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Human equilibrative nucleoside transporter 1 (hENT1) expression is a potential predictive tool for response to gemcitabine in patients with advanced cholangiocarcinoma

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ABSTRACT

Background: Cholangiocarcinoma (CC) is a rare cancer of the liver. Surgery offers the only chance for cure. When surgery is unfeasible, chemotherapy is the backbone of treatment. The combined administration of cisplatin and gemcitabine is considered standard of care. Human equilibrative nucleoside transporter 1 (hENT1) is the major transporter responsible for gemcitabine uptake into cells. hENT1 expression is associated with an increased survival for patients receiving gemcitabine after pancreatic cancer surgery, suggesting that hENT1 is predictive of response to gemcitabine.

Aim: To determine whether there is a correlation between the expression of hENT1 and disease outcome in CC.

Methods: A retrospective study on 43 patients treated at our centre with a locally advanced or metastatic CC, who received first line treatment with gemcitabine, was performed.

Results: For the whole population, median Progression Free Survival (PFS) and overall survival (OS) were 4.0 (95% Confidence Interval 2.7–5.3 months) and 10.0 months (95% CI 6.8–13.2 months), respectively. From the 26 samples available for hENT1 staining, 18 (69%) and 8 (31%) patients had high and low hENT1 immunostaining, respectively. The median PFS were 2.0 versus 6.0 months for low versus high staining respectively ($p = 0.012$). The median OS were 5.0 versus 11.0 months for low versus high staining, respectively ($p = 0.036$). On multivariate analysis, hENT1 expression was the single independent predictive factor associated with prolonged PFS (HR 0.35, $p = 0.023$) and OS (HR 0.41, $p = 0.046$).

Conclusion: In this study we show the potential of hENT1 expression as a predictor of outcome in CC treated with gemcitabine. Larger studies are necessary to confirm these promising results.

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1. Introduction

Cholangiocarcinoma (CC) is the second most frequent primary cancer of the liver. Worldwide, CC accounts for 3% of all gastrointestinal cancers. Its incidence has been rising over the past two decades in the United States and France.[1,2] It arises from the epithelial lining of the intrahepatic or extrahepatic bile duct. The anatomic location of CC can be described as intrahepatic, perihilar or distal extrahepatic. Five year survival rates are around 10–40% for CC.[3] Complete surgical resection offers the only chance for cure. However, only 10–20% of patients present with early stage disease are considered surgical candidates. Among these resected patients, recurrence rates are high, thus for the vast majority of CC patients, systemic chemotherapy is the mainstay of their treatment plan. Patients with unresectable or metastatic CC have a poor prognosis with a median overall survival (OS) of <1 year, as shown by a comprehensive pooled analysis of published clinical trials.[4] These authors also showed that chemotherapy with gemcitabine combined with cisplatin or oxaliplatin could increase response rate and tumour control rate in CC. Very recently, a randomised controlled phase III study, the ABC-02 study[5], has shown a clear benefit of a combination regimen over gemcitabine alone, considered by many as the standard chemotherapy. Authors show the superiority of the combination of cisplatin and gemcitabine over gemcitabine alone, in terms of Progression Free Survival (PFS) and OS, with a nearly 4 month absolute benefit in survival and a hazard ratio of death of 0.63 ($p < 0.0001$). This combination can thus be considered the new standard for advanced non-operable or metastatic CC.

However, chemotherapy is administered to patients without knowledge of the genetic background of the disease, which may affect drug efficacy.

Genetically determined variability of key enzymes has been shown to influence response and toxicity of cytotoxic agents including 5-fluorouracil, irinotecan and 6-mercaptopurine.[6] Potential candidates to predict which patients are likely to respond to treatment are genes encoding proteins involved in metabolism, transport across membranes, or target of anticancer drugs.[7] It is likely that genetic variability of key enzymes in gemcitabine transport and metabolism may therefore have an impact on treatment response and toxicity of gemcitabine. Because the biochemical targets of gemcitabine are intracellular, the mandatory first step in the production of cytotoxicity is permeation across the cell membrane. Gemcitabine and physiologic nucleosides are hydrophilic, and diffusion through the cell membrane lipid layer is slow. Therefore, efficient cellular uptake requires the presence of specialised integral membrane nucleoside transporter proteins.[8] Two general processes of nucleoside transport have been identified: the equilibrative bi-directional facilitators (human equilibrative nucleoside transporter 1) and the concentrative sodium/nucleoside symporters (hCNT1 and 3). The major routes for transporting gemcitabine are human equilibrative nucleoside transporter (hENT1) and to lesser extent, hCNT1 and hCNT3.[9–11] Authors have shown that cells lacking hENT1 are highly resistant to gemcitabine.[12] The abundance and distribution of the hENT1 protein can be evaluated using immunohistochemistry and has been assessed in a number of malignant and benign tissues.[13–17] Very interestingly, a recent randomised phase III trial showed that hENT1 protein expression was associated with an increased overall and disease-free survival in patients operated for a pancreatic cancer who received adjuvant gemcitabine, but not in those who received 5-FU and radiotherapy after surgery, suggesting that hENT1 expression may be predictive of response to gemcitabine.[18] These results have been reproduced by others.[19]

On the other hand, two retrospective studies showed that overexpression of hENT1 protein was significantly correlated with poor prognosis in patients with resected ampullary and gastric cancers that did not receive any chemotherapy,[13,17] suggesting that hENT1 expression could also be a prognostic marker.

On the basis of these findings, we reviewed the files of 43 patients treated at our centre with a locally advanced or metastatic CC, out of 60 patients who received first line treatment with gemcitabine alone. The aim of the study was to determine whether there was a correlation between the expression of hENT1 and disease outcomes in CC. We excluded 17 patients treated with gemcitabine for a gallbladder cancer, because their prognosis and sensitivity to chemotherapy is different.[4] Although increasing knowledge suggests that extrahepatic (including perihilar) CC and intrahepatic CC could have different pathophysiology, they share common molecular features, as described by a recent review,[20] and are treated the same way. We therefore decided to analyse together both extra- and intrahepatic CC in this study.

2. Materials and methods

2.1. Clinical data and tumour specimen acquisition

This retrospective study was restricted to patients with a CC, excluding gallbladder and ampullary cancer, followed-up and treated with gemcitabine as first-line therapy, at the Cliniques universitaires Saint-Luc, Université catholique de Louvain in Bruxelles, between July 1998 and November 2007. Data on clinical variables, including sex, age, and TNM classification were gathered from patient record files. Tumour samples were obtained from surgery blocks (23% of patients had prior surgery) or from local biopsies or fine-needle aspiration (FNA) obtained through endosonography.

2.2. Tissue preparation and immunostaining

Among the 43 patients, 26 tumour samples were available and analysable. The other 17 samples were too small because these were generally obtained by FNA and thus not containing enough cancer cells to allow semi-quantitative analysis. Anti-hENT1 monoclonal antibodies were produced and characterised as described previously.[15] Goat anti-mouse antibodies and horseradish peroxidase-labelled dextran polymer (DAKO EnVision+) were purchased from DAKO Corporation (Carpinteria, CA). The formalin-fixed, paraffin embedded CC tumour sections were deparaffinised with three immersions in xylene baths (10 min) each followed by serial washes in graded alcohol from 100% to 50%. After rinsing in water, slides

were placed in 250 ml of high pH 1×DAKO target antigen retrieval solution and microwaved in TT-mega Mileston (ESBE Scientific, Markham, Ontario, Canada) under control temperature and high pressure for 10 min at 100 °C. After cooling in water for 6 min, the slides were rinsed with water; peroxidase was blocked in 3% H_2O_2 solution with methanol for 10 min, and then washed in running water for 10 min. Phosphate-buffered saline was used for rinsing before incubation with appropriate dilutions of anti-hENT1 monoclonal antibodies. Slides with anti-hENT1 were incubated in a humidified chamber overnight at 4 °C. The sections were rinsed with PBS, immersed in a buffer for 5 min, incubated with goat anti mouse dextran conjugate (DAKO envision) for 30 min, followed by the soaking in PBS. DAKO diaminobenzidine liquid chromagen was placed on the samples for 5 min and rinsed, after which the slides were soaked in 1% $CuSO_4$ for another 5 min. Subsequently, the sections were rinsed, counterstained with haematoxylin, dehydrated through graded alcohol and xylene, and finally coverslipped. Negative controls were provided by omitting the primary antibodies.

Two pathologists (R.L. and C.S.), blinded to clinical characteristics and outcomes, assessed and scored the hENT1 immunostaining intensities. Scoring for hENT1 was based on relative intensities of staining of the CC with reference to the normally strong hENT-1 staining within lymphocytes, as previously described.[21] As an adaptation from the score described by Farrell et al.,[18] a *high* hENT1 staining score was given for weak and/or strong reactivity in greater than 50% of neoplastic cells. A *low* hENT1 staining score was given if there was no staining in greater than 50% of cells. Overall interobserver agreement was very good, with only 2/26 samples scored differently. In these 2 cases, a second interpretation was given by the most experienced pathologist (R.L.), who made the final scoring.

Examples of high and low tumoural stainings and a positive control (lymphoid tissue) are shown in Figs. 1 and 2.

2.3. *Statistical analysis*

Demographic and clinical information was obtained from medical records. Overall survival (OS) was calculated from the date of first treatment with gemcitabine to the date of death or last follow-up. Progression-free survival (PFS) according to clinical judgement was calculated from the date of first treatment to the date of first progression or death from any event, whichever was the first. OS and PFS were calculated according to the Kaplan–Meier method and compared by the log-rank test based on the pattern of hENT1 immunostaining. A multivariate analysis using Cox regression model was built to identify potential independent variables for PFS and OS. We analysed sex, T, N and M stages, tumour size, previous surgery, vascular invasion at surgery, R0 resection, extra- or intra-hepatic CC and hENT1 status. Results were considered significant at $p < 0.05$.

3. Results

3.1. *Patient population*

The main patients' characteristics are summarised in Table 1. The cohort consisted of 43 patients (20 males), with pathology-proven diagnosis of a locally advanced (i.e. deemed unresectable) or metastatic CC. The median age at diagnosis was

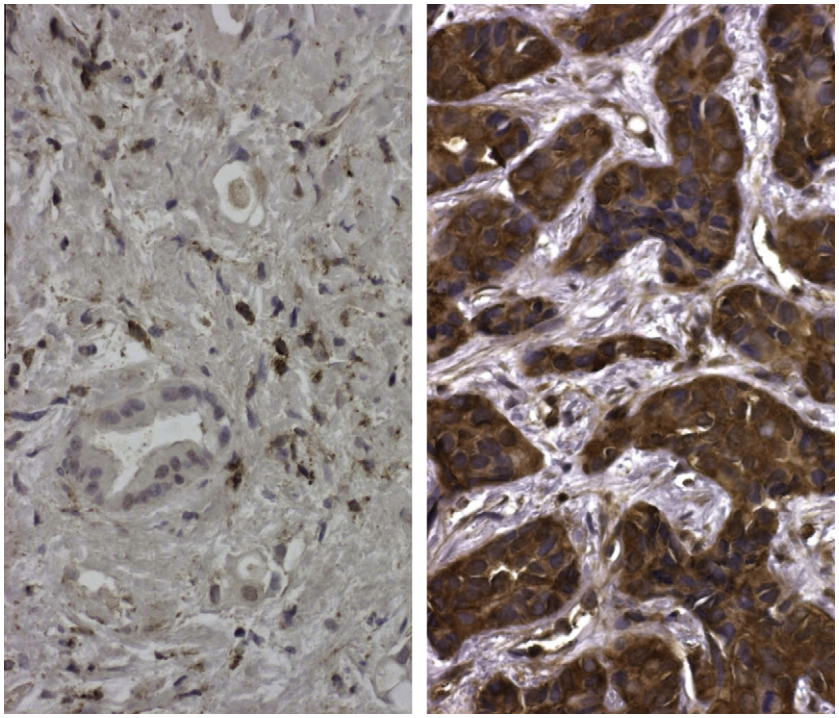


Fig. 1 – Left: representative cholangiocarcinoma classified as 'low' for human equilibrative nucleoside transporter 1 (hENT1) expression. Note the scarce staining in rare cells. Right representative cholangiocarcinoma classified as 'high' for hENT1 expression. Note the strong staining in the majority of malignant cells.

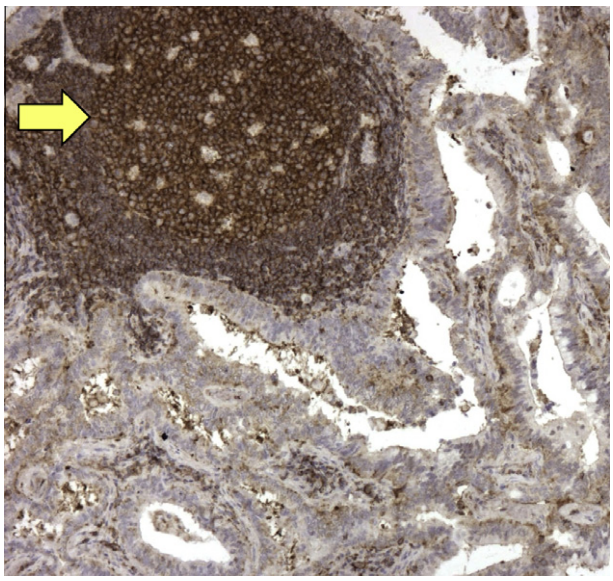


Fig. 2 – Lymph node showing a high staining, depicted by the yellow arrow, surrounded by a cholangiocarcinoma classified as 'low' for human equilibrative nucleoside transporter 1 (hENT1) expression. Note the scarce staining in rare cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1 – Study population main characteristics.

Total number	43
Median age (min.–max.)	62 (43–80)
Female (%)	23 (53)
Extra-hepatic cholangiocarcinoma (CC) (%)	17
Intra-hepatic CC (%)	26
T stage	
T2 (%)	4 (9)
T3 (%)	36 (84)
T4 (%)	3 (7)
N stage	
N0 (%)	9 (21)
N1-2 (%)	19 (44)
Nx (%)	15 (35)
M stage	
M0 (%)	14 (32)
M1 (%)	29 (68)
Human equilibrative nucleoside transporter 1 (hENT1) status (n = 26 pts)	
Positive (%)	18 (69)
Negative (%)	8 (31)

62 years (range 43–80). Seventeen patients had an extrahepatic CC (Klatskin and distal tumours), 26 having an intrahepatic tumour; 29 had metastatic disease at the time of initiation of the treatment with gemcitabine. Twelve patients (28%) had had prior surgery, and relapse prompted chemotherapy initiation. Chemotherapy consisted of 1000 mg/m^2 gemcitabine every week during 7 weeks with 1 week rest, then on days 1, 8 and 15 every 28 days for two cycles. Patients were evaluated for response after completion of at least 8 weeks of treatment, then every two cycles (i.e. 2 months). The median follow-up was 9.5 months (1–120 months).

3.2. Outcome

For the whole population, median PFS and OS were 4.0 (95% CI 2.7–5.3 months) and 10.0 months (95% CI 6.8–13.2 months), respectively. These results compare favourably with previous studies that assessed the efficacy of gemcitabine as monotherapy in treatment of CC[4] and are in line with the results of the phase III ABC-02 study[5].

3.2.1. Univariate analysis

We further analysed PFS and OS according to anatomic localisation. We found that extrahepatic CC ($n = 17$) had a median PFS of 4.0 months (95% CI 2.9–5.1 months) and a median OS of 8.0 months (95% CI 0–16.0 months), whereas intrahepatic CC ($n = 26$) tend to have a slightly better median PFS of 5.0 months (95% CI 3.6–6.4 months) and a median OS of 10.0 months (95% CI 6.8–13.2 months). This better outcome was not statistically different ($p = 0.64$). Among other factors analysed, only previous surgery was borderline significant for PFS, with a median PFS of 4.0 months (95% CI 2.7–5.3) versus 6 months (95% CI 0.91–11.1) for non-operated versus operated patients, respectively (HR 0.534, $p = 0.093$), and for survival with median OS of 10.0 months (95% CI 7–13.0) versus 12 months (95% CI 7.3–14.7) for non-operated vs operated patients (HR 0.579, $p = 0.15$).

To determine the role of hENT1 expression on gemcitabine effect, patients were analysed according to hENT1 staining and compared. From the 26 analysed samples, 18 patients (69%) had high hENT1 immunostaining and eight patients (31%) had low hENT1 immunostaining, which is in line with results obtained in pancreatic cancer[18] and in biliary tract cancer.[22]

Results of the PFS and OS curves are shown in Figs. 3 and 4. The median PFS were 2.0 versus 6.0 months for low versus high staining, respectively ($p = 0.012$) The median OS was 5.0 months in patients with low hENT1 staining versus 11.0 months in patients with high staining ($p = 0.036$). On univariate analysis, looking at PFS, only hENT1 expression was linked to a significantly prolonged survival (HR = 0.35, $p = 0.023$). Regarding OS, only T stage (T1 + T2 versus T3 + T4, $p = 0.046$) and hENT1 expression ($p = 0.046$) were linked to a statistical difference.

3.2.2. Multivariate analysis (Tables 2 and 3)

Using a Cox regression model for multivariate analysis, we found that hENT1 expression was the single independent predictive factor significantly associated with prolonged PFS (HR 0.35, 95% CI 0.14–0.87, $p = 0.023$) and OS (HR 0.41, 95% CI 0.17–0.98, $p = 0.046$).

4. Discussion

In this study, we analysed the potential role of hENT1 expression as a predictive marker of gemcitabine efficacy given as first-line exclusive therapy to patients with advanced CC, excluding gallbladder cancer. Our findings support the

Table 2 – Progression-free survival.

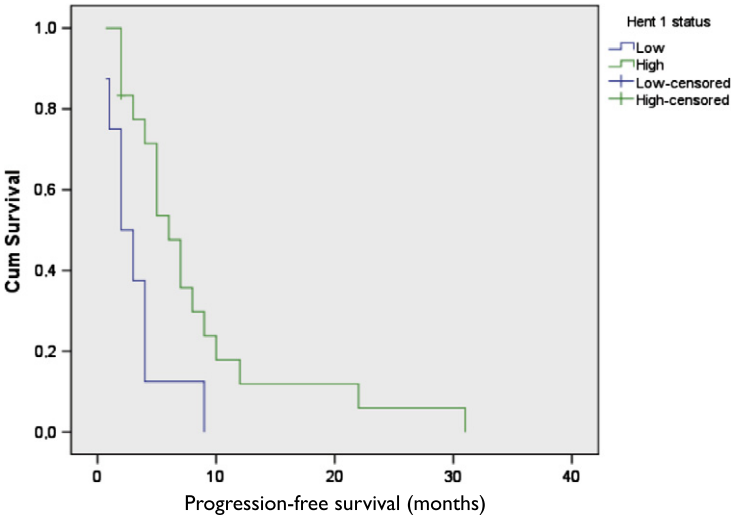
Predictor	Category	Number	Univariate analysis		Multivariate analysis	
			Hazard ratio (95% Confidence Interval)	*p* Value	Hazard ratio (95% CI)	*p* Value
Sex	Male	23	1	0.66		
	Female	20	0.93 (0.68–1.27)			
T stage	T1 + T2	4	1	0.12	3.2 (0.34–29.8)	0.31
	T3 + T4	39	2.6 (0.78–8.53)			
N stage	N0	9	0.61 (0.26–1.41)	0.25		
	N+	19	1			
M stage	M0	14	0.98 (0.5–1.92)	0.94		
	M1	29	1			
Surgery	Yes	12	0.53 (0.26–1.11)	0.093		
	No	29	1			
Vascular invasion	Yes	18	1	0.25		
	No	11	0.61 (0.27–1.4)			
R0 resection	Yes	5	0.69 (0.2–2.4)	0.56		
	No	7	1			
Intrahepatic CC	Yes	26	0.88 (0.47–1.66)	0.69	0.72 (0.25–2.06)	0.54
	No	17	1			
Human equilibrative nucleoside transporter 1 (hENT1) status	High	17	0.35 (0.14–0.87)	0.023	0.35 (0.14–0.87)	0.048
	Low	9	1			

Table 3 – Overall survival.

Predictor	Category	Number	Univariate analysis		Multivariate analysis	
			Hazard ratio (95% Confidence Interval)	*p* Value	Hazard ratio (95% CI)	*p* Value
Sex	Male	23	1	0.62		
	Female	20	0.86 (0.46–1.59)			
T stage	T1 + T2	4	1	0.046	3.3 (0.37–29.8)	0.29
	T3 + T4	39	3.43 (1.02–11.6)			
N stage	N0	9	0.58 (0.24–1.39)	0.22		
	N+	19	1			
M stage	M0	14	0.64 (0.33–1.27)	0.2		
	M1	29	1			
Surgery	Yes	12	0.58 (0.28–1.21)	0.15		
	No	29	1			
Vascular invasion	Yes	18	2.09 (0.92–4.8)	0.08	2.39 (0.59–9.6)	0.22
	No	11	1			
R0 resection	Yes	5	0.85 (0.25–2.95)	0.65		
	No	7	1			
Intrahepatic CC	Yes	26	0.5 (0.22–1.1)	0.1	0.6 (0.22–1.65)	0.32
	No	17	1			
Human equilibrative nucleoside transporter 1 (hENT1) status	High	17	0.41 (0.17–0.98)	0.046	0.41 (0.17–0.98)	0.046
	Low	9	1			

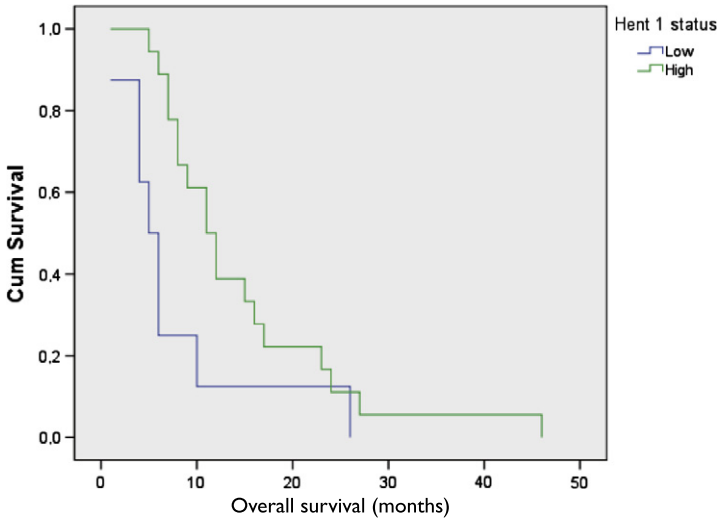
assumption that high expression of hENT1 confers an increase both in PFS and OS to these patients. These findings were made both in univariate and multivariate analysis, after adjustment for standard clinicopathologic prognostic factors. There is a substantial interest in identifying and validating molecular markers to select patients with a high likelihood of benefiting from specific chemotherapy regimens. Gemcitabine requires the NT protein hENTs to efficiently enter cells and hENT1 deficiency confers resistance to gemcitabine *in vitro*. Recently it has been demonstrated that hENT1 expression could be both a relevant predictive[18] and prognostic[19] marker of benefit from gemcitabine in patients with resected pancreatic cancer.

In Biliary tract cancer (BTC), only one study recently explored hENT1 expression as a predictive factor for gemcitabine efficacy. The study failed to show statistical differences in OS, although they showed a better TTP in patients with high hENT1 expression, only in univariate analysis.[22] That study has several limitations: first, authors mixed patients with different organ origin. Indeed from the 31 patients



No. at risk	0 months	5 months	10 months	15 months	20 months	25 months
high hENT1	17	14	9	4	2	1
Low hENT1	9	2	0			

Fig. 3 – Actuarial curves for progression-free survival. Cholangiocarcinoma positive for human equilibrative nucleoside transporter 1 (hENT1) expression are shown by the green curve, hENT1 negative cholangiocarcinoma by the blue curve. The median PFS is 2.0 versus 6.0 months for negative versus positive hENT1 tumours respectively ($p = 0.012$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



No. at risk	0	6 months	12 months	18 months	24 months	30 months
high hENT1	17	17	9	4	3	1
Low hENT1	9	4	2	1	1	0

Fig. 4 – Actuarial curves for overall survival. Cholangiocarcinoma positive for human equilibrative nucleoside transporter 1 (hENT1) expression are shown by the green curve, hENT1 negative cholangiocarcinoma by the blue curve. The median overall survival is 5.0 versus 11.0 months for negative versus positive hENT1 tumours respectively ($p = 0.036$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

studied, nine had gallbladder cancer, five ampullary, with only 17 'pure' biliary tract cancers. However, as already stated, it is known that gallbladder cancer has a different natural prognosis, but also distinct sensitivity to chemotherapy. The same authors have published data suggestive of worse prognosis in hENT1 high ampullary tumours,[13] so adding those tumours to their paper assessing hENT1 expression in BTC added further heterogeneity .to the population studied.[22]

Second, chemotherapy consisted of a mix of doublet regimens, including capecitabine or 5-fluorouracile for 19 patients and oxaliplatin for four patients. So only nine patients received Gem alone. As these authors state, hENT1 seems to play a role in the efficacy of fluoropyrimidines,[23] indicating their conclusions may be influenced by this confounding factor. For all these reasons, any conclusion in this paper would be based on a very limited dataset. In contrast, despite the small sample-size of the present study, we have assessed a homogenous population of CC patients, excluding gallbladder and ampullary tumours, treated with gemcitabine in monotherapy. We believe the results we obtain in a homogeneous population are strongly suggestive of a predictive effect of the hENT1 status. As the combination of gemcitabine and cisplatin is considered the new standard in advanced CC since the results of the ABC-02 trial,[5] it is critical to better define those patients that are really benefiting from gemcitabine.

However, we do acknowledge that our findings do not allow making a distinction between a predictive and/or a prognostic effect of hENT1 expression on CC outcome treated with gemcitabine. Although this would be highly interesting, it is unlikely that a prospective trial with a BSC arm, which would help us answering this question, will ever be launched. In the mean time, we believe the potential of hENT1 expression as a predictor of outcome in CC treated with gemcitabine should further be explored. As for any biomarker, a first step would be further replication of our findings in independent and, ideally, bigger set of patients. Subsequently, ideally, biomarker driven randomised protocols should be undertaken, but the rarity of this disease will render this approach difficult to realise.

Conflict of interest statement

None declared.

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