Afatinib in combination with GEMOX chemotherapy as the adjuvant treatment in patients with ErbB pathway mutated, resectable gallbladder cancer: study protocol for a ctDNA-based, multicentre, open-label, randomised, controlled, phase II trial

Mao Yang,1,2 Yuhao Zhao,1,2 Yongsheng Li,1,2 Xuya Cui,1,2 Fatao Liu,2 Wenguang Wu,1,2 Xu-An Wang,1,2 Maolan Li,1,2 Yun Liu,2 Yingbin Liu1,2

ABSTRACT

Introduction Gallbladder cancer (GBC) is an aggressive type of digestive system cancer with a dismal outcome. Given the lack of effective treatment options, the disease rapidly recurs and 5-year survival rate is <5%. Our team previously found that a significant percentage of GBC tissues harboured mutations of the ErbB-related pathway. Afatinib is a chemically synthesised drug specifically targeting the ErbB pathway mutations. However, its efficacy in the treatment of patients with GBC remains unknown. Circulating tumour DNA (ctDNA) refers to a proportion of cell-free DNA in the blood which is released by apoptotic and necrotic cells from tumours in situ, metastatic foci or circulating tumour cells. ctDNA-based liquid biopsy is a non-invasive pathological detection method that offers additional value to evaluate the therapeutic efficacy of antitumour drugs.

Methods and analysis We conduct a multicentre and randomised study on afatinib combined with gemcitabine and oxaliplatin (GEMOX) in patients with ErbB pathway mutated GBC. Clinical and biological evaluation involving ErbB pathway ctDNA detection will be made during the 3-year follow-up after participation. The primary objective of this clinical trial is to evaluate the clinical efficacy of afatinib. Disease-free survival is the primary end point and will be correlated with plasma ctDNA of patients in the treatment with afatinib. In addition, we will evaluate the sensitivity and specificity of plasma ctDNA for monitoring tumour recurrence and progression. Finally, we will assess the safety of afatinib by keeping an eye on the safety indicators.

Ethics and dissemination The study was approved by the medical-ethical review committee of Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine and Renji Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. The clinical trials results, even inconclusive, will be published in peer-reviewed journals.

Trial registration number NCT04183712.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ This is the first clinical trial to evaluate the efficacy of afatinib as an adjuvant therapy regimen in patients with gallbladder cancer.
⇒ This is a prospective multicentre trial with a randomised, controlled design to ensure the reliability of the data.
⇒ Only patients with ErbB pathway mutations are enrolled in this study, as these mutations are the target of afatinib.
⇒ After surgery, both image and circulating tumour DNA detection are used to monitor tumour recurrence and evaluate the efficacy of afatinib.
⇒ There is a lack of sample size due to the low incidence of gallbladder cancer with ErbB pathway mutations.

INTRODUCTION

Gallbladder cancer (GBC) is one of the most common and lethal tumours of the biliary tract system with 5% of 5-year survival rate.1,2 GBC lacks typical symptoms at early stages but rapidly undergoes cancer malignant transformation that is characterised by rigorous tumour infiltration and metastasis.3 To date, while no powerful means are available for curing GBC, surgical treatment is the mainstay of this intractable malignancy. Unfortunately, previous studies from our group reported that the resection rate of GBC in China is only 44.7% after diagnosis and the radical resection rate is even less than half (18.84%-21.59%).13 Thereof, a large population of cancers fall into non-surgical therapy including radiotherapy, chemotherapy and

targeted therapy.\textsuperscript{6} We recently have completed a clinical trial that comparing efficacy of gemcitabine combined with oxaliplatin (GEMOX) versus modified fluorouracil, leucovorin, irinotecan and oxaliplatin (mFOLFIRINOX) in the prognosis of patients with unresectable locally advanced or metastatic GBC. The result is still unsatisfactory as the median survival time of patients treated with GEMOX is 7 months versus mFOLFIRINOX 9 months.\textsuperscript{7} Therefore, it is critically important to find alternative therapeutic strategies for GBC.

Targeted therapy is emerging as more effective interventions than traditional radiotherapy and chemotherapy. A number of clinical trials have demonstrated the efficacy of targeted therapy in haematological tumours, lung cancer as well as biliary tract cancer (BTC). For example, phase II clinical trials with cetuximab\textsuperscript{8,9} and panitumumab,\textsuperscript{10} targeted to EGFR and KRAS, respectively, can improve the prognosis of patients with biliary tract cancer. However, other phase II clinical trials of multitargeted tyrosine kinase inhibitors (TKI), sorafenib and EGFR-targeted TKI, erlotinib, did not yield significant improvement in the prognosis of patients with BTC.\textsuperscript{11,12} These outcomes may be attributed to the insufficient sample size from a single cancer centre or lack of subgroup settings based on their genetic features such as ERBB family somatic gene mutations.\textsuperscript{6} Our team previously found that high-frequency somatic mutations in the ErbB pathway (including EGFR, ERBB2, ERBB3, ERBB4 and their downstream genes) up to 36.8% accounted for the occurrence and development of GBC.\textsuperscript{13} These patients with GBC were associated with tumour proliferation, invasion, immune escape and poor prognosis.\textsuperscript{13,14} At present, afatinib, a targeted drug for the ErbB pathway, has been approved for clinical treatment in EGFR-positive lung cancer and also engaged for clinical research on cholangiocarcinoma.\textsuperscript{15,16} Preclinical studies have discovered that afatinib can inhibit the invasiveness of GBC cell lines and reduce the tumour size of GBC xenografts.\textsuperscript{17} Given these evidences, here, we set up a clinical trial to test the hypothesis that afatinib may help improve the prognosis of patients with ErbB pathway mutated GBC.

Liquid biopsy is a non-invasive pathological detection method which was first reported by Sorrells in 1974 in the diagnosis of synovitis from synovial fluid.\textsuperscript{18} The advantage of this new technology such as easy accessibility and non-invasive approach offers significant value in the cancer research including cancer screening, early diagnosis, finding treatment targets and monitoring disease progress. As the result, this innovation was listed as one of the top 10 technological breakthroughs in 2015 and considered to be of great clinical significance and application prospect.\textsuperscript{19} Liquid biopsy mainly includes circulating tumour cells (CTCs), circulating tumour DNA (ctDNA), circulating tumour RNA, tumour-associated platelets and exosomes, among which ctDNA and CTCs are the most well-studied and have been approved by the US Food and Drug Administration for clinical application.\textsuperscript{19-21} ctDNA, first reported in 1948, refers to cell-free DNA (cfDNA) released into the blood by apoptotic and necrotic cells from tumours in situ, metastatic foci or CTCs.\textsuperscript{22} Currently, based on the rapid development of advanced detection technology, such as digital PCR and the next-generation sequencing (NGS), ctDNA-based liquid biopsy has received remarkable attention to monitor tumour burden and response to therapy.\textsuperscript{23,24} Several studies in gastrointestinal cancers reported that ctDNA dynamic changes may be predictive markers to monitor therapy efficacy.\textsuperscript{25-27} However, the application of ctDNA in evaluation of GBC diagnostic and therapeutic studies remains to be established. Therefore, we add ctDNA detection of participants to monitor disease progression and evaluate the therapeutic effects of afatinib on the recurrence and metastasis of GBC.

**Aim of the study**

The aim of this study is to evaluate the clinical efficacy and safety of afatinib in combination with GEMOX as an adjuvant therapy in patients with resectable GBC with ErbB pathway mutation by monitoring the dynamic changes of ctDNA.

**METHODS AND ANALYSIS**

**Study design**

The study is designed as a randomised, open-label and multicentre clinical trial with a combined regimen of afatinib and chemotherapy drugs compared with chemotherapy drugs alone in patients with GBC with ErbB pathway mutation who underwent surgical removal (see figure 1 for an overview of the study design). A minimum of 102 patients will be enrolled from national four top-ranked hospitals in Shanghai, China (Renji Hospital, Ruijin Hospital and Xinhua Hospital, all affiliated to Shanghai Jiao Tong University School of Medicine, and Zhongshan Hospital Affiliated to Fudan University). The study has started on 1 June 2020 and the recruitment is expected to last 36 months. Medical records and biological samples including ctDNA detection will be collected and evaluated during the 3-year follow-up after diagnosis (see online supplemental appendix 1 for a detailed time schedule of study). Disease-free survival (DFS) and overall survival (OS) will also be evaluated.

**Objectives**

**Primary objective**

To assess the efficacy of afatinib combined with GEMOX chemotherapy. Three-year DFS and 3-year OS will be used as the primary and secondary end points, respectively.

**Secondary objectives**

To assess the correlation between plasma ctDNA level and OS and DFS of patients.

To evaluate the sensitivity and specificity of plasma ctDNA for monitoring tumour recurrence and progression.

To assess the safety of afatinib in this study population.

**Secondary purposes**

To assess the safety of afatinib in this study population.
Study population

Inclusion criteria

Participants must:
- be pathologically diagnosed with GBC that is resectable;
- have ErbB pathway mutations (EGFR, ERBB2, ERBB3, ERBB4) both on surgical tumour tissue samples and preoperative blood samples based on NGS;
- sign written informed consent (if the participant is unable to read or sign, the legal representative shall sign the informed consent form. For participants who are incapable of expressing consent, their legal representative shall be told the introduction and explanation above, and sign the informed consent);
- age: 18–80 years old;
- have stable vital signs and an Eastern Cooperative Oncology Group performance status ≤1;
- show pathologically at least stage T2 or positive lymph nodes or R1 resection, according to the 8th American Joint Committee on Cancer tumour, node, metastases staging system, and have an evaluation of survival >18 weeks;

Figure 1  A clinical trial flow diagram. The study was designed as a randomised, open-label, multicentre clinical trial with a combined regimen of afatinib and chemotherapy drugs compared with chemotherapy drugs alone in patients with GBC with ErbB pathway mutations. ctDNA, circulating tumour DNA; GBC, gallbladder cancer; GEMOX, gemcitabine and oxaliplatin.
Exclusion criteria
Participants with any of the following conditions or characteristics are excluded:

- All without presence of ErbB pathway mutations either in tumour tissue samples or in blood samples.
- Have targeted therapy or chemotherapy before enrolment. Have experienced radiotherapy but have not progressed prior to this study.
- Participate in other therapeutic/interventional clinical trials.
- Have not been disease-free for at least 5 years of other cancers prior to registration, except for curatively treated cervical cancer in situ and non-melanoma skin cancer.
- Have uncontrolled concurrent illness including but not limited to: uncontrolled congestive heart failure (New York Heart Association class ≥3), unstable angina pectoris, uncontrolled cardiac arrhythmia, uncontrolled hypertension (defined by systolic blood pressure >160 mm Hg or diastolic blood pressure >100 mm Hg despite optimal medical management).
- Are ongoing or active infection.
- Have uncontrolled diabetes.
- Have active autoimmune system diseases requiring long-term use of steroids.
- Have any history of organ allograft.
- Experience substance abuse, medical, psychological or social conditions that may interfere with the patient’s ability to understand informed consent and participation in the study or evaluation of the study results.
- Keep any serious illness or medical conditions that are not suitable for the study.

Withdrawal criteria

- Participants or their legal representative (such as a parent or legal guardian) withdraw the informed consent.
- Participants loss to follow-up.

The sponsor suspends the study.

Termination criteria

Termination of study does not mean withdrawal from the study. Participants who terminate the study must continue to complete the remaining follow-up as required by the protocol. Participants must stop receiving any treatment from the study when any of the following conditions occurs:

- Participants withdraw from the trial on their own: consciously think the effect is not as good as prospect; cannot tolerate some adverse events (AEs).
- GBC relapse or metastasis during the treatment cycles.
- Participants whose condition changes after inclusion and do not meet the inclusion criteria anymore.
- Unexpected and intolerable AEs happen after the doctor’s judgement.
- If an uncontrollable factor affects the trial process and/or the interpretation of the trial results significantly, the researcher must suspend the treatment.

Study contents

Sample size calculation

We expect that the 3-year DFS rate for patients in the experimental group can rise to about 52% given that the study indicates that the 3-year DFS rate for patients with GBC in the control group is approximately 21%. The trial is designed with a two-sided significance alpha level of 0.05 and an estimated 90% power. Calculated by PASS 11, this trial requires 46 patients to be enrolled in each group. Considering a 10% drop-off rate, a total sample size of 102 is required in order to have a 90% probability of drawing a conclusive conclusion about the difference in the 3-year DFS rate between two groups.

The annual average number of visits for resectable GBC is about 40 in each hospital and the frequency of ErbB pathway mutations is roughly 36.8%. About 59 patients from 4 hospitals are expected to have GBC with ErbB pathway mutations each year. Within 3 years, it is anticipated that at least 102 patients will have signed up for the study, taking into account factors for all potential reasons for non-participation, such as patient refusal or abrupt termination.

Randomisation

Patients are randomly assigned in a ratio of 1:1 to either the control or intervention arm in a double-blinded manner. Briefly, administrators at each centre assign a random number in an envelope. When the patient is enrolled and given an envelope, the commissioner will provide the participant’s medical record required for the study to a new file with corresponding number. Researchers will be only accessible to this new file with assigned number throughout the study.

Intervention to be measured

The treatment involves up to six cycles of a 21-day cycle. All participants will receive GEMOX, as conventional chemotherapy. For participants in experimental group, they will...
also receive afatinib, as defined in table 1. When the clinician observes that the participant shows the indication of terminating chemotherapy and has completed for more than four cycles, the participant is deemed to finish the trial. If the participant during the course requests to end the test, the case will be terminated at any time.

**Sample collection**
In our study, both blood and tissue samples are collected to detect ErbB pathway mutations (EGFR, ERBB2, ERBB3, ERBB4) through NGS.

**Blood samples**
An amount of 10 mL of venous blood samples is collected from patients with GBC at scheduled timepoints (see online supplemental appendix 1 for a detailed time schedule of study) in Cell-Free DNA Blood Collection Tubes (Streck, USA). Samples are centrifuged to extract cfDNA to detect mutations.

**Tissue samples**
Tumour and paracancerous tissue samples are collected from resectable patients during surgery, cryopreserved in liquid nitrogen and stored in the biobank of Renji Hospital, Shanghai Jiao Tong University School of Medicine. Formalin-fixed and paraffin-embedded tumour sections are collected as well for further study.

**Study end points**

**Primary end point**
The primary end point was 3-year DFS rate. The DFS will be reached when GBC relapses indicated by contrast-enhanced MRI/CT in the enrolled cases. And the 3-year DFS rate was considered as the proportion of patients without recurrence in 3-year follow-up.

**Secondary end points**
Secondary end points included 3-year OS rate, safety and exploratory translational end points of cfDNA prognostic value. After tumour recurrence, the patients will continue to be followed up every 3 months till death or reaching 3 years after enrolment (see online supplemental appendix 1 for a detailed time schedule of study for participants) to assess 3-year OS rate. Safety was evaluated according to AEs, serious AEs (SAEs) and adverse drug reactions (ADR) rate.

**Statistical analysis**

**Effectiveness analysis**
The 3-year DFS rate and the 3-year OS rate of the two groups will be calculated, respectively. The log-rank test and the Kaplan-Meier curve of the DFS and OS are used for comparison between the two groups. Stratification factors and other important data related to clinical prognosis can be transformed into categorical variables for univariate Cox regression analysis; variables with clinical and statistical significance are incorporated as covariates into the multivariate Cox proportional hazard model to calculate HR and 95% CI.

Sensitivity, specificity, kappa value and correlation coefficient will be used to evaluate the prognostic value of plasma ctDNA test for patients with GBC treated with afatinib.

**Safety analysis**
Descriptive study will be mainly performed to analyse AEs, SAEs and ADR according to the Common Terminology Criteria for Adverse Events V.5.0. Definitions of different types of AEs and ADR are listed in online supplemental appendix 2. The incidence of AEs and ADR and their 95% CIs will be calculated. And every AE, SAE and ADR case will be listed in detail.

**Data management**
Source data from the trial are locally stored in electronic case report forms (eCRF) via Oracle Clinical/Remote Data Capture (OC/RDC) system by clinical researchers. The eCRF is activated when the patient is enrolled. After all subjects complete the trial and the medical records load into the system, the principal investigator, sponsors, statistical analysts and data administrator will review the data and confirm that the database is accurate and complete. Finally, the data are stored in a computer that needs authorised personnel to access once providing a code number.

During the study, the clinical monitors appointed by the sponsor regularly perform on-site audit to ensure the authenticity of the documents and the protocol to be strictly followed.

**Ethics and dissemination**

**Ethical considerations**
The principal investigator ensures that this study conforms to the Declaration of Helsinki or the laws and regulations of the country, whichever provides the greater protection to the patient. Ethical approval has been obtained from both Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (XHEC-C-2019-023-2) and Renji Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (RA-2021-041). Participants are required to provide written informed consent (see an example of informed consent form in online supplemental materials).

**Dissemination**
The protocol and the trial results, even inconclusive, will be presented at national and international scientific meetings, and published in peer-reviewed journals. Genomic data will be made available in public open data sets.

**Patient and public involvement**
Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

**Author affiliations**

1Department of Biliary-Pancreatic Surgery, Shanghai Jiao Tong University School of Medicine Affiliated Renji Hospital, Shanghai, Shanghai, China
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Contributors YSL is the principal investigator steering the study, and responsible for funding acquisition and supervision of the study. MY, YZ and YSL wrote and revised the manuscript. MY, YZ, YSL and XC are responsible for data collection and coordination. FL is responsible for data analysis. MY, YSL, WW, X-AW, ML and YL are responsible for the study design. All authors reviewed the manuscript for intellectual content and approved the final version of the report.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement No data are available.

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ORCID iD Yingbin Liu http://orcid.org/0000-0001-6110-0185

REFERENCES
INFORMED CONSENT FORM
(English Version)

Participant Information Page

Study title: A ctDNA-based, multicentre, open-label, randomized, controlled, phase II trial of afatinib in combination with GEMOX chemotherapy as the adjuvant treatment in patients with ErbB pathway mutated, resectable gallbladder cancer
Version: V1.0, 2020-5-20
Principal Investigator: Yingbin Liu, Renji Hospital Affiliated to Shanghai Jiaotong University School of Medicine

Dear participant:
You have been diagnosed with ErbB-pathway mutated gallbladder cancer, and will be invited to participate in a randomized, open-label, and multicentre study of afatinib in combination with GEMOX as adjuvant therapy after surgery. The study will be conducted at Renji Hospital, Xinhua Hospital, and Ruijin Hospital, all affiliated to Shanghai Jiao Tong University School of Medicine, and Zhongshan Hospital Affiliated to Fudan University, and at least 102 subjects are expected to participate. This study has been reviewed and approved by the Ethics Committee of Renji Hospital, Shanghai Jiaotong University School of Medicine.

The information provided below will help you decide whether to participate in this study or not. Your participation in this study is completely voluntary and your decision will not affect your normal treatment at our hospital. If you choose to participate in this study, our research team will make every effort to ensure your safety and rights during the study!

Please read this informed consent carefully and ask any questions you may have to your study doctor or the investigator. The background, purpose, process and other important information of this study are as follows:

1. BACKGROUND

Gallbladder cancer (GBC) is one of the most common and lethal tumors of the biliary tract system with 5% of 5-year survival rate. GBC lacks typical symptoms at early stages but rapidly undergoes cancer malignant transformation that is characterized with rigorous tumor infiltration and metastasis. To date, while no powerful means are available for curing GBC, surgical treatment is the mainstay of this intractable malignancy. Unfortunately, previous studies from our group reported that the resection rate of GBC in China is only 44.7% after diagnosis and the radical resection rate is even less than half (18.84%-21.59%). Thereof, a large population of cancers fall into non-surgical therapy including radiotherapy, chemotherapy and targeted therapy. Our group recently have completed a clinical trial that comparing efficacy of GEMOX (gemcitabine combined with oxaliplatin) vs. modified FOLFIRINOX (fluorouracil, leucovorin, irinotecan and oxaliplatin) in the prognosis of patients with unresectable locally advanced or metastatic GBC. The result is still unsatisfactory as the median survival time of
patients treated with GEMOX is 7 months vs. mFOLFIRINOX 9 months. Therefore, it is critically important to find alternative therapeutic strategies for GBC. Targeted therapy is emerging as more effective interventions than traditional radiotherapy and chemotherapy. A number of clinical trials have demonstrated the efficacy of targeted therapy in hematological tumors, lung cancer as well as biliary tract cancer (BTC). Our team previously found that high-frequency somatic mutations in the ErbB pathway (including EGFR, ERBB2, ERBB3, ERBB4 and their downstream genes) up to 36.8% accounted for the occurrence and development of GBC. At present, afatinib, a targeted drug for the ErbB pathway, has been approved for clinical treatment in EGFR-positive lung cancer and also engaged for clinical research on cholangiocarcinoma. Preclinical studies have discovered that afatinib can inhibit the invasiveness of GBC cell lines and reduce the tumor size of GBC xenografts. Given these evidences, here, we set up a clinical trial to test the hypothesis that afatinib may help improve the prognosis of patients with ErbB pathway mutated GBC.

cDNA, refers to cell free DNA released into the blood by apoptotic and necrotic cells from tumors in situ, metastatic foci or CTCs. Currently, based on the rapid development of advanced detection technology, such as digital PCR and the next-generation sequencing (NGS), cDNA-based liquid biopsy has received remarkable attention to monitor tumor burden and response to therapy. Several studies in gastrointestinal cancers reported that cDNA dynamic changes may be predictive markers to monitor therapy efficacy. Therefore, we add cDNA detection of participants to monitor disease progression and evaluate the therapeutic effects of afatinib on the recurrence and metastasis of GBC.

2. STUDY PURPOSE

The aim of this study is to evaluate the clinical efficacy and safety of afatinib in combination with GEMOX as an adjuvant therapy in resectable GBC patients with ErbB pathway mutation by monitoring the dynamic changes of cDNA.

3. STUDY PROCESS

(1) How many people will participate in the study?

A minimum of 102 patients will be enrolled from national four top-ranked hospitals in Shanghai, China (Renji Hospital, Ruijin Hospital, and Xinhua Hospital, all affiliated to Shanghai Jiao Tong University School of Medicine, and Zhongshan Hospital Affiliated to Fudan University).

(2) What are the study procedures?

A. Screening

Before you are enrolled in the study, your medical history will be asked, and you will be screened for ErbB pathway mutations by NGS using blood and tumor tissue samples. Inclusion and exclusion criteria are listed below in details:

Inclusion criteria
Participants must:
Be pathologically diagnosed with GBC that is resectable.

Have ErbB pathway mutations (EGFR, ERBB2, ERBB3, ERBB4) both on surgical tumor tissue samples and preoperative blood samples based on NGS.

Sign written informed consent. (If the participant is unable to read or sign, the legal representative shall sign the informed consent form. For participants who are incapable of expressing consent, their legal representative shall be told the introduction and explanation above, and sign the informed consent)

Age: 18-80 years old

Have stable vital signs and an Eastern Cooperative Oncology Group (ECOG) performance status ≤1;

Show pathologically at least stage T2 or positive lymph nodes or R1 resection, according to the 8th American Joint Committee on Cancer (AJCC) TNM staging system, which is fit for adjuvant therapy, and have an evaluation of survival greater than 18 weeks;

Have important organs to be functional including bone marrow, kidney and liver: leucocytes >3000/μL, with an absolute neutrophil count >1500/μL, platelets >75000/μL, hemoglobin ≥ 9 g/dL, total bilirubin ≤ 3.0 × institutional upper limit of the normal (ULN), aspartate aminotransferase (AST)/alanine transaminase levels (AST) ≤ 5 × institutional ULN, creatinine clearance ≥ 30 mL/min;

Agree to use adequate contraception prior to and during the study specific for women bearing child and men.

Exclusion criteria

Participants with any of the following conditions or characteristics are excluded:

- ALL without presence of ErbB pathway mutations either in tumor tissue samples or in blood samples.
- Have targeted therapy or chemotherapy before enrollment. Have experienced radiotherapy but have not progressed prior to this study.
- Participate in other therapeutic/interventional clinical trials;
- Have not been disease-free for at least 5 years of other cancers prior to registration, EXCEPT for curatively treated cervical cancer in situ and non-melanoma skin cancer.
- Have uncontrolled concurrent illness including but not limited to: uncontrolled congestive heart failure (New York Heart Association (NYHA) class ≥ 3), unstable angina pectoris, uncontrolled cardiac arrhythmia, uncontrolled hypertension (defined by systolic blood pressure > 160 mm Hg or diastolic blood pressure > 100 mm Hg despite optimal medical management).
- Are ongoing or active infection;
- Have uncontrolled diabetes;
- Have active autoimmune system diseases requiring long-term use of steroids;
- Have any history of organ allograft;
- Experience substance abuse, medical, psychological or social conditions that may interfere with the patient’s ability to understand informed consent and participation in the study or evaluation of the study results.
- Keep any serious illness or medical conditions that are not suitable for the study.

B. Intervention

After determining that you are eligible to participate in the study based on inclusion and exclusion criteria, you will be collected and randomly assigned to treatment:
Test group: **afatinib** 40mg once daily (afatinib 40mg from Day 1 to Day 21, *Boehringer-Ingelheim*) combined with **GEMOX** chemotherapy (gemcitabine 1000 mg/m² on days 1 and 8 of each cycle by IV infusion, *Eli Lilly and Company* and oxaliplatin 100 mg/m² on day 1 of each cycle by IV infusion, *Jiangsu Hengrui Medicine Co., Ltd.*)

Control group: **GEMOX** chemotherapy (gemcitabine 1000 mg/m² on days 1 and 8 of each cycle by IV infusion, and oxaliplatin 100 mg/m² on day 1 of each cycle by IV infusion)

C. Follow-Up

After the last time receiving therapy, follow-up data will be collected during your visits to the hospital every 3 months till death or reaching 3 years after enrollment.

(3) How long will the study last?

This study will last for 3 years from the time you receive treatment, and the end of the study will be reached when GBC relapses indicated by contrast enhanced MRI/CT in the enrolled cases. If the patient participates in the trial **treatment for 6 cycles** without recurrence, the endpoint will also be reached. After tumor recurrence, and the patients will continue to be followed up **every 3 months** till death or reaching 3 years after enrollment.

You may drop out of the study at any time without losing any benefits to which you are entitled. However, if you decide to withdraw during the study, you are encouraged to talk to your doctor first. If you experience a serious adverse event, or if your study doctor feels it is not in your best interest to continue in the study, he or she may decide to withdraw you from the study. The sponsor or regulatory agency may also terminate during the study period. However, your withdrawal will not affect your normal medical treatment and rights.

If you withdraw from the study for any reason, you may be asked about your participation in the study. You may also be asked for a medical examination and follow-up questionnaire if your doctor deems it necessary.

(4) Information and biological specimens collected during the study

Within 28 days prior to starting treatment, your doctor will take and record your medical history, combined medications and adverse events, also perform an ECHO and enhanced contrast CT or MRI. Within 7 day prior to treatment, your vital signs and weight will be measured, and an ECOG PS score will be performed in conjunction with the following tests:

- complete blood counts
- urinalysis
- hemostasis(PT, APTT, TT, FIB, INR)
- electrolytes(Na, K, P, Ca, Mg)
- liver/renal function(ALT, AST, ALP, GGT, TBIL, DBIL, Alb, BUN, Cr)
- tumor markers (CA19-9, CEA, CA125)
- myocardial enzyme spectrum (cTnI, CK, LDH)
- thyroid function (FT3, FT4, TSH, TT3, TT4)

These clinical assessments are also obtained at a series of scheduled timepoints (see the study schedule for details).

In addition, both blood and tissue samples are collected for this trial to detect ErbB pathway mutations(EGFR, ERBB2, ERBB3, ERBB4) through NGS.
1) Blood samples
10 milliliters of venous blood samples is collected from you at scheduled timepoints (see the study schedule for details) in Streck Cell-Free DNA Blood Collection Tubes. Samples are centrifuged to extract cell-free DNA (cfDNA) to detect mutations.

2) Tissue Samples
Tumor and para-cancerous tissue samples are collected during surgery, cryopreserved in liquid nitrogen, and stored in the biobank of Renji hospital, Shanghai Jiao Tong University School of Medicine to detect mutated tissue DNA. Formalin-fixed and paraffin-embedded tumor sections are collected as well for further study.

All data obtained will be kept strict and stored electronically on a database with secured and restricted access. An encryption will be used for data transfer, with removal for any information able to identify individuals. Data will be only deidentified for analysis at the completion of this study.

4. RISKS AND BENEFITS

(1) What are the risks of participating in this study?

The risks you may incur by participating in this study are as follows. You should discuss these risks with your study doctor.

1) Drug Adverse Reaction
Toxic side effects caused by gemcitabine, oxaliplatin, and afatinib may occur during any phase of the study.
Adverse reactions related to the chemotherapy drugs (gemcitabine and oxaliplatin) include: nausea, vomiting, bloating, diarrhea, constipation, loss of appetite, fever, malaise, joint and muscle pain, hair loss, peripheral neuritis, impairment of liver and kidney function, and bone marrow suppression (decreased white blood cells, platelets, and hemoglobin), etc.
Conduted clinical studies have reported that most common adverse events associated with afatinib are diarrhea and skin rash.
Investigational medicinal product (IMP) are subject to unanticipated or unpredictable risks in clinical trials, including unpredictable types and severity of risks, such as rapid disease progression, unanticipated serious adverse events related or unrelated to treatment, which may cause you harm. If you experience any discomfort, or new changes in your condition, or any unexpected events during the treatment, whether it is related to the drug or not, you should promptly notify your doctor, who will make a judgment and provide medical treatment.

2) ctDNA based liquid biopsy
The ctDNA detection does not cause any harm to the body, but the sample collection is still minimally invasive to the body. Therefore, we collect an additional 10 ml of peripheral blood along with the routine blood test to avoid unnecessary inconvenience and harm to you. There may be minor discomfort, including temporary pain, local bruising, a few cases of mild dizziness, or, rarely, needle infection. If you experience any discomfort after a blood sample collection, whether it is related to the test or not, you should promptly notify your doctor, who will make a judgment and provide medical treatment.

3) Others
You will need to come to the hospital for regular follow-up visits and assessments (imaging included) to evaluate efficacy and safety of afatinib, which may cause inconvenience and more frequent exposure to low doses of radiation.

(2) What are the benefits of participating in the study?

The IMP may decrease the disease progression in some participants, but we cannot guarantee this. You can get information about your health from the physical examination and laboratory tests done in the study. Although there may be no direct benefit to you from participation in this study, your participation may benefit future patients who are suffering from the same disease. This study will give you free NGS reports of both ctDNA and tumor tissue, which will provide genetic information related to tumor development and prognosis. This information, especially from continuous ctDNA detection, can be very helpful in guiding your subsequent treatment and monitor tumor recurrence and metastasis.

5. ALTERNATIVE TREATMENT OPTIONS

In addition to participating in this study, you may receive other treatments as adjuvant therapy for GBC, including radiation therapy, chemotherapy, and immunotherapy. Your study doctor will discuss the possible risks, the advantages and disadvantages of other treatment options with you. Please decide whether to participate in this study after fully discussion. You do not have to participate in this study to get treatment for your disease. Other alternative treatments may be available to you which are defined as follows:

- Other clinical studies that your doctor may know of to your disease.
- The best supportive treatment to minimize your pain or discomfort, etc.

6. USE OF RESEARCH RESULTS AND CONFIDENTIALITY OF PERSONAL INFORMATION

Results conducted through this program may be published in medical journals with the understanding and assistance of you and other participants, but we will keep your study records confidential as required by law.

The personal information of study participants will be kept strictly confidential, and your personal information will not be disclosed unless required by relevant laws.

If necessary, government administrative departments, hospital ethics committees and other relevant researchers can access your data according to regulations.

7. RESEARCH EXPENSES AND RELATED COMPENSATION

(1) Cost of drugs used in the study and related examinations

Afatinib is provided for free until the end of the treatment specified in the study protocol. GEMOX chemotherapy is at your own expense. In addition, you will be solely responsible for the expenses incurred by you for any examination, hospitalization, and treatment other than this study, as well as for the routine treatment and examination required for any concurrent disease.
(2) Compensation for participation in the study

There are no additional compensation for this study.

(3) Compensation/compensation after damage

For participants who suffer damage related to this study, the sponsor will bear the treatment cost and corresponding economic compensation in accordance with Chinese laws and regulations.

8. RIGHTS OF PARTICIPANTS AND RELEVANT MATTERS NEEDING ATTENTION

(1) Your rights

Your participation in the study is voluntary throughout the entire process. If you decide not to participate in this study, it will not affect other treatments you should receive. If you decide to participate, you will be asked to sign this written informed consent. You have the right to withdraw from the trial at any stage without discrimination or unfair treatment, and your medical treatment and rights will not be affected.

(2) Matters needing attention

Before enrollment, you will undergo screening to confirm if you are eligible for the study. You are required to provide true information about your medical history and current medical condition. During treatment and follow-up visits, you must come to the hospital at the scheduled timepoints. Your follow-up visits are very important because your doctor will determine if the treatment you are receiving is actually working. You should not use other chemotherapy, targeted or immunotherapy drugs for GBC during the study. If you need other treatments, please contact your doctor in advance.

9. RELEVANT CONTACT INFORMATION

If there is any significant new information during the study that may affect your willingness to continue to participate, your doctor will inform you promptly. If you are interested in your own study data, or you would like to know the findings after this study, you may ask any questions about this study at any time and receive answers accordingly, Please contact doctor Mao Yang at **********.
Participant Signature Page

Participant Consent Statement:
☐ I have been informed of the purpose, background, process, risks and benefits of this study. I have plenty of time and opportunity to ask questions, and I am satisfied with the answers.
☐ I am also told who to contact when I have questions, want to report difficulties, concerns, suggestions for research, or want further information, or to help with research.
☐ I have read this informed consent and agree to participate in this study.
☐ I understand that I may choose not to participate in the study or withdraw from the study at any time during the study without any reason.
☐ I already know that if I get worse, or if I have a serious adverse event, or if my study doctor decides it's not in my best interest to continue, he or she will decide to withdraw me from the study. The funder or regulatory agency may terminate during the study without my consent. If this happens, the doctor will inform me and the study doctor will discuss other options with me.
☐ I will be provided with a copy of the informed consent which contains my signature and that of the investigator.

Participant Signature:
Date:
Tel:

(NOTE: If participant has no capacity/limited capacity, legal representative signature and date will be required)

Legal Representative's Signature:
Date:
Tel:

Investigator Statement:
I have explained the entire process of this study in detail, particularly, the possible risks and benefits of participating.

Investigator Signature:
Date:
Tel:
## Supplementary Appendix 1

### Study schedule

<table>
<thead>
<tr>
<th></th>
<th>Screening</th>
<th>Treatment (21-day cycle)</th>
<th>End of study treatment</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before surgery</td>
<td>After surgery</td>
<td>Cycle 1</td>
<td>Cycle 2</td>
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<tr>
<td><strong>Basic information</strong></td>
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<tr>
<td>Informed consent</td>
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<tr>
<td>Randomization</td>
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<tr>
<td>Medical history(^1)</td>
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<tr>
<td><strong>Clinical assessments</strong></td>
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<td>Physical examination</td>
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<td>✓</td>
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<tr>
<td>ECG</td>
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<td>✓</td>
<td>✓</td>
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<tr>
<td>ECHO(^2)</td>
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<tr>
<td><strong>Laboratory Tests</strong></td>
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<td>Complete blood count</td>
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<td>Urinalysis</td>
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<td>Serum chemistry(^3)</td>
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<tr>
<td>Hemostasis(^4)</td>
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<tr>
<td>Thyroid function(^5)</td>
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<table>
<thead>
<tr>
<th></th>
<th>Screening</th>
<th>Treatment(^a) (21-day cycle)</th>
<th>End of study treatment(^b)</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before surgery</td>
<td>After surgery</td>
<td>Cycle 1</td>
<td>Cycle 2</td>
</tr>
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<td>Pregnancy test</td>
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<td>Disease assessments</td>
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<td>Tumor marker(^b)</td>
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<td>√</td>
<td>√</td>
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<tr>
<td>Imaging(^b)</td>
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<td>√</td>
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<td></td>
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<td>Treatment</td>
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</tr>
<tr>
<td>Afatinib</td>
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</tr>
<tr>
<td>Gemcitabine</td>
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<tr>
<td>Oxaliplatin</td>
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<td>Safety assessments</td>
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<td>Adverse events</td>
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<td>Dispense drug</td>
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<tr>
<td>Survival assessments</td>
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<td>Disease progression</td>
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<td>√</td>
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<tr>
<td>Survival status</td>
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<tr>
<td>Tumor tissue DNA</td>
<td>√</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. All assessments during treatment may be performed within the 1 day prior to the scheduled treatment date of each cycle.

b. The End of Treatment assessment visit should be performed within 14 days of the patient’s last cycle of treatment.
1. Medical history includes: demographics, prior treatment, allergy history and concomitant diseases.
2. An ECHO should be performed at screening and the end of study treatment. Repeat one as clinically indicated.
3. Serum chemistry includes: Glucose, cardiac troponin I(cTnI), LDH and creatine kinase (CK) at screening only. Total Bilirubin(TB), direct bilirubin(DBIL), AST, ALT, ALP, albumin(ALB), $\gamma$-glutamyltransferase( $\gamma$-GT), blood urea nitrogen(BUN), serum creatinine(Cr), Sodium, Potassium, Total Calcium, Phosphate, Magnesium at all timepoints. The investigator may perform additional testing, if necessary.
4. Hemostasis includes: PT, APTT, TT, FIB, INR at screening. Repeat one as clinically indicated.
5. Thyroid function includes: FT3, FT4, TSH, TT3, TT4 at screening. Repeat one as clinically indicated.
6. Tumor marker includes: CA19-9, CEA, CA125 at all timepoints. The investigator may perform additional testing, if necessary.
7. Imaging test should be enhanced contrast CT/MRI or PET-CT.
8. Research samples are collected to detect ErbB pathway mutations-EGFR, ERBB2, ERBB3, ERBB4. Both blood and tissue samples must confirm presence of ErbB pathway activating mutations before treatment.
**Supplementary appendix 2**

**Adverse Event (AE)**
Any untoward medical occurrence in a patient or clinical trial subject administered a medicinal product and which does not necessarily have a causal relationship with this treatment. An AE can be any unfavourable and unintended sign (including abnormal laboratory findings), symptom or disease temporally associated with the use of an investigational medicinal product, whether or not related to the investigational medicinal product (IMP). In this trial, any adverse event that occurs from the time the subject receives the study drug until 30 days after the last dose is judged to be an AE.

**Adverse drug reaction (ADR)**
All untoward and unintended responses to an IMP related to any dose administered. In this trial, an ADR is an adverse event that is judged to be related to the IMP.

<table>
<thead>
<tr>
<th>Causality term</th>
<th>Assessment criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Certain</td>
<td>• Event or laboratory test abnormality, with plausible time relationship to drug intake</td>
</tr>
<tr>
<td></td>
<td>• Cannot be explained by disease or other drugs</td>
</tr>
<tr>
<td></td>
<td>• Response to withdrawal plausible (pharmacologically, pathologically)</td>
</tr>
<tr>
<td></td>
<td>• Event definitive pharmacologically or phenomenologically (i.e.an objective and specific medical disorder or a recognised pharmacological phenomenon)</td>
</tr>
<tr>
<td></td>
<td>• Rechallenge satisfactory, if necessary</td>
</tr>
<tr>
<td>Probable/Likely</td>
<td>• Event or laboratory test abnormality, with reasonable time relationship to drug intake</td>
</tr>
<tr>
<td></td>
<td>• Unlikely to be attributed to disease or other drugs</td>
</tr>
<tr>
<td></td>
<td>• Response to withdrawal clinically reasonable</td>
</tr>
<tr>
<td></td>
<td>• Rechallenge not required</td>
</tr>
<tr>
<td>Possible</td>
<td>• Event or laboratory test abnormality, with reasonable time relationship to drug intake</td>
</tr>
<tr>
<td></td>
<td>• Could also be explained by disease or other drugs</td>
</tr>
<tr>
<td></td>
<td>• Information on drug withdrawal may be lacking or unclear</td>
</tr>
<tr>
<td>Unlikely</td>
<td>• Event or laboratory test abnormality, with a time to drug intake that makes a relationship improbable (but not impossible)</td>
</tr>
<tr>
<td></td>
<td>• Disease or other drugs provide plausible explanations</td>
</tr>
<tr>
<td>Conditional/</td>
<td>• Event or laboratory test abnormality</td>
</tr>
<tr>
<td>Unclassified</td>
<td>• More data for proper assessment needed, or • Additional data under examination</td>
</tr>
<tr>
<td>Unassessable/</td>
<td>• Report suggesting an adverse reaction</td>
</tr>
<tr>
<td>Unclassifiable</td>
<td>• Cannot be judged because information is insufficient or contradictory</td>
</tr>
<tr>
<td></td>
<td>• Data cannot be supplemented or verified</td>
</tr>
</tbody>
</table>

An AE which meet the criteria of “Certain”, “Probable/Likely” and “Possible” is judged to be an ADR.

**Serious Adverse Event (SAE)**
Any untoward medical occurrence or effect that at any dose:
- Results in death
- Is life threatening
- Requires hospitalisation or prolongation of existing inpatients’ hospitalisation
- Results in persistent or significant disability or incapacity
- Is a congenital anomaly/birth defect
Or is otherwise considered medically significant by the Investigator. The occurrence of pregnancy in female patients is considered as a SAE. Cases of disease progression due to the development and progression of the tumor itself (PD according to the protocol) should not be included as AE. Obstructive jaundice or cholangitis or pancreatitis caused by GBC is not considered as a SAE.