focused on TGF modulators or anti-SMAD4 agents in GBC. In the context of colorectal cancer, mutations in the *SMAD4* gene are associated with poor prognosis. Xu et al., identified *SMAD4* mutations as tumor suppressors in cholangiocarcinoma with a prevalence of 45.2 % in ICC cases, showing a significant association with clinical stage and prognosis [64].

The *CTNNB1* protein plays a crucial role in the Wnt/β-catenin signalling pathway. Currently, various inhibitors targeting the Wnt pathway are undergoing clinical trials for multiple tumor types, with celecoxib and sulindac being the only FDA-approved inhibitors of the Wnt/β-catenin pathway. The mutation rate of the *CTNNB1* gene has been documented to range from 4.0 % to 21.21 % [35,65]. In GBC, research showed mutation frequency for *CTNNB1* in range from 4.4 % to 9.0 % [66,67].

1.2. Molecular marker of GBC using liquid biopsy

GBC is associated with a poor prognosis and a lack of effective treatment options. Diagnoses are often only made at an advanced stage of the disease. There is limited research on the role of liquid biopsy in identifying diagnostic and prognostic biomarkers for GBC. Kim et al., analysed the *PIK3CA* mutation in metastatic bile duct cancer by digital droplet PCR and found a strong correlation for *PIK3CA* mutation status between tumor DNA and cfDNA [68]. The detection of low levels of *PIK3CA* mutations in serum suggests that cfDNA may serve as a useful source for identifying cancer-related mutations in metastatic biliary cancer [68]. Furthermore, Kinugasa et al. examined 49 oncogenes in 2018 and reported a concordance rate of 85.7 % between ctDNA from bile and tissue DNA samples, with sensitivities of 58.3 % for biliary ctDNA and 45.8 % for cytological DNA in patients with GBC [69]. A recent study by Jovelet et al. examined a hotspot gene panel in both tumor and cfDNA and found a sensitivity of 49.90 % and a specificity of 99.80 % for patients with advanced or metastatic solid tumors [70]. At the 2019 ASCO meeting, Mody K et al. presented results that delineated the genomic alteration landscape of ctDNA in bile duct cancer and suggested that 55 % of patients had targeted genetic alterations [71]. Additionally, a blood-based genomic profiling study suggested that certain patients with bile duct cancer may benefit from targeted therapies, with *TP53* and *KRAS* identified as the most commonly altered genes [72].

GBC exhibits significant genetic diversity and the exact number of driver genes involved in its carcinogenesis and tumor progression remains unclear. Examining histological and molecular subtypes can provide valuable insights into identifying different prognostic groups [73,74]. Our research group has identified various epigenetic and genetic changes, that contribute to the development and clinical behaviour of GBC [35,75–77]. Recent advances in epigenome analysis of cfDNA in serum have emerged as a minimally invasive diagnostic tool for disease classification, even in early-stage cancers [78]. In recent decades, changes in miRNA levels in serum and plasma have been recognized as non-invasive biomarkers of disease [79]. Nevertheless, the diagnostic effectiveness of individual miRNAs is limited by tumor heterogeneity [80–82]. To address this limitation, recent studies have focused on the combined expression of multiple miRNAs to improve the specificity and sensitivity of cancer diagnoses [83,84]. A study by Ueta et al., identified miR-1246, transported by extracellular vesicles in serum, as a promising diagnostic and prognostic biomarker, while miR-451a was proposed as a new therapeutic target for GBC patients [85]. There are currently no clinically established biomarkers for the diagnosis of GBC. Mishra et al. (2024) analysed a panel of five lncRNAs and observed significant changes in their expression in GBC serum, which could effectively distinguish between early and late stages of the disease [77]. The integration of two lncRNAs into diagnostic panels could provide a promising serum-based biomarker for the early diagnosis of GBC in addition to radiological examinations.

1.3. Technical limitation in adopting liquid biopsy in GBC

Liquid biopsy is being used to detect clinically relevant variants. However, monitoring treatment by quantifying cfDNA/ctDNA, CTC and exosomes is difficult because factors such as tissue origin, tumor type, stage and heterogeneity require high sensitivity, specificity, positive and negative predictive power and robustness of current technologies. Furthermore, separating the ctDNA from the cfDNA (mixture of non-mutant tumor DNA, normal DNA and tumor DNA) is challenging depending on the grade and stage of the tumor. The false negative rate is related to allele frequency and tumor heterogeneity. Early-stage tumors have a very low frequency of different alleles, which increases the diagnostic power of the assay to detect these variants [86,87]. The ctDNA used for genetic testing represents a very small portion of the tumor, sometimes as little as 0.01 %. Another component of liquid biopsy, known as CTC, is present in low abundance in early-stage tumors, making detection and quantification difficult. Another limiting factor is the larger blood volume required for CTC detection. Targeted analysis, sequencing, and molecular characterization of CTCs and cfDNA may only capture a limited aspect of tumor intratumoral heterogeneity. Attempting to fully represent this heterogeneity through ctDNA runs the risk of including DNA from normal cells, which may obscure the true nature of the tumor due to the background presence of leukocytes that may overlap with cancer cells in the genes of interest. In addition, the integrity of the samples, the short lifetime of the CTCs and the influence of circulating nucleic acids are crucial factors that influence the final analytical results.

It is expected that continued development of NGS technologies and other pre-analysed variables will improve both the cost-effectiveness and accuracy of ctDNA over time. However, some challenges remain that need to be addressed. It remains uncertain whether performing both blood and tumor NGS at baseline provides better insight for treatment decision-making or patient selection for clinical trials than using either

modality in isolation, particularly given the intratumoral effects observed in gastrointestinal cancers Heterogeneity. Furthermore, there is a notable lack of consensus on the most biologically relevant thresholds for ctDNA-related metrics, such as maximum variant allele frequency (maxVAF) and percent change in VAF, which are essential for guiding oncologists in clinical practice. In the context of postoperative surveillance, it is unlikely that ctDNA will completely replace conventional diagnostic and staging methods, including imaging and protein-based serum cancer markers, in the treatment of gastrointestinal cancer. Nevertheless, in clinical trials, ctDNA has the potential to accelerate drug development by enabling molecular genotyping of patients for novel targeted therapy trials, identifying early indicators of drug response, and monitoring the emergence of clonal resistance.

1.4. ctDNA/cfDNA and tissue based assay

The diagnosis of cancer is mainly based on tumor tissue biopsies and is an important tool for molecular testing [88]. However, taking a tumor tissue biopsy is invasive and anatomically difficult. Traditional tissue biopsies are not always feasible, they have to be repeated frequently, and it is not easy to obtain sufficient material of the right quality for genomic profiling of the cancer. On the other hand, ctDNA analysis can overcome the above limitations because it can capture spatial and temporal tumor heterogeneity and enable real-time monitoring. The use of liquid biopsy has emerged as a non-invasive diagnostic biomarker and has moved from the laboratory to the bedside [89]. ctDNA analysis is quick and easy compared to classic tissue biopsy, as blood, urine and saliva are easier to assess than a tissue biopsy. Liquid biopsy includes cfDNA, CTCs, circulating microRNAs, circulating proteins and extracellular vesicles. The presence of cfDNA/ctDNA has been reported from serum, plasma, induced sputum, bronchial lavage, milk, urine and stool, cerebrospinal fluid, bile, ascites and pleural fluid. Whole liquid biopsy offers a personalized oncology approach, allowing disease monitoring during treatment and minimal residual disease monitoring, and has the potential to capture tumor heterogeneity, thus providing a comprehensive view of the tumor [90–92].

1.5. Clinical applications of ctDNA/cfDNA analysis

Analysis of cfDNA is an attractive approach to interrogate the circulating cancer genome. CfDNA quantification is of greatest interest in clinical practice in oncology and prenatal diagnostics. However; cfDNA monitoring has promising value in the areas of transplant medicine, cardiovascular care, traumatology, and monitoring of certain autoimmune and microbial diseases [93]. CfDNA analysis offers advantages in characterizing molecular profiles when tissue is not available, reflects tumor heterogeneity, allows monitoring response to therapy, detecting residual disease after therapy, and assessing tumor evolution during therapy. Analysis of genetic changes in cfDNA could serve as a better alternative biomarker to traditional protein estimation such as CEA or CA19-9, which are expressed in both tumor cells and normal cells.

Significantly different and higher cfDNA levels have been reported in various malignancies, including lung, breast, colon, hepatocellular, ovarian, prostate and melanoma, than in healthy individuals [94–96]. However; Elevated cfDNA can also be found in non-cancer patients or in patients with pathological inflammation. Depending on the tumor type, there can also be significant differences in cfDNA levels [97]. This variation in cfDNA levels limits the importance of studying cfDNA in each individual cancer. CfDNA includes coding and non-coding gDNA, also enables the monitoring of tumor-specific changes and enables the detection of genetic and epigenetic changes in the patient. Analysis of microsatellite instability, loss of heterozygosity, mutations, polymorphisms, methylation and integrity can also be performed in cfDNA samples. These have been extensively studied in various tumor types, including breast, cervical, bladder, lung, cervical, colorectal, hepatocellular carcinoma, non-Hodgkins's lymphoma, melanoma, ovarian, pancreatic and prostate cancer [89].

CfDNA estimation has proven effective in monitoring disease treatment effectiveness and disease progression in patients undergoing surgery or chemotherapy or radiation therapy. In most studies, reduced cfDNA levels predict better patient response to treatment, whereas consistently high cfDNA levels indicate the presence of residual cancer cells, lack of response to treatment, or systemic spread of the disease [89, 98]. Numerous studies have found an association between cfDNA level and overall survival rates. Study by Gal et al. reported better survival in breast cancer patients with lower cfDNA levels [99]. Study by Ren et al., reported a worse prognosis of hepatocellular carcinoma with higher cfDNA levels [100]. CfDNA as a prognostic marker has recently been investigated in nasopharyngeal carcinoma, non-small cell lung carcinoma and breast carcinoma [101,102]. Study by Gautschi et al., found that tumor progression in patients with non-small cell lung cancer was significantly correlated with increasing plasma DNA concentrations [103].

CfDNA integrity in serum/plasma using real-time quantitative PCR for ALU repeats has been shown to be a reliable biomarker for cancer detection. Recent studies have evaluated tumor-derived cfDNA as a diagnostic tool for early detection, prediction of tumor progression, or monitoring treatment success. A change in the ratio of short and long DNA fragments in cancer patients reflects relatively more apoptotic long fragments of circulating DNA and provides a more sensitive distinction between normal and cancer patients. Study by Umetani et al. (2006a) on breast cancer showed that cfDNA integrity is a biomarker for tumors. Studies have also shown that cfDNA integrity is an informative marker for studying disease progression in patients at different stages, grades, lymph node metastasis, as well as tumor size and lymphovascular invasion. The development of malignancy is associated with greater cell proliferation, which is initially balanced by apoptosis and later by passive necrosis as the tumor differentiates and becomes invasive. An increased proportion of longer DNA fragments (ALU247) and higher DNA integrity index values are considered a potential non-invasive biomarker for the detection of malignant tumors. Quantitative amplification of the cyclin D1 oncogene in Esophageal squamous cell carcinoma showed a relative increase in copy number in both tissue and plasma with prognostic significance [102].

Qualitative studies on cfDNA have shown mutations in various genes such as KRAS and p53, microsatellite instability, loss of heterozygosity (LOH), aberrant methylation of several genes and also tumor-specific cell-free mRNA, suggesting a tumorigenic origin of cfDNA, providing high specificity Detection of cancer DNA [104–106]. Methylation markers could be very useful as an assistive technology in disease detection or progression. Studies reported cfDNA methylation by bisulfite modification and methylation-specific PCR of various genes. The milestone in cfDNA research was the study of tumor suppressor mutations and the monitoring of oncogenes. Alterations in global genome hypomethylation and hypermethylation of tumor suppressor genes at CpG islands in the promoter region are commonly found in various tumor types. The methylation pattern of mutated genes such as p16, DAPK, GSTP1, O6-MGMT etc. by methylation-specific PCR (MSP) proves to be a specific tool in lung cancer diagnosis [107,108]. Changes in the hypermethylation pattern of the DAP kinase gene in the serum and plasma DNA of patients with small cell lung cancer were found in 80 % and 40 % of these cases, respectively [109]. Consistent results were obtained between target tissue DNA and cfDNA (0–75 %) in the gene with high mutation frequency, i.e. h. KRAS and TP53, reported. With regard to cancer-specific methylation markers, certain prototypical genes that exhibit aberrant methylation, e.g. p16 in lung and breast cancer and SEPT9 and APC in colorectal cancer, have proven to be promising diagnostic and prognostic tools. However, the maximum diagnostic sensitivity (80 %) is achieved when several methylation markers are assessed simultaneously [89,98]. A potentially contradictory factor in cfDNA methylation analysis is that 8–20 % of healthy

individuals also have abnormal methylation status. Such aberrant methylation can be attributed to smoking, chewing alcohol and tobacco, or carcinogenic environmental factors. The presence of cfDNA has also been reported in a cancer with viral etiological involvement. CNA of Epstein-Barr virus (EBV) was first reported by Lo et al. discovered. al. (1999) in patients with nasopharyngeal carcinoma [110]. Study by Hoque et al., 2004, reported aberrant cfDNA methylation as an attribute of human hepatitis B virus cfDNA, while human papillomavirus cfDNA was found to be responsible for cervical, head and neck cancer, and hepatocellular carcinoma [111]. Whole-genome hypomethylation may also contribute to the process of oncogenesis, allowing increased replication of cancer-promoting proteins. Colorectal adenocarcinoma showed a significant decrease in circulating lymphocyte DNA.

1.6. Analysis approach for ctDNA/cfDNA

cfDNA/ctDNA detection methods vary significantly in terms of identifying genomic alterations, required variant allele frequency (VAF), and cost. For most solid tumors, the gene amplification technique is most commonly used to mitigate the low ctDNA levels, especially in the early stages of the disease. The most commonly used techniques include quantitative real-time polymerase chain reaction (qPCR), digital PCR (dPCR), digital droplet PCR (ddPCR), and next-generation sequencing (NGS). All of these techniques have their own advantages and limitations. QPCR is the first and most widely used technique to quantify cfDNA levels with high sensitivity and reproducibility. In ctDNA quantification, sensitivity is limited and generally requires a VAF of 10 % to identify genetic alterations. Digital PCR alleviates some of these limitations by dividing a DNA sample into thousands of individual compartments that can contain zero, one, or more DNA strands [112]. These compartments are then subjected to parallel PCR amplification, allowing precise quantification of the original DNA strands. This method significantly reduces background noise and enables the detection of genetic variants with an allele frequency of 0.1 % [113]. Furthermore, ddPCR, a method that uses water-oil emulsion droplets to divide a DNA sample into tens of thousands of droplets, enables the detection of VAF as low as 0.01 % [114].

NGS platforms are associated with higher costs but provide more valuable information, including the ability to detect unknown mutations, structural changes, and copy number variations that are not identifiable using PCR-based techniques [115]. New NGS technologies enable the detection of gene mutations at different allele frequencies, similar to those achievable with digital droplet PCR (ddPCR) [116,117]. A major disadvantage of NGS is the current cost, which typically reaches thousands of dollars per sample; However, these costs decrease significantly. In addition, NGS-based RNA sequencing of tumor and peripheral blood using whole transcriptome sequencing platforms has recently become commercially available and facilitates the identification of differentially expressed genes, fusions, transcript variants, and point mutations. However, epigenetic changes play an important role in the development and progression of GBC; NGS can easily detect these changes.

The development of high-throughput quantitative methylation assays enables rapid and precise identification of tumor DNA methylation from blood samples [118]. Furthermore, DNA methylation profiling has demonstrated consistent reliability in predicting tumor origin in patients with cancers of unknown primary location [119]. Recently, the application of epigenome and ATAC sequencing has enabled the simultaneous profiling of gene expression and open chromatin regions in conjunction with genome-scale DNA methylation analysis using reduced representation bisulfite sequencing (RBBS) [120,121]. Furthermore, the process of isolating cell-free methylated DNA by immunoprecipitation can be effectively combined with NGS and PCR-based sequencing techniques to improve specificity and reduce background noise [78]. For solid tumors, there are several FDA-approved or approved liquid biopsy platforms that can detect genetic alterations in ctDNA and are now being used to guide clinical decisions. Examples include GuardantTM (breast, colon and lung cancer and multiple cancer detection), FoundationOne (multiple cancer detection), SignateraTM (colorectal cancer), Galleri (multiple cancer detection), CancerSEEK (multiple cancer detection) and TempusTM (multiple cancer detection). In addition, Carisnow offers bioinformatic testing of both circulating DNA and RNA.

1.7. The use of cfDNA/ctDNA in GBC

Liquid biopsy could be an attractive non-invasive diagnostic biomarker in early-stage GBC, but there are very few studies. Due to the non-standardized collection, isolation and detection method for cfDNA/ctDNA, progress has been hindered and sometimes low cfDNA/ctDNA level in patients with localized disease is a major limiting factor. As outlined above, obtaining cytological material to confirm the diagnosis and genomic testing in GBC is challenging, resulting in late diagnosis and poorer prognosis. Therefore, cfDNA/ctDNA analysis could play an important role in GBC as traditional methods have shown limited sensitivity and biopsy samples are often not suitable for genomic analysis.

For the first time, Kumari et al., 2019, analyse cfDNA level for GBC diagnosis. Serum samples were collected from histologically confirmed primary GBC patients (n = 34), cholecystitis patients (n = 22) as disease controls, and healthy controls (n = 17) [122]. The cfDNA was isolated using a magnetic bead-based kit and the content was quantified using Sybr Green real-time quantitative PCR by amplification of the beta-globin gene. The cfDNA content was reported in ng/ml. Interestingly, cfDNA level was significantly higher in GBC compared to normal control (p=<0.001). In ROC curve analysis, the cfDNA value of 74.37 ng/ml could clearly distinguish cholecystitis from normal control with a sensitivity and specificity of 81.82 % and 64.71 %, respectively. In distinguishing between GBC and normal control, the sensitivity and specificity of cfDNA were 100.0 %. Furthermore, the sensitivity and specificity in distinguishing between GBC and cholecystitis were 88.24 % and 100.05, respectively. Interestingly, cfDNA level was associated with clinical stage, lymph node status and jaundice. cfDNA level was significantly lower in stage II + III patients compared to stage IV GBC patients (p = 0.002), but the number of stage IV cases was higher (n = 29 vs. n = 05). Thus, cfDNA analysis hold a promise to serve as non-invasive biomarker for diagnosis and as a marker of inflammatory process in GBC [122].

In another series of GBC cases, Kumari et al., analysed cfDNA level and cfDNA integrity index in GBC diagnosis. Serum samples were collected from GB C (n = 60), xanthogranulomatous cholecystitis (n = 09), chronic cholecystitis (n = 12) and healthy controls (n = 15). Real-time quantitative PCR using ALU115 and ALU247 was used and the integrity index was calculated using the ratio of ALU115/ALU247. The ALU115-presented smaller cfDNA fragment could distinguish GBC from normal control with a sensitivity and specificity of 71.7 %, 66.7 % and 69.7 %, respectively. ALU247, presenting the larger cfDNA fragment, could distinguish GBC from healthy controls with a sensitivity and specificity of 80.0 % and 86.1 %, respectively. The cfDNA integrity index showed a sensitivity and specificity of 78.3 % and 80.6 %, respectively [123].

In another study, Hua Ying et al., prospective analysis of cfDNA levels in a cohort of 228 participants. Serum sample was collected from GBC (n = 83), cholecystitis (n = 75) and healthy donors (n = 70) using chemiluminescence DNA biosensing system and beta-actin gene expression. There was no difference in cfDNA content between the two methods used. The cfDNA level was higher in GBC than in cholecystic and healthy donors. They reported a sensitivity and specificity of 100.0 % in distinguishing GBC from normal control. In distinguishing between GBC and cholecystitis, the sensitivity and specificity were 93.5 % and 100.0 %, respectively. The cfDNA level also differentiates cholecystitis from normal control with a sensitivity and specificity of 75.325 and 69.14 %, respectively. Further cfDNA level was also associated with

TNM stage, lymph node involvement, metastasis and jaundice [124].

Katsunori Sakamoto et al., used cfDNA levels to preoperatively identify T2 invasion in patients with suspected GBC. Peripheral blood was collected preoperatively from 24 patients with suspected GBC. cfDNA was isolated from plasma using the MagMaxTM cell-free DNA isolation kit and cfDNA was quantified using TapeStation2200 and the High Sensitivity D5000 kit. The longer cfDNA fragments were significantly lower in the pT2 group than in the <pT2 groups, but shorter cfDNA fragments showed no difference between both comparisons. In the pT2 group, the concentration of the longer cfDNA fragment was lower than in the benign/<pT2 GBC group, but the difference was not significant [125].

As previously mentioned, the sequencing process for tissue samples may encounter limitations due to insufficient tumor contents, leading to the introduction of liquid biopsy for genomic profiling in GBC. For the first time in GBC, Mishra et al., 2024 genomic alteration using a targeted NGS panel in paired serum cfDNA and tissue DNA [77]. The overall agreement was 66.67 %. Concordance for individual genes ranged from 44.44 to 82.0 %. Considering tissue DNA mutation as the gold standard, the diagnostic sensitivity of commonly mutated genes in cfDNA was analysed. The sensitivity and specificity of *TP53* were 94.44 % and 100.05, respectively, in distinguishing GBC from cholecystitis and normal control. The *EGFR* and *MET* gene showed a sensitivity of 88.89 % and 85.71 %, respectively, with a specificity of 90.0 %. These results highlight the use of cfDNA as a source for mutation testing using liquid biopsy [77].

1.8. Bile as source of liquid biopsy

The use of bile as a source of ctDNA/cfDNA has been demonstrated in BTC as bile is another component of liquid biopsy. In GBC, Kinugasa et al. analysed the cytologic and biliary ctDNA in GBC and found a sensitivity of 45.8 % and 58.3 %, respectively, with an agreement rate of 87.5 %, showing higher sensitivity of bile ctDNA in GBC [69]. Shen et al., 2018, analysed the bile ctDNA for somatic mutation identification in GBC (n = 04) and found sensitivity and specificity of 94.7 % and 99.9 % respectively using targeted deep sequencing highlighting the promising role of bile cfDNA as a source for somatic mutation identification [78]. Further studies on larger samples size are needed to confirm these results and for clinical and routine use of ctDNA/cfDNA in GBC. Uchida et al. analysed the bile to quantify the human telomerase reverse transcriptase mRNA in GBC (n = 12) and cholecystitis (n = 08) and found sensitivity and specificity of 83.3 % and 100.0 % respectively. This is the only study reported the bile encoding RNA as a diagnostic biomarker [126].

1.9. Clinical trails using ctDNA in GBC

Cholangiocarcinoma's (CC), including IHCC, EHCC, and gallbladder cancer, are often diagnosed at an advanced stage, resulting in an increased risk of postoperative recurrence [73]. Of note, FGFR1-3 fusions and IDH1/2 mutations are present in approximately 15–20 % of IHCC cases, with concordance of tumor and ctDNA findings in IHCC (92 %) is higher compared to EHCC (55 %) [73]. The application of targeted panel next-generation sequencing (NGS) for ctDNA detection has facilitated monitoring of clonal evolution during chemotherapy and has shown that over 60 % of patients can acquire new driver mutations as disease progresses [73]. Significant progress has been made in the development of targeted CC therapies, including *IDH1* inhibitors for tumors with *IDH1* mutations [127] and FGFR inhibitors for tumors with *FGFR2* fusions [128,129]. The role of ctDNA has been investigated in selecting patients for these therapies and in monitoring the emergence of secondary resistance. Goyal et al. were pioneers in elucidating the molecular mechanisms underlying acquired resistance to the *FGFR2* antibody BGJ39 through serial monitoring of cfDNA during treatment [130]. They identified an acquired V564F mutation in three of four patients who experienced disease progression, while two patients had multiple FGFR point mutations. Strong concordance between tissue samples and ctDNA was observed in the identification of these resistant variants, suggesting that this research could serve as a basis for larger studies aimed at using ctDNA as a guide for anti-*FGFR2* therapy in CC [130].

1.10. Clinical applications of CTC analysis

CTC detection in blood serves as a liquid biopsy that would be useful for numerous prognostic applications and would avoid the need for tumor tissue biopsies. The use of such a liquid biopsy offers the possibility of repeatedly taking blood samples and thus tracking the changes in the CTC during the natural course of metastasis or during cancer treatment. CTC is an alternative to invasive biopsy for early detection of metastatic tumors and monitoring response to treatment as well as determining additional treatments [27]. Measurement of CTC evolved as liquid biopsy, a better alternative to invasive biopsy for predicting and monitoring treatment response in metastatic cancer. The presence of CTC in cancer patients with metastatic or apparently localized disease is an important indicator of metastatic disease and poor prognosis. These cells could represent new targets for therapy to prevent their spread to distant locations. For clinical purposes, collecting blood samples for analysis of CTCs without surgical intervention is a more attractive alternative. CTCs have been characterized as epithelial tumor cells with a high nuclear-to-cytoplasmic ratio with irregular nuclear shape, large size and non-proliferative nature.

Clinically, the appearance of CTCs suggests a negative prognostic impact and highlights the role of these cells as biomarkers of disease progression and drug response. However; Due to their aggressive biology, heterogeneous metastatic potential, and variable growth and invasion capabilities, conventional drug therapies are less effective for treatment. Tumor cells can metastasize independently of the primary tumor due to a genetic change. Therefore, targeting the primary tumor and metastatic tumor is not enough to target cancer treatment. During the course of therapy, CTC measurement can provide information about tumor development under therapeutic natural selection and enable the identification of biological determinants of drug resistance or progression (e.g. secondary mutations) [131,132]. The effectiveness of post-therapy response is easily achieved by changing CTCs, as studies have shown that the number of CTCs changes before changes in anatomical imaging. CTCs are found in patients with or without evidence of a primary tumor, in recurrent cases CTCs are present in a significant proportion of patients, and CTCs persist after removal of the primary tumor. The presence of CTCs may reflect tumor burden at all stages of tumor development. These play an important role in characterizing immunophenotypic and genetic changes in tumor development. Circulating tumor cells in the peripheral blood of patients with solid tumors are associated with more aggressive disease, increased risk of local or distant metastases, and decreased overall survival [133].

1.11. The use of CTC in GBC

There are very few studies analysed the CTC as a diagnostic and prognostic marker of GBC. In 2012, Omar Al Ustwan et al. reported the CTC in 33.33 % (1/3) GBC patients [134]. NP Awasthi et al., 2017, for the first time analysed the CTC in peripheral preoperative blood of treatment naïve GBC (n = 27) and reported the diagnostic potential. CTC were detected using negative immunomagnetic bead separation followed by flow cytometric detection of EpCAM positive and CD45 negative cells. The CTC was detected in 92.59 % (25/27) GBC with median count of 4 CTCs/ml (range 0–20). ROC analysis showed a sensitivity and specificity of 92.6 % and 91.7 % respectively. Moving to next step, CTC count could discriminate early-stage disease from late stage and metastatic disease from nonmetastatic disease. CTC count was correlated with clinical prognostic indicators such as T stage, M stage

and with disease stage (p = 0.024, 0.022 and 0.013) [135]. Yan et al., 2023, analysed the CTC in 101 GBC for prognostication of disease. The overall CTC detection rate was 19.80 % (20/101), while in subgroup analysis showed CTC positivity in 61.54 % (08/13) patients in inoperable group and 13.64 % (12/88) in operation group. The CTC positivity was independent predictor of poorer prognosis after resection [136].

Another study of Wang et al., 2021, analysed the CTC in 45 unresectable locally advanced or metastatic GBC [137]. The CTC was detected in all GBC before treatment and was associated with metastatic disease. The CTC count was declined post treatment (n = 02) compared to pre-treatment (n = 04). The higher CTC count during chemotherapy was associated with worse outcome [120]. Xiaoguang Wang et al., 2018, analysed the CTC in 51 GBC using nano microfluidic chip and correlated the CTC count with clinicopathological features and prognosis. The CTC was detected in 43.1 % (22/51). The CTC count was correlated with liver metastasis and staging. The 1- and 2-year survival was higher in patient whom no CTC was detected compared to patients with detectable CTC [138].

1.12. Non-coding RNA as liquid biopsy in GBC

Recently, miRNA has been analysed in various solid tumors and plays an important role in cell invasion, proliferation migration and apoptosis. miRNA also acts as a tumor suppressor and oncogenic [139–141]. These results have also been reported in GBC, as the possible association between various miRNAs and GBC was first reported in 2010. Research has shown that abnormal expression of miRNAs significantly influences cancer-related mechanisms by targeting specific genetic alterations. These changes serve as valuable biomarkers for the diagnosis, treatment and prognosis of GBC [142–144].

Recently, Ueta E et al., 2021 investigated the miRNAs associated with serum extracellular vesicles in the diagnosis and prognosis of GBC and found a possible use of miRNA as a diagnostic and prognostic marker for GBC [85]. Peripheral blood was collected from GBC (n = 50), benign gallbladder disease (n = 55), and healthy controls (n = 14). The relative fold change expression of miR-1246 and miR451a, were significantly up- and down-regulated, respectively, in GBC (p = 0.005) compared to healthy control. ROC curve analysis revealed an AUC of 0.646 for miR1246 and an AUC of 0.664 for miR-451a in distinguishing the GBC shape control. The combination of miR-1246 with CEA and CA19-9 showed sensitivity and specificity of 72.0 % and 90.8 %, respectively. Furthermore, the fold change of miR1246 was an independent risk predictor for GBC (HR: 0.05p = 0.017). miR-451a has been shown to have a therapeutic effect in GBC. and miR-1246 was an independent prognostic marker [85].

Yang P et al., 2022 analysed circulating exosomal miRNA as a diagnostic biomarker for GBC and found that the signature of these exosomal miRNAs can be used in developing a non-invasive tool for the diagnosis, screening and prognosis of GBC [145]. The relative fold change for miR-551b-3p, miR-552-3p, miR-581, miR-4433a-3p, miR-496 and miR-203b-3p was analysed in GBC (n = 102) and chronic cholecystitis (n = 112). The diagnostic sensitivity of miR-4433a-3p was the highest (96.67 %) and the specificity of miR-551b-3p was the highest (96.67 %). Combining these miRNAs with CEA and CA19-9 increased the diagnostic sensitivity and specificity to 80.0 % and 90.05, respectively. The exosomal miRNAs expression was also correlated with clinicopathological features and was negatively correlated with overall survival and disease-free survival in GBC. These findings highlight the use of miRNA as diagnostic as well as prognostic biomarker of GBC [145].

Ganghua Yang et al., 2022, analysed miR141 in paired tissue and plasma samples from GBC (n = 98) and healthy controls (n = 60) [146]. miR-141 expression was upregulated in GBC compared to healthy controls, and a trend toward upregulation was evident in plasma samples. The AUC for miR-41 was 0.894, which was more valuable than other established CEA, CA19-9 and CA-125 biomarkers. miR-141 expression also correlated with tumor invasion (p = < 0.0001) and TNM stage (p = 0.009). The higher expression of miR141 was an indicator of worse overall survival and may induce apoptosis and inhibit proliferation of GBC cells, highlighting miR-141 as a potential therapeutic target [146].

Taking a step forward, Srivastava et al. analysed the set of 5 miRNAs in paired serum and tissue from GBC (n = 34) and compared the mean relative fold change with cholecystitis (n = 19) and a healthy control group (n = 21) [75]. The expression of miR-21 and 182 in serum was upregulated and miR-130, miR-146 and miR-182 were downregulated in GBC compared with normal and cholecystitis. Interestingly, the trend of up- and down-regulation was evident in paired tissue samples, highlighting serum as a potential source of miRNA. The expression changes for miR-1, miR21, miR-182 and miR-146 could clearly distinguish early-stage GBC from cholecystitis and normal control. Among these miRNAs, the diagnostic sensitivity of miR-1 (85.71 %) was the highest and the specificity of miR-21 was 92.73. In addition, the combined diagnosis was calculated and ranged from 73.13 % (CI: 60.90–83.24 %) to 98.63 % (CI: 89.0–99.61 %). These results highlight the diagnostic potential of miRNAs in GBC in combination with radio diagnostic scans [75].

Mishra et al., 2024 analysed the set of 5 lncRNAs in paired serum and tissue from GBC (n = 34) and compared the mean relative fold change with cholecystitis (n = 19) and a healthy control group (n = 21) [76]. The expression of serum HOTAIR, ANRIL and H19 was upregulated and CCAT1 and MEG3 were downregulated in GBC compared to normal control and cholecystitis. Interestingly, the trend of up- and down-regulation was evident in paired tissue samples, highlighting serum as a potential source of miRNA. The expression changes for HOTAIR and MEG3 could clearly distinguish early-stage GBC from cholecystitis (p = 0.0371, 0.0020), and H19 could distinguish early-stage GBC from late-stage GBC. The expression changes for ANRIL correlated with the M stage (p = 0.0488), H19 with the stage (p = 0.009), M stage (p=<0.0001) and stage (0.009) and CCAT1 with the M stage (0.044), highlighting their role in development and progression of GBC. When differentiating GBC from normal control, the AUC for HOTAIR was 0.75, ANRIL 0.78, H19 0.74, CCAT1 0.80, and 0.96 for MEG3. The combination of lncRNAs increased the diagnostic sensitivity and ranged from 84.13 % to 100.0 %. Overall, the authors concluded that a panel of two lncRNAs with radio diagnostic scanning could be a potential non-invasive GBC biomarker and could be useful in identifying early-stage diseases [76].

2. Conclusion

The application of liquid biopsy that include cfDNA/ctDNA, CTC, exosomes, extracellular vesicles, microRNA (miRNA) and long non-coding RNA (lncRNA) in the context of tumor detection, classification and genomics analysis is promising for the Further development of cancer management. Despite the limited data currently available on cfDNA/ctDNA analysis in GBC, this method offers a cost-effective, rapid and non-invasive approach that will facilitate the introduction of precision medicine and improve clinical outcomes for a disease characterized by could be its aggressive nature and increasing incidence. Furthermore, miRNA analysis has demonstrated significant diagnostic, therapeutic and prognostic capabilities. These liquid biopsy biomarkers have increased diagnostic potential and require further validation for their clinical application.

Ethical approval/patient consent

No ethical approvals or patient consent were necessary for the review article.

Declaration of generative AI in scientific writing

All authors certify that AI and AI-assisted technologies were not used

for preparation of this manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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