



Clinicopathological prognostic factors for gallbladder carcinoma: a retrospective study

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Background: Gallbladder carcinoma (GBC) is an uncommon neoplasm with poor long-term survival. Worldwide the incidence rates vary according to geographic area. The multifactorial aetiology and the rarity of the disease limits the studies to improve outcomes in patients, since the treatment remains mostly surgical. The aim of this study was to identify clinicopathological prognostic factors for survival in patients with GBC submitted to surgery in our institution—a tertiary centre in Portugal. Also, to assess the expression of possible biomarkers (HER2, CD44 and ALDH1) in GBC, as well as the frequency of microsatellite instability (MSI) tumours.

Methods: Clinicopathological characteristics of 41 consecutive patients that underwent surgical resection for GBC (2008–2019) at our hospital were retrospectively reviewed. Clinicopathological factors were assessed and an immunohistochemical (IHC) analysis was done. Microsatellite stability (MSS) was considered if there was maintenance of nuclear expression of MLH1, MSH2, MSH6 and PMS2. Human epidermal growth factor receptor 2 (HER2) expression was evaluated according to the rules applied for gastric cancer and expression of CD44 and ALH1 was evaluated in order to detect cancer stem cells (CSC). Survival analysis was conducted using Kaplan-Meier and Cox regression was used to find prognostic factors.

Results: Incidence of GBC in our cohort of patients was 0.45%, most commonly affecting females. Median overall survival (OS) was 23 months with a 39.6% 5-year survival rate. Stage > II [hazard ratios (HR) =8.58; P=0.007], lymphovascular invasion (LVI) (HR =4.06; P=0.045) and hepatic resection (HR =0.288; P=0.034) independently influenced survival. HER2 positivity and high expression of CD44 or ALDH1 did not show significant influence in survival (P=0.649, P=0.868 and P=0.914, respectively), although HER2 and ALDH1 positive patients showed a tendency to a shorter OS, compared to negative patients. We found no relation between these biomarkers expression and disease stage. All analysed samples had MSS.

Conclusions: GBC patients with a worse prognosis can be identified. The overexpression of HER2 could select patients for targeted therapy and prompt tissue sampling in unresectable patients.

Keywords: Gallbladder cancer; microsatellite instability (MSI); HER2; CD44; ALDH1

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Introduction

In developed countries gallbladder carcinoma (GBC) is an uncommon neoplasm, whose incidence depends on geographic, age-, gender- and ethnicity-related genetics and/or environmental factors (1-3). GBC is highly aggressive, with a low 5-year survival rate, varying between 5% to 32%, and the only effective treatment depends on an early diagnosis (2,4). Half of the patients are detected incidentally during or after a cholecystectomy and fewer than 20% with non-incidental GBC are potential candidates for surgery, as it progresses silently, consequently associated with a worse prognosis (5).

Surgical resection is the only effective treatment and the benefit of systemic adjuvant therapy following resection remains uncertain (6,7). Some data suggest a survival benefit for adjuvant chemoradiation therapy in locally advanced disease (T2b, T3 and T4) (8). Even with palliative chemotherapy, the median overall survival (OS) in advanced disease was less than 9.5 months (9). As such, the search for new therapeutic targets is of utmost importance. However, due to the rarity of GBC, solid information about the pathophysiology and the clinical outcomes is limited. GBC is linked to multiple genetic and environmental factors, such as chronic infection, gallstones and gender (10). The information regarding genetic and molecular alterations remains limited. Human epidermal growth factor receptor 2 (HER2) amplification is one of the most frequent molecular abnormalities found in GBC (11). The prognostic significance of HER2 amplification is still unknown, however has been associated to lower survival time (12). Furthermore, cancer stem cells (CSC) have been related to high tumorigenicity and resistance to treatments in different solid tumours, as well as, poor clinical outcomes (13,14). Among these CSC, cluster of differentiation 44 (CD44) and aldehyde dehydrogenase 1 (ALDH1) have been demonstrated to contribute to cell proliferation, invasion and metastasis (15,16). The carcinogenesis role of microsatellite instability (MSI) is controversial, since varies accordingly with the GBC population prevalence (17,18). MSI is a useful molecular predictive marker of response in colorectal cancer, since these tumours exhibit different histopathologic patterns and a less aggressive behaviour compared with tumours without this phenotype (19).

In the present study, we aimed to identify the clinicopathological factors that influence survival outcomes of GBC patients submitted to surgical resection at our hospital. Furthermore, to assess the proportion of different biomarkers: HER2, CD44 and ALDH1, as well as the frequency of MSI tumours, and to estimate the incidence of GBC in our Portuguese region. We present the following article in accordance with the REMARK reporting checklist (available at <https://jgo.amegroups.com/article/view/10.21037/jgo-22-61/rc>).

Methods

Study design

This clinical retrospective study included 41 consecutive patients who underwent surgery for GBC between January 2008 and December 2019 at Centro Hospitalar e Universitário de Coimbra (CHUC), in Portugal. The patients' clinical histories were reviewed, using the hospital database and office records. Data regarding demographic features, clinical presentation and potential clinicopathological prognostic factors were obtained. The study was conducted in accordance with Declaration of Helsinki (as revised in 2013) (20). The study was approved by the Centro Hospitalar e Universitário de Coimbra Ethics Committee (No. CHUC-165-20). The requirement for informed consent was waived, since this was an exclusively observational study, with no risks for subjects.

Treatment and follow-up

The therapeutic approach was always debated and decided on in a multidisciplinary meeting. Pre- or post-operative staging was based on computed tomography scans of the thorax, abdomen and pelvis and/or magnetic resonance imaging. If the diagnosis of GBC was first detected in the surgical specimen by a pathologist, after a cholecystectomy, it was classified as "incidental". The surgical treatment included cholecystectomy alone, cholecystectomy with simultaneous hepatic resection and hepatic pedicle lymphadenectomy (HPL) and hepatic resection with HPL after simple cholecystectomy, in case the diagnosis was incidental. Hepatic resection comprised, in most

patients, an anatomical or atypical (at least 2 cm of hepatic parenchyma) resection of segments 4b and 5. Some patients received adjuvant therapy.

Follow-up data were recorded from the day of surgery until the day of death or until the date of analysis, if the patient was alive. Postoperative mortality was defined as the one that occurred until 1 month after surgery, irrespective of the cause. OS was defined as the time (in months) from the day of surgery until death of any cause or to the date of analysis.

Histopathological evaluation and immunohistochemistry

The diagnosis and staging of the GBC were reviewed based on the 2019 WHO classification of tumours of the digestive system, the American Joint Commission on Cancer (AJCC) cancer staging of the gallbladder (eighth edition). All the specimens were reviewed and histopathologic findings were recorded, including depth of invasion, vascular invasion, nodal involvement, and presence of invasion of the adjacent liver parenchyma. The location of the tumour was defined macroscopically and histologically. Regarding stage, we were unable to restage five patients with T2 tumours according to the eighth edition of the AJCC cancer staging of the gallbladder, so we decided not to divide the T2 into T2a and T2b tumours.

Respecting immunohistochemistry, only 31 formalin-fixed paraffin-embedded sample blocks could be analysed. Microsatellite stability (MSS) was considered if there was maintenance of nuclear expression of MLH1, MSH2, MSH6 and PMS2. HER2 expression was evaluated according to the rules applied for gastric cancer (21). Expression of CD44 and ALDH1 was evaluated in order to detect CSC; a high CSC population was considered if there was the expression in more than 50% of the tumour cells. All samples were observed under an optical microscope—Nikon Eclipse 50i and pictures were taken with Nikon-Digital Sight DS-Fi1 camera.

Statistical analysis

Statistical analysis was performed using IBM SPSS software version 25.0 (IBM corporation, Armonk, NY, USA). Initially, a descriptive analysis of the results was done. Secondly, relational statistics was conducted. Metric variables were described by mean whenever there was a normal distribution of the values and by median if not. Survival analysis was conducted using the Kaplan-Meier

method and the corresponding log-rank tests. Univariate Cox regression analysis was conducted with the statistically significant variables from the survival analysis in order to identify predictors of mortality. Later, multivariate Cox regression was conducted. Hazard ratios (HR) and corresponding 95% confidence intervals (CI) and P values were reported. In all the tests used for statistical analysis, a P value of ≤ 0.05 (two-tailed test) was considered statistically significant.

Results

Clinical characteristics and treatment

Over the 11-year study period (2008–2019), a total of 9,150 adult patients underwent cholecystectomy either by laparoscopy or laparotomy at our hospital; 41 of them were diagnosed with GBC, representing an incidence of, approximately, 0.45%. Our GBC patients had a mean age of 70.1 (range, 45–96) years, 26 were female (63.4%) and 28 (68.3%) had gallstones; 27 (65.9%) of the 41 patients were symptomatic: 11 (26.8%) had jaundice and 11 also presented with acute cholecystitis.

Regarding the diagnosis, 17 (41.5%) patients were diagnosed incidentally by a pathologist, after a cholecystectomy, while in 20 (48.8%) patients the diagnosis was suspected by a radiology exam and confirmed by a pathologist. Only four (9.8%), had a suspected diagnosis perioperatively, which was also confirmed by a pathologist. Median carcinoembryonic antigen (CEA) was 3.05 ng/mL (IQR, 1.6–7.67) and median carbohydrate antigen (CA) 19.9 was 15 U/mL (IQR, 7–140).

Of the 41 patients, 16 (39.0%) underwent cholecystectomy alone, 14 (34.1%) underwent cholecystectomy and simultaneous hepatic resection (4b/5 subsegmentectomy) with HPL, while 11 (26.8%) patients underwent hepatic resection with HPL after simple cholecystectomy. Mortality in the first month after surgery was 2.4% (only 1 patient); 12 (29.3%) patients underwent adjuvant chemotherapy.

Histopathological characteristics

Of the 41 GBC, 35 (85.4%) were adenocarcinomas and six (14.6%) were other carcinomas: two undifferentiated carcinomas, two adenosquamous/squamous carcinomas and two neuroendocrine carcinomas; 13 (31.7%) patients also had a preneoplastic lesion. For 19 (46.3%) patients diagnosis was at stage > II. Globally, we were unable to stage 9 patients due to missing information on nodal staging or metastasis.

Table 1 Histopathological characteristics of patients with GBC

Variables	N=41
Preneoplastic lesion	13 (31.7%)
Type of neoplastic lesion	
Adenocarcinoma	35 (85.4%)
Other carcinoma	6 (14.6%)
Undifferentiated	2 (4.9%)
Epidermoid	1 (2.4%)
Neuroendocrine	2 (4.9%)
Adenosquamous	1 (2.4%)
Adenocarcinoma grade	
Well differentiated	14 (34.1%)
Moderately differentiated	12 (29.3%)
Poorly differentiated	3 (7.3%)
NOS	4 (9.8%)
T	
T1a	5 (12.2%)
T1b	6 (14.6%)
T2	17 (41.5%)
T3	12 (29.3%)
T4	0
Nodal staging	
N0	22 (53.7%)
N1	12 (29.3%)
N2	0
Stage	
≤ II	13 (31.7%)
> II	19 (46.3%)
LVI	19 (46.3%)
Hepatic parenchyma invasion	11 (26.8%)
Bile duct invasion	4 (9.8%)
Resection margin	
R0	23 (56.1%)
≥R1	10 (24.4%)

GBC, gallbladder carcinoma; NOS, not otherwise specified; LVI, lymphovascular invasion.

Table 2 IHC markers

Markers	N=41
MSS (MLH1, MSH2, MSH6 and PMS2 ⁺)	31 (75.6%) (26 adenocarcinomas)
HER2 ⁺	6 (14.6%) (6 adenocarcinomas)
CD44 ⁺	8 (19.5%) (4 adenocarcinomas)
ALDH1	17 (41.5%) (15 adenocarcinomas)

IHC, immunohistochemical; MSS, microsatellite stability.

At least 12 (29.3%) patients had nodal invasion (N⁺). Lymphovascular invasion (LVI) was verified for 19 (46.3%) patients, 11 (26.8%) had hepatic parenchyma invasion, four (9.8%) bile duct invasion and 10 (24.4%) had a ≥R1 resection. Histopathological characteristics can be found resumed on *Table 1*. Regarding immunohistochemistry (see *Table 2*), all analysed blocks showed MSS (75.6% from the total patients). Six patients were HER2 positives, eight were CD44 positives and seventeen were ALDH1 positives (see *Figure 1*). There was no statistically significant relation between HER2 positivity or high expression of CD44 or ALDH1 and stage. Considering adenocarcinomas only, we also did not find a statistically significance relation with histological grade.

Follow-up and survival

With a median follow-up period of 20.5 months (IQR, 8.8–53.8), 24 (58.5%) patients died and the remaining 17 (41.5%) survived at the time of analysis. Median OS was 23 months and the 3- and 5-year survival rates of the entire cohort were estimated at 43.2% and 39.6%, respectively.

For adenocarcinoma patients only (n=35) median OS was 31 months and the 3- and 5-year survival rates were 47.8% and 43.5%, respectively. On the other hand, considering other carcinoma patients only (n=6), median OS was 2 months with a 3-year survival rate of 16.7% and a 5-year survival rate of 0%.

Prognostic factors

For the entire cohort of GBC patients, presenting with acute cholecystitis or jaundice, or having a CA 19.9 ≥500 U/mL or a CEA ≥5 ng/mL were significant predictors of mortality

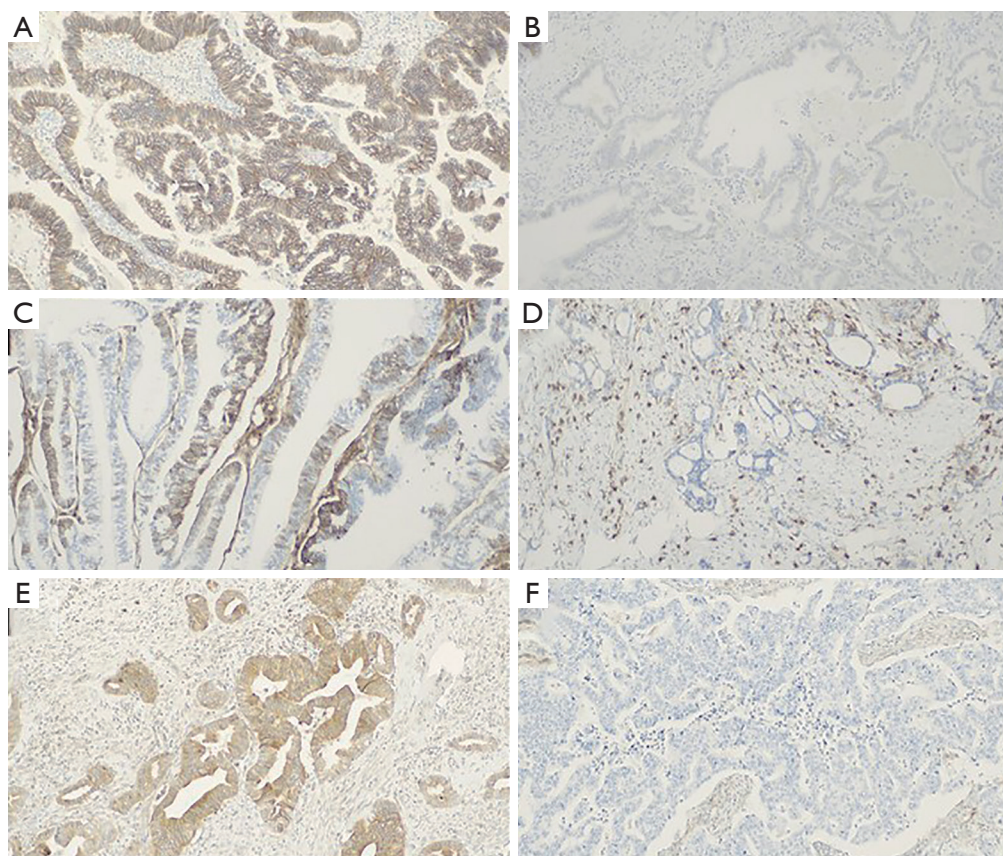


Figure 1 Immunohistochemistry results from GBC, corresponding to positive (A, C and E) and negative (B, D and F) HER2, CD44 and ALDH1 staining, respectively. Magnification is $\times 100$ in all cases.

(HR =2.59, $P=0.028$; HR =3.18, $P=0.007$; HR =2.5, $P=0.039$ and HR =2.99, $P=0.032$, respectively). Other carcinomas (versus adenocarcinomas), stage > II, N^+ , LVI, hepatic parenchyma invasion, bile duct invasion and an R1 or R2 resection were also statistically significant predictors of mortality (HR =3.32, $P=0.022$; HR =10.35, $P=0.002$; HR =6.55, $P<0.001$; HR =8.96, $P<0.001$; HR =6.86, $P<0.001$; HR =3.50, $P=0.033$ and HR =11.79, $P<0.001$, respectively). On the other hand, patients submitted to hepatic resection were 76% less likely to die than patients not submitted to hepatic resection (HR =0.24, $P<0.001$). On multivariate analysis, stage > II, LVI and hepatic resection, independently influenced survival (results presented on *Table 3*). For adenocarcinomas only, presenting with acute cholecystitis or having a CEA ≥ 5 ng/mL didn't show an association with survival.

In our cohort of patients, HER2 positivity didn't show a significant influence in survival ($P=0.649$), although these

patients showed a tendency to a shorter global survival (18 *vs.* 20 months). High CD44 and ALDH1 expression also didn't show statistically significant influence in survival ($P=0.868$ and 0.914 , respectively), although high expression ALDH1 patients showed a tendency to a shorter global survival, compared to low expression patients (13 *vs.* 23 months, respectively). On the other hand, high CD44 expression patients had a longer median global survival (20 *vs.* 18 months).

Discussion

Our retrospective study found a 0.45% incidence of GCB, which is similar to other European countries, such as Italy and Iceland, and slightly higher compared with the United Kingdom and Greece (22,23). The geographical variations in the incidence of GBC suggest that there are important genetic and environmental factors in the development of the

Table 3 Prognostic factors for GBC patients at our unit

Variables	Univariate regression			Multivariate regression		
	HR	95% CI	P value	HR	95% CI	P value
Acute cholecystitis	2.59	1.11–6.06	0.028	–	–	–
Jaundice	3.18	1.37–7.334	0.007	–	–	–
CA ≥ 19.9 ≥ 500 U/mL	2.5	1.05–5.98	0.039	–	–	–
CEA ≥ 5 ng/mL	2.99	1.10–8.14	0.032	–	–	–
Other carcinomas	3.32	1.19–9.23	0.022	–	–	–
Stage > II	10.35	2.34–45.75	0.002	8.58	1.786–41.171	0.007
N+	6.55	2.38–18.03	<0.001	–	–	–
LVI	8.96	2.78–28.89	<0.001	4.06	1.035–15.918	0.045
Hepatic invasion	6.86	2.51–18.75	<0.001	–	–	–
Bile duct invasion	3.50	1.11–11.02	0.033	–	–	–
$\geq R1$ resection	11.79	3.8–36.6	<0.001	–	–	–
Hepatic resection	0.24	0.11–0.67	<0.001	0.288	0.091–0.910	0.034

GBC, gallbladder carcinoma; CA, carbohydrate antigen; CEA, carcinoembryonic antigen; LVI, lymphovascular invasion.

disease. Besides incidence variations, there are also survival variations between countries, as shown for T2 tumours (24).

Of the 41 GBC patients, 26 (63.4%) were female. The higher incidence of GBC in women seems to be related to higher oestrogen levels, such as higher age at menarche, a higher number of childbirths and a higher number of pregnancies (25). Endogenous and exogenous oestrogen is thought to promote gallstone formation and cholecystitis (26). Estrogen receptor 1 (ESR1) seems to play a key role in mediating oestrogen lithogenic action at a cellular and molecular level (27). The mean age was 70.1 years, which is in agreement with the previous published studies (28,29). Older age is a predictor factor for malignancy (30). Gallstones have been the strongest risk factor for gallbladder malignancy. In fact, in our study, 68.3% (28/41) patients have a history of gallstones. Furthermore, GBC rates correlate with the prevalence of gallstone disease, which more commonly affects certain populations (31–33). However, only a small proportion (1–3%) of patients with cholelithiasis develop GBC, probably because other risk factors play a role (31). Among 41 patients 41.5% were accidentally diagnosed. GBC has no specific clinical manifestations and, frequently, it is difficult to diagnose. When symptomatic, may be difficult to distinguish from cholelithiasis or cholecystitis (34). In our cohort of patients, 27 (65.9%) were symptomatic. The

most common clinical manifestations were jaundice (11/41) and cholecystitis (11/41), which were consistent with other studies (35). Pathological examination showed that the majority of patients (85.4%) had adenocarcinomas, which was consistent with other studies (36,37).

Although chemotherapy and surgical techniques have improved during recent years, the 5-year survival rate in patients with GBC is still low (8). At the time of diagnosis, 46.3% of patients submitted to resection were already at a high stage > II. In the present study, the 3- and 5-year survival rates were 43.2% and 39.6%, respectively, with better survival results for adenocarcinomas only (47.8% and 43.5%, respectively). Rarer histological types of GBC had worse 3- and 5-year survival rates of 16.7% and a 5-year survival rate of 0%, compared with adenocarcinoma. Our results are in agreement with the literature (38).

Radical resection is the only effective treatment for GBC. Kang *et al.* (39) suggested that simple cholecystectomy is sufficient for stage I and II GBC. Cholecystectomy and simultaneous hepatic resection (4b/5 subsegmentectomy) are preferred in locally advanced diseases (T2b, T3 and T4) and rare histological GBC types (8). In this study, the majority of GBC was diagnosed at stage T2 (41.5%) and T3 (29.3%). Some of these patients, underwent cholecystectomy alone without subsegmentectomy (39%) due to various reasons, including the advanced patient age and the presence of

major comorbidities. For instance, patients submitted to hepatic resection were 76% less likely to die than patients not submitted to hepatic resection. This result can probably be interpreted as a study bias, since patients who did not undergo resection had more comorbidities and were older, probably having a worse performance status, than those who underwent hepatectomy. Only a study that compares identical groups will be able to eliminate this bias. However, there may be other reasons for the decrease in the risk of death, such as reducing the risk of local recurrence by increasing the disease-free margins, or justified by the pattern of dissemination of the disease itself, which should be studied in our population.

In past years, different models to predict the survival of GBC patients after surgical resection was developed (40,41). Zhang *et al.* (40) that used the SEER database with 1,422 patients identified eight factors associated with survival: age, gender, tumour size, histological grade, pT stage, pN stage, lymph node dissection and chemotherapy. In our study, we found other factors related to survival, namely acute cholecystitis or jaundice, CA 19.9 ≥ 500 U/mL or a CEA ≥ 5 ng/mL. Furthermore, the rare histological types of GBC were additional significant predictors of mortality, such as stage > II, N⁺, LVI, hepatic parenchyma invasion, biliary duct invasion and an R1 or R2 resection.

Given the poor OS and the fact that the genetic abnormalities involved in the pathogenesis of GBC remain unclear, it is crucial to search for new biomarkers. DNA mismatch repair (MMR) deficiency is a major pathway of genomic instability that leads to the accumulation of numerous mutations, resulting in a phenotype known as MSI. MSI tumours are more responsive to immune checkpoint blockade, particularly antibodies against the immune checkpoint PD-1 or PD-L1 (42). MSI has been described in different carcinomas, but little is known about the significance of MMR in GBC carcinogenesis (43). We did not found MMR deficiency, as it is already reported in other studies (44).

On the other hand, it is well established the important role of HER family of tyrosine kinases in the pathogenesis of cancer. Among the four HER family proteins, HER2 has the strongest catalytic kinase activity and, therefore, is used as an important target in cancer therapy (45). HER2 is an oncoprotein that is overexpressed in several cancers including breast and stomach (46). Overexpression drives malignant transformation in cell culture and in animal models (47). In the literature, the frequencies of HER2 expression vary according to ethnic backgrounds,

ranging from 9.4% to 44% (12). The present study showed that about 14% of patients with GBC exhibited HER2 amplification [all displayed HER2 immunohistochemical (IHC) score of 3+], which is in agreement with preliminary results published in the literature. Roa *et al.* (48) conducted a study with the aim to determine the frequency of HER2 overexpression on GBC. They reported a prevalence of HER2 overexpression in 12.8% of cases, which have a trend to show a worse OS. Since trastuzumab has been used as an effective therapy anti-HER2, patients with HER2 amplification/overexpression are good candidates, as previously shown, with promising results (49). In our cohort, these patients also showed a tendency to a shorter OS survival (P=0.649). We did not find a statistically significant relation between HER2 and stage or histological grade.

More recently, CSC markers have been studied in different gastrointestinal tumours (13,50). Preliminary results in GBC patients showed that CSC are correlated with aggressive tumour behaviour and resistance to treatments in different solid tumours (13,50,51). CD44 and ALDH1 are two potential prognostic markers and therapeutic targets of CSC. However, few studies have studied their presence in GBC. Our study showed positivity to CD44 in 19.5% of the tumours and 41.5% were positive for ALDH1. No statistically significant relation was found between high CD44 or ALDH1 and stage or histological grade (considering, in this case, only adenocarcinomas). ALDH1 positive patients showed a tendency to shorter global survival and CD44 positive patients had a longer median OS, although without statistically significant results (P=0.914 and 0.868, respectively). Liu *et al.* (16) described that ALDH1 expression was significantly higher in GBC than in peritumoral tissues, adenomatous polyps and chronic cholecystitis, also with a negative correlation with OS (P<0.001). Contrary to our results, He *et al.* (14) showed a correlation between positivity to CD44 and the aggressiveness of the tumour behaviour. In order to clarify this relation between CSC and GBC behaviour, more studies should be done. Expression of CD44 and ALDH1 might be related not only to carcinogenesis but also to clinical biological behaviour and prognosis.

There are several potential limitations in this study. First, it is a retrospective study from a single institution, with a small sample size and an heterogeneous group of patients. This may be due to the low incidence of GBC and the fact that most patients are probably not surgical candidates, and could limit the extrapolation of the results. Prospective

studies with bigger sample sizes are needed to clarify our results. Second, it was only done with GBC submitted to surgical resection, although the majority of GBC patients are not submitted to surgical treatment. It would be important to analyse that cohort of patients in future studies and also identify prognostic factors that could characterize them.

Conclusions

The incidence of GBC in our cohort is 0.45%, most commonly affecting females. GBC patients with a worse prognosis can be identified, based on the results of our study. The overexpression of HER2 could select patients for targeted therapy and prompt tissue sampling in unresectable patients, and ALDH1 and CD44 could represent prognostic markers and therapeutic inhibition targets.

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at <https://jgo.amegroups.com/article/view/10.21037/jgo-22-61/rc>

Data Sharing Statement: Available at <https://jgo.amegroups.com/article/view/10.21037/jgo-22-61/dss>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://jgo.amegroups.com/article/view/10.21037/jgo-22-61/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with Declaration of Helsinki (as revised in 2013) and was approved by Centro Hospitalar e Universitário de Coimbra Ethics Committee (No. CHUC-165-20). The requirement for informed consent was waived, since this was an exclusively observational study, with no risks for subjects.

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