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Prognostic effect of hENT1, dCK and HuR expression by morphological type in periampullary adenocarcinoma, including pancreatic cancer

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ABSTRACT

Background: Putative biomarkers of gemcitabine response have been extensively studied in pancreatic cancer, but less so in other types of periampullary adenocarcinoma. The most studied biomarker is human equilibrative nucleoside transporter 1 (hENT1), and the activating enzyme deoxycytidine kinase (dCK) has also been linked to treatment response. The RNA-binding protein human antigen R (HuR) has been demonstrated to confer increased dCK levels in vitro and to predict gemcitabine response in vivo. Here, we investigated the prognostic impact of hENT1, dCK and HuR in pancreatobiliary (PB) and intestinal (I) type periampullary cancers, respectively. *Material and methods*: Immunohistochemical expression of hENT1, dCK and HuR was evaluated in tissue microarrays with all primary tumours and 103 paired lymph node metastases from a consecutive retrospective cohort of 175 patients with resected periampullary adenocarcinomas. *Results*: In patients with PB-type tumours, neither hENT1 nor dCK expression was prognostic. A high HuR cytoplasmic/nuclear ratio was associated with a significantly reduced five-year overall survival (OS) in patients receiving adjuvant gemcitabine (HR 2.07, 95% CI 1.03–4.17) but not in untreated patients ($p_{interaction} = 0.028$). In patients with I-type tumours receiving adjuvant chemotherapy, high dCK expression was significantly associated with a prolonged recurrence-free survival (RFS) (HR 0.09, 95% CI 0.01–0.73, $p_{interaction} = 0.023$). Furthermore, HuR expression was associated with a prolonged OS and RFS in unadjusted but not in adjusted analysis and hENT1 expression was an independent predictor of a prolonged RFS (HR 0.24, 95% CI 0.10–0.59), regardless of adjuvant treatment. *Conclusion*: hENT1 expression is a favourable prognostic factor in I-type, but not in PB-type tumours. High dCK expression is a favourable prognostic factor in patients with I-type tumours receiving adjuvant treatment and a high cytoplasmic/nuclear HuR ratio is a negative prognostic factor in gemcitabine-treated PB-type tumours. Morphological subtype should always be considered in biomarker studies on periampullary cancer.

Gemcitabine is an antimetabolite commonly used for treatment of pancreatic cancer and other periampullary adenocarcinomas, in the adjuvant as well as palliative setting. Several putative biomarkers predictive of gemcitabine response have been examined, with varying and sometimes conflicting results.

Human equilibrative nucleoside transporter 1 (hENT1) provides the major route for gemcitabine to enter a cell, and is one of the most extensively studied biomarkers in the context of gemcitabine response. In a meta-analysis encompassing 10 studies on 399 patients with resected pancreatic cancer, hENT1 expression was found to be predictive of gemcitabine response [1]. Results from a retrospective study on 413 consecutive, unselected cases of resected pancreatic cancer showed that hENT1 had no prognostic value in patients receiving non-gemcitabine-based adjuvant therapy, whereas high hENT1 predicted a longer overall survival (OS) among patients who

had received gemcitabine [2]. In a study on 196 pancreatic cancer cases from the prospective RTOG 9704 trial, high hENT1 expression was found to correlate with an increased OS and disease-free survival in patients treated with gemcitabine but not with 5-FU [3]. Another study on 380 pancreatic cancer cases from the ESPAC-3 trial demonstrated that resected cases with high tumour-specific hENT1 expression receiving adjuvant gemcitabine had a significantly longer OS than those with low expression, using the median hENT1 score as cut off, but also that patients with low hENT1 had a longer OS after 5-FU therapy than after gemcitabine [4]. The majority of studies have been performed on pancreatic cancer, but in a study on patients with resected ampullary adenocarcinomas, who did not receive adjuvant chemotherapy (n=41), hENT1 expression was found to be higher in intestinal type (I-type) than in pancreatobiliary type (PB-type) tumours [5] and to be associated with a shorter OS [6].

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In a first, rate-limiting step, gemcitabine is phosphorylated by deoxycytidine kinase (dCK), which is required for its incorporation into DNA and subsequent masked chain termination and apoptosis [7]. Expression of dCK is required for gemcitabine sensitivity and cell lines with induced resistance show decreased dCK RNA levels, while influx of gemcitabine into the cells is unaffected [8]. In a cohort of 416 patients with resected pancreatic cancer, high dCK expression correlated with a significantly longer OS in patients treated with gemcitabine, but not in patients receiving no adjuvant treatment or non-gemcitabine-based adjuvant chemotherapy [2]. In patients with resected pancreatic cancer (n=165) who received adjuvant 5-FU chemoradiation followed by either 5-FU or gemcitabine, high dCK expression correlated with a longer OS in the 5-FU arm but not in the gemcitabine arm [9]. The loss of a treatment predictive effect of dCK in the gemcitabine arm was proposed to be an effect of radiation disrupting the complex of human antigen R (HuR) and dCK-mRNA, leading to lower levels of dCK protein. This hypothesis does however not explain the observed association between high dCK and longer survival in the 5-FU arm. In a meta-analysis including four studies of either protein or gene expression of dCK, high dCK levels predicted a longer OS and recurrence-free survival (RFS) in gemcitabine-treated patients with pancreatic cancer [1]. To our knowledge, the prognostic or predictive value of dCK has not yet been studied in I-type periampullary adenocarcinoma.

HuR is an RNA binding protein that performs post-transcriptional regulation of several proteins in response to stress or growth signals, thereby stabilising mRNAs related to proliferation, angiogenesis and evasion of apoptosis [10,11]. Cytoplasmic HuR (referred to as HuR) is also increased in malignant cells as compared with corresponding normal cells, and has been found to be associated with adverse clinico-pathological factors and a shorter OS in several different cancer forms [12], e.g. gastric cancer, gallbladder cancer, breast cancer, urothelial cancer and non-small cell lung cancer. In pancreatic cancer, however, two small studies found high HuR expression to be associated with a longer OS in patients treated with gemcitabine, and HuR was also demonstrated to bind dCK-mRNA, which might explain a greater sensitivity to gemcitabine in tumours with high levels of HuR [13,14]. Low nuclear HuR expression has not been associated with prognosis or prediction of response to chemotherapy, but a high cytoplasmic to nuclear ratio of HuR (HuR C/N ratio) was demonstrated to be associated with a shorter OS in 560 cases of colorectal cancer [15]. The expression of HuR has, to the best of our knowledge, not been studied in I-type periampullary adenocarcinoma before.

Overall, mechanisms and markers of sensitivity to chemotherapy in I-type periampullary adenocarcinomas remain less studied. Therefore, the aim of the present study was to examine the associations between protein levels of hENT1, dCK and HuR, and their prognostic and potential treatment predictive values, in both PB-type periampullary adenocarcinomas, and in I-type periampullary adenocarcinomas.

Patients

The study cohort is a previously described retrospective consecutive series of pancreaticoduodenectomy specimens from all patients ($n = 175$) with periampullary adenocarcinoma, including pancreatic cancer, resected at the university hospitals of Lund and Malmö, Sweden, from 1 January 2001 until 31 December 2011 [16–19]. Data on survival were gathered from the Swedish National Civil Register. Follow-up started at the date of surgery and ended at death, at five years after surgery or at 31 December 2013, whichever came first. Information on neoadjuvant and adjuvant treatment and recurrence was obtained from patient records. All haematoxylin and eosin stained slides from all cases were re-evaluated by one pathologist (JEL), blinded to the original report and outcome, as previously described [16].

The study has been approved by the Ethics Committee of Lund University (ref no 445/07).

Tissue microarray construction

Tissue microarrays (TMAs) were constructed using a semi-automated arraying device (TMArrayer, Pathology Devices, Westminister, MD, USA). A standard set of three tissue cores (1 mm) were obtained from each of the 175 formalin-fixated paraffin-embedded primary tumours and from lymph node metastases from 105 of the cases, whereby one to three lymph node metastases were sampled in each case.

Immunohistochemistry and staining evaluation

For immunohistochemical analysis of dCK and HuR expression, 4 μm TMA-sections were automatically pre-treated using the PT Link system and then stained in an Autostainer Plus (DAKO, Glostrup, Copenhagen, Denmark) with the mouse monoclonal dCK antibody 16G6 (OriGene Technologies, Inc., Rockville, MD, USA) and the mouse monoclonal HuR (G-8) antibody sc-365816 (Santa Cruz Biotechnology Inc., Dallas, TX, USA). For immuno-histochemical analysis of HENT1, 4 μm TMA-sections were pre-treated using Cell Condition Solution 1 (Ventana Medical Systems, Tucson, AZ, USA) and stained with the ready-to-use rabbit monoclonal HENT1 antibody SP120 on a Ventana BenchMark stainer (Ventana Medical Systems Inc.).

The staining of dCK and hENT1 was annotated by one pathologist (JEL) and HuR was independently annotated by two observers (JEL and LBD) and consensus was reached in discordant cases. For dCK, only the nuclear staining was scored, HuR and nuclear HuR staining was assessed separately, and for hENT1, cytoplasmic and membranous staining was assessed together. A multiplier of the fraction of stained cells for each level of staining intensity (0=negative, 1=weak, 2=moderate and 3=strong) was calculated for each core (H-score, 0–300) and the mean value of assessable cores was used for further analysis. HuR however often showed varying intensities of weak staining, making it necessary to fine tune the scoring of intensity (0=negative, 1=very weak, 2=weak, 3=weak moderate, 4=strong moderate, 5=strong and 6= very strong), creating a score ranging from 0 to 600. The HuR C/N ratio

was calculated using the formula HuR C/N ratio=HuR+0.1/HuRn+0.1, to make cases with no staining computable.

Lymphocytes served as a positive internal control for dCK, endocrine pancreatic islets for HuR, and endocrine pancreatic islets and endothelial cells for hENT1. The median scores of hENT1, dCK, HuR and HuR C/N ratio were calculated separately for PB- and I-type adenocarcinomas, and were used as cut-offs to create groups of high and low expression.

Statistical analysis

χ^2-test was applied to analyse the relationship between the dichotomised expression of each biomarker and clinicopathological parameters. Two patients with PB-type adenocarcinomas who had received neoadjuvant chemotherapy were excluded from the correlation and survival analyses. Three additional patients were excluded from the survival analyses; two with I-type adenocarcinomas who died within one month from surgery due to complications and one with PB-type adenocarcinoma who emigrated five months after surgery.

Kaplan Meier estimates of five-year OS and RFS and log rank test were applied to evaluate survival differences in strata according to high and low expression for each biomarker combined with given adjuvant treatment; gemcitabine versus none/other for PB-type and any versus none for I-type tumours. Hazard ratios (HR) for death and recurrence within five years were calculated by Cox regression proportional hazard's modelling in unadjusted analysis and in a multivariable model adjusted for expression of hENT1, dCK, HuR and HuR C/N ratio as well as age, T-stage, N-stage, differentiation grade, lymphatic invasion, vascular invasion, perineural invasion, and adjuvant chemotherapy. A backward conditional method was used for variable selection in the adjusted model. To estimate the interaction effect for survival between given adjuvant treatment and the biomarker expression, the following interaction variables were constructed; any adjuvant chemotherapy (+/−) × biomarker (high/low) for I-type, and gemcitabine-based adjuvant treatment (+/−) × biomarker (high/low) for PB-type tumours.

All tests were two sided. p-Values <0.05 were considered significant. All statistical analyses were performed using IBM SPSS Statistics version 20.0 (SPSS Inc., Chicago, IL, USA).

The proportional hazards assumption was tested by examining log-log survival curves.

In the planning and execution of this study, efforts were made to follow the REMARK-criteria to increase comparability between studies and enable results to be reproduced [20].

Results

Patient population

In the group of 109 patients with PB-type tumours, 50 received gemcitabine-based adjuvant therapy (45 gemcitabine, 3 gemcitabine + 5-FU analogue and 2 gemcitabine + oxaliplatin) and 59 did not receive adjuvant gemcitabine (50 no adjuvant, 8 5-FU and 1 5-FU + oxaliplatin). Among the 63 patients with I-type tumours, 18 received adjuvant therapy (7 gemcitabine, 5 5-FU, 4 5-FU + oxaliplatin, 1 gemcitabine + 5-FU analogue and 1 gemcitabine + oxaliplatin) and 45 received no adjuvant chemotherapy.

Seven patients with PB-type tumours received adjuvant radiotherapy, six together with 5-FU and one together with gemcitabine. Two patients with I-type tumours received adjuvant radiotherapy, one together with 5-FU and one without chemotherapy.

Median follow up time, from surgery to death, censoring or at the most 60 months, was 25.4 months for PB-type and 38.8 months for I-type tumours.

Median OS for 109 patients with PB-type tumours (84 events) was 25.4 months [95% confidence interval (CI) 22.2–28.7]; 28.1 months (95% CI 26.3–29.9) for 50 patients (37 events) receiving gemcitabine-based adjuvant therapy and 23.1 months (95% CI 19.2–27.0) for 59 patients (47 events) not receiving adjuvant gemcitabine.

Median OS for 63 patients with I-type tumours (32 events) was 52.9 months (95% CI 34.0–71.9); 46.6 months (95% CI 28.9–64.4) for 45 patients not receiving adjuvant treatment, and median OS was not reached for 18 patients who received adjuvant chemotherapy.

hENT1, dCK and HuR expression

Sample immunohistochemical images of hENT1, dCK and HuR stainings are shown in Figure 1. H-score expression levels in PB-type and I-type primary tumours and metastases are shown in Supplementary Figure 1 (available online at http://www.informahealthcare.com).

Independent samples t-test showed a higher expression of hENT1 and HuR, and also a higher HuR C/N ratio in I-type as compared with PB-type primary tumours (all three comparisons p<0.001) while there was no difference in expression of dCK by morphological type (p=0.725). In PB-type tumours paired samples t-test showed an increased expression of dCK and hENT1 in metastases, as compared with corresponding primary tumours, while in I-type tumours there was a decreased expression of HuR in metastases (Supplementary Figure 1). The HuR C/N ratio did not differ between primary tumours and paired metastases in either morphological type (data not shown).

Paired samples t-test in the full cohort showed an increased dCK H-score from primary tumours to metastases (p=0.003) and a decreased HuR H-score (p=0.002) while there were no differences in HuR C/N ratio or H-score of hENT1 between primary tumours and metastases (data not shown).

Expression levels of hENT1, dCK and HuR did not differ according to adjuvant treatment (data not shown).

Associations of hENT1, dCK and HuR expression with clinicopathological parameters

Associations between the dichotomised expression of hENT1, dCK and HuR and clinicopathological parameters are shown in Table I for PB-type and in Table II for I-type adenocarcinomas.

In PB-type tumours, dichotomised dCK expression was not significantly associated with other parameters. There were no associations between the dichotomised or continuous H-score
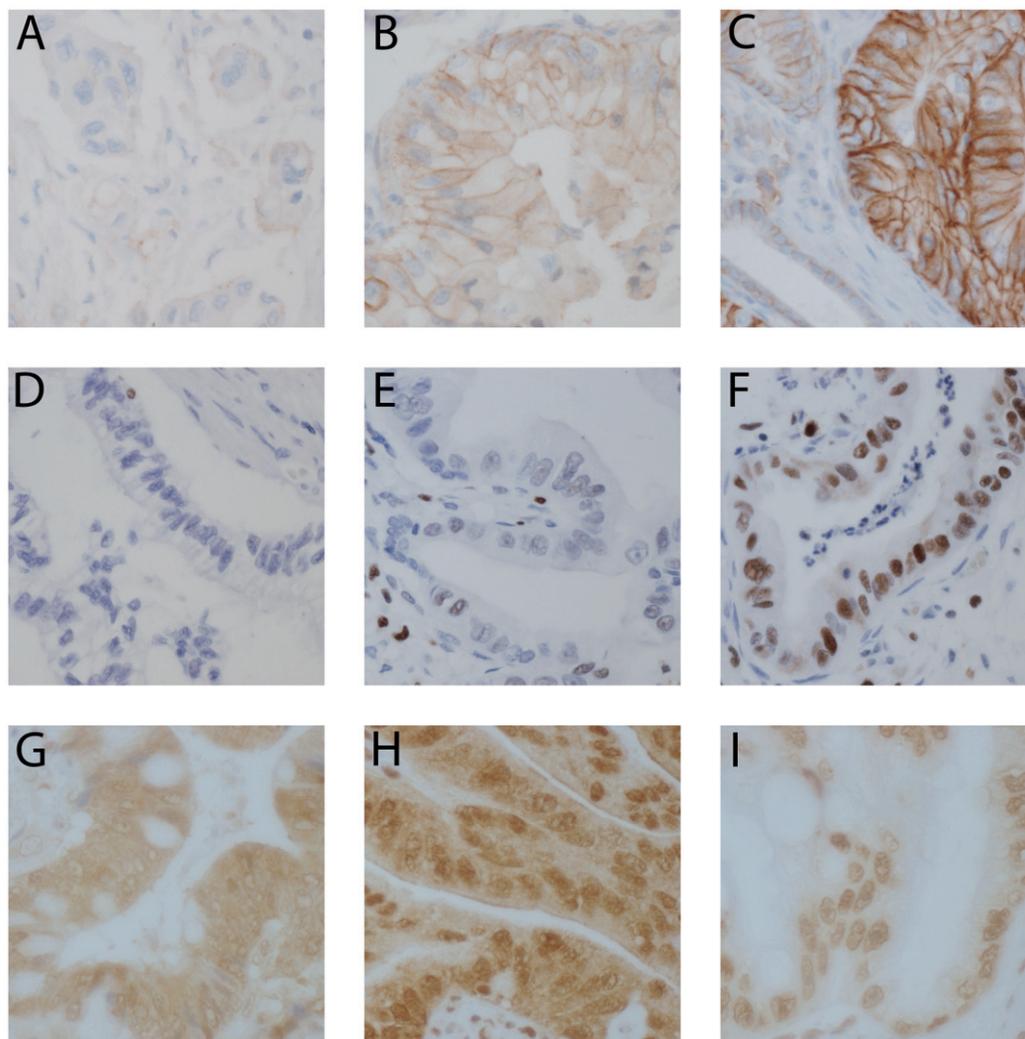
Figure 1. Examples of immunohistochemical staining of hENT1 (A–C), dCK (D–F) and HuR (G–I).

of dCK, HuR or HuR C/N ratio in the full cohort or when excluding the nine patients who received adjuvant radiotherapy. HuR was significantly associated with male sex, and hENT1 with well/moderately differentiated PB-type tumours (Table I). As demonstrated in Supplementary Table I (available online at http://www.informahealthcare.com), a high HuR C/N ratio was associated with male sex, high HuR expression and positive or unassessable margins (R1-Rx vs. R0).

In I-type tumours dCK expression was significantly associated with a higher proportion of uninvolved margins, while HuR was associated with hENT1 expression and a lower proportion of perineural growth, and hENT1 was associated with duodenal origin, larger tumour size and uninvolved lymphatic vessels (Table II). There were no significant associations between HuR C/N ratio and any clinicopathological parameter apart from HuR in I-type tumours (data not shown).

Prognostic value of hENT1, dCK and HuR expression

Kaplan–Meier analysis revealed that in the entire group of patients with PB-type tumours, including both those receiving

and not receiving adjuvant gemcitabine, there were no differences in OS or RFS according to high or low hENT1, dCK, HuR and HuR C/N ratio (Figure 2A–F, and Supplementary Figure 2, available online at http://www.informahealthcare. com). These findings were confirmed in univariable and multivariable Cox regression analysis for RFS (Table III) and five-year OS (Supplementary Table II, available online at http://www.informahealthcare.com).

Kaplan–Meier analysis revealed that in the entire group of patients with I-type tumours, high hENT1 expression was significantly associated with a longer RFS but not OS, with similar findings in patients not receiving adjuvant therapy (Figure 3A and B). These findings were confirmed in univariable analysis for RFS (HR 0.33, 95% CI 0.15–0.72), and remained significant in multivariable analysis (HR 0.24, 95% CI 0.10–0.59) (Table IV). High hENT1 also had a similar, but borderline significant, prognostic effect for RFS when considering only I-type tumours of ampullary origin, and thus excluding tumours of duodenal origin (data not shown).

In patients with I-type tumours, there was no significant difference in OS or RFS according to high and low dCK expression, neither in the entire group nor in untreated patients (Figure 3C and D). These findings were confirmed in

Table I. Associations between hENT1, dCK, HuR and clinicopathological parameters in pancreatobiliary type periampullary adenocarcinomas.

	hENT1			dCK			HuR		
	Low (n=55)	High (n=54)	p-value	Low (n=54)	High (n=55)	p-value	Low (n=55)	High (n=54)	p-value
Excluded, neoadjuvant treatment	1	1					1	1	
Lost to follow-up				1					
hENT1						0.336		1	0.564
Low				30 (56%)	24 (44%)		29 (54%)	25 (46%)	
High				24 (45%)	29 (55%)		25 (47%)	28 (53%)	
dCK			0.336		2				0.442
Low	30 (56%)	24 (44%)					25 (46%)	29 (54%)	
High	24 (45%)	29 (55%)					29 (55%)	24 (45%)	
HuR C			0.564			0.442		1	
Low	29 (54%)	25 (46%)		25 (46%)	29 (54%)				
High	25 (47%)	29 (55%)		29 (55%)	24 (45%)				
Year of surgery, M (IQR)	2009 (2005–2010)	2009 (2007–2011)	0.634	2009 (2005–2010)	2009 (2006–2011)	0.783	2008 (2004–2010)	2009 (2007–2010)	0.054
Age, M (IQR)	66 (61–71)	67 (62–73)	0.805	67 (61–73)	66 (62–72)	0.246	67 (61–73)	67 (62–72)	0.177
Sex			0.563			0.123			**0.004**
Women	27 (54%)	23 (46%)		21 (42%)	29 (58%)		33 (66%)	17 (34%)	
Men	27 (47%)	30 (53%)		33 (58%)	24 (42%)		21 (37%)	36 (63%)	
Tumour origin			0.765			0.351			0.623
Ampulla Vateri	11 (58%)	8 (42%)		11 (58%)	8 (42%)		8 (42%)	11 (58%)	
Distal bile duct	21 (47%)	24 (53%)		19 (42%)	26 (58%)		25 (56%)	20 (44%)	
Pancreas	22 (51%)	21 (49%)		24 (56%)	19 (44%)		21 (49%)	22 (51%)	
Tumour size, mm, M (IQR)	30 (25–40)	30 (21–30)	0.349	30 (23–36)	30 (25–35)	0.224	30 (25–35)	30 (22–40)	0.546
Differentiation grade			**0.001**			0.689			0.550
Well/moderate	11 (28%)	28 (72%)		21 (54%)	18 (46%)		18 (46%)	21 (54%)	
Poor	43 (63%)	25 (37%)		33 (49%)	35 (51%)		36 (53%)	32 (47%)	
T-stage			0.556			1.000			1.000
T1/T2	5 (42%)	7 (58%)		6 (50%)	6 (50%)		6 (50%)	6 (50%)	
T3/T4	49 (52%)	46 (48%)		48 (51%)	47 (49%)		48 (51%)	47 (49%)	
N-stage			0.283			0.830			1.000
N0	18 (60%)	12 (40%)		16 (53%)	14 (47%)		15 (50%)	15 (50%)	
N1	36 (47%)	41 (53%)		38 (49%)	39 (51%)		39 (51%)	38 (49%)	
Perineural growth			0.059			0.347			0.812
No	7 (32%)	15 (68%)		9 (41%)	13 (59%)		12 (55%)	10 (45%)	
Yes	47 (55%)	38 (45%)		45 (53%)	40 (47%)		42 (49%)	43 (51%)	
Growth in lymphatic vessels			0.676			0.094			0.528
No	15 (47%)	17 (53%)		12 (38%)	20 (62%)		18 (56%)	14 (44%)	
Yes	39 (52%)	36 (48%)		42 (56%)	33 (44%)		36 (48%)	39 (52%)	
Growth in blood vessels			0.224			0.685			1.000
No	32 (46%)	38 (54%)		34 (49%)	36 (51%)		35 (50%)	35 (50%)	
Yes	22 (59%)	15 (41%)		20 (54%)	17 (46%)		19 (51%)	18 (49%)	
Growth in peripancreatic fat			0.347			0.639			1.000
No	9 (41%)	13 (59%)		10 (45%)	12 (55%)		11 (50%)	11 (50%)	
Yes	45 (53%)	40 (47%)		44 (52%)	41 (48%)		43 (51%)	42 (49%)	
Margins			0.437			0.437			0.678
R0	2 (33%)	4 (67%)		2 (33%)	4 (67%)		4 (67%)	2 (33%)	
R1/Rx	52 (51%)	49 (49%)		52 (51%)	49 (49%)		50 (50%)	51 (50%)	
Adjuvant treatment			0.246			0.847			0.246
No gemcitabine	33 (56%)	26 (44%)		29 (49%)	30 (51%)		33 (56%)	26 (44%)	
Gemcitabine	21 (44%)	27 (56%)		25 (52%)	23 (48%)		21 (44%)	27 (56%)	
Recurrence			0.354			0.507			0.354
None	9 (47%)	10 (53%)		11 (58%)	8 (42%)		9 (47%)	10 (53%)	
Local	18 (62%)	11 (38%)		12 (41%)	17 (59%)		18 (62%)	11 (38%)	
Distant	27 (46%)	32 (54%)		31 (53%)	28 (47%)		27 (46%)	32 (54%)	

IQR, interquartile range; M, median. Bold text indicates significant values.

Table II. Associations between hENT1, dCK, HuR and clinicopathological parameters in intestinal type periampullary adenocarcinomas.

	hENT1			dCK			HuR		
	Low (n = 32)	High (n = 31)	p-value	Low (n = 32)	High (n = 31)	p-value	Low (n = 33)	High (n = 30)	p-value
Excluded from survival analysis	1	1		2			1	1	
hENT1						0.466			**0.021**
Low				17 (55%)	14 (45%)		21 (68%)	10 (32%)	
High				13 (43%)	17 (57%)		11 (37%)	19 (63%)	
dCK			0.466						0.611
Low	17 (57%)	13 (43%)					17 (57%)	13 (43%)	
High	14 (45%)	17 (55%)					15 (48%)	16 (52%)	
HuR			**0.021**			0.611			
Low	21 (66%)	11 (34%)		17 (53%)	15 (47%)				
High	10 (34%)	19 (66%)		13 (45%)	16 (55%)				
Year of surgery, M (IQR)	2007 (2003–2009)	2007 (2003–2010)	0.969	2006 (2002–2009)	2007 (2005–2010)	0.140	2008 (2005–2010)	2005 (2002–2010)	0.659
Age, M (IQR)	66 (59–69)	67 (60–72)	0.372	67 (62–72)	65 (56–70)	0.137	67 (61–70)	66 (59–72)	0.140
Sex			1.000			0.616			0.317
Women	17 (50%)	17 (50%)		16 (47%)	18 (53%)		20 (59%)	14 (41%)	
Men	15 (52%)	14 (48%)		16 (55%)	13 (45%)		13 (45%)	16 (55%)	
Tumour origin			**0.016**			1.000			0.068
Duodenum	3 (21%)	11 (79%)		7 (50%)	7 (50%)		4 (29%)	10 (71%)	
Ampulla Vateri	29 (59%)	20 (41%)		25 (51%)	24 (49%)		29 (59%)	20 (41%)	
Tumour size, mm, M (IQR)	28 (14–35)	27 (15–45)	**0.019**	30 (16–40)	25 (15–40)	0.683	25 (15–40)	30 (15–45)	0.331
Differentiation grade			0.454			0.617			0.133
Well/moderate	14 (45%)	17 (55%)		17 (55%)	14 (45%)		13 (42%)	18 (58%)	
Poor	18 (56%)	14 (44%)		15 (47%)	17 (53%)		20 (63%)	12 (37%)	
T-stage			0.774			1.000			0.769
T1/T2	7 (47%)	8 (53%)		8 (53%)	7 (47%)		7 (47%)	8 (53%)	
T3/T4	25 (52%)	23 (48%)		24 (50%)	24 (50%)		26 (54%)	22 (46%)	
N-stage			0.629			0.665			1.000
N0	15 (45%)	18 (55%)		16 (48%)	17 (52%)		17 (52%)	16 (48%)	
N1	10 (53%)	9 (47%)		9 (47%)	10 (53%)		10 (53%)	9 (47%)	
N2	7 (64%)	4 (36%)		7 (64%)	4 (36%)		6 (55%)	5 (45%)	
Perineural growth			0.585			1.000			**0.031**
No	21 (48%)	23 (52%)		22 (50%)	22 (50%)		19 (43%)	25 (57%)	
Yes	11 (58%)	8 (42%)		10 (53%)	9 (47%)		14 (74%)	5 (26%)	
Growth in lymphatic vessels			**0.023**			0.210			0.133
No	10 (34%)	19 (66%)		12 (41%)	17 (59%)		12 (41%)	17 (59%)	
Yes	22 (65%)	12 (35%)		20 (59%)	14 (41%)		21 (62%)	13 (38%)	
Growth in blood vessels			1.000			0.355			0.054
No	29 (50%)	29 (50%)		28 (48%)	30 (52%)		28 (48%)	30 (52%)	
Yes	3 (60%)	2 (40%)		4 (80%)	1 (20%)		5 (100%)	0 (0%)	
Growth in peripancreatic fat			0.430			0.188			0.111
No	19 (46%)	22 (54%)		18 (44%)	23 (56%)		18 (44%)	23 (56%)	
Yes	13 (59%)	9 (41%)		14 (64%)	8 (36%)		15 (68%)	7 (32%)	
Margins			0.164			**0.050**			0.395
R0	6 (35%)	11 (65%)		5 (29%)	12 (71%)		7 (41%)	10 (59%)	
R1/Rx	26 (57%)	20 (43%)		27 (59%)	19 (41%)		26 (57%)	20 (43%)	
Adjuvant treatment			0.585			1.000			1.000
None	24 (53%)	21 (47%)		23 (51%)	22 (49%)		24 (53%)	21 (47%)	
Any	8 (44%)	10 (56%)		9 (50%)	9 (50%)		9 (50%)	9 (50%)	
Recurrence			0.022			0.125			0.145
None	12 (35%)	22 (65%)		15 (44%)	19 (56%)		14 (41%)	20 (59%)	
Local	3 (75%)	1 (25%)		4 (100%)	0 (0%)		2 (50%)	2 (50%)	
Distant	17 (68%)	8 (32%)		13 (52%)	12 (48%)		17 (68%)	8 (32%)	

IQR, interquartile range; M, median. Bold text indicates significant values.
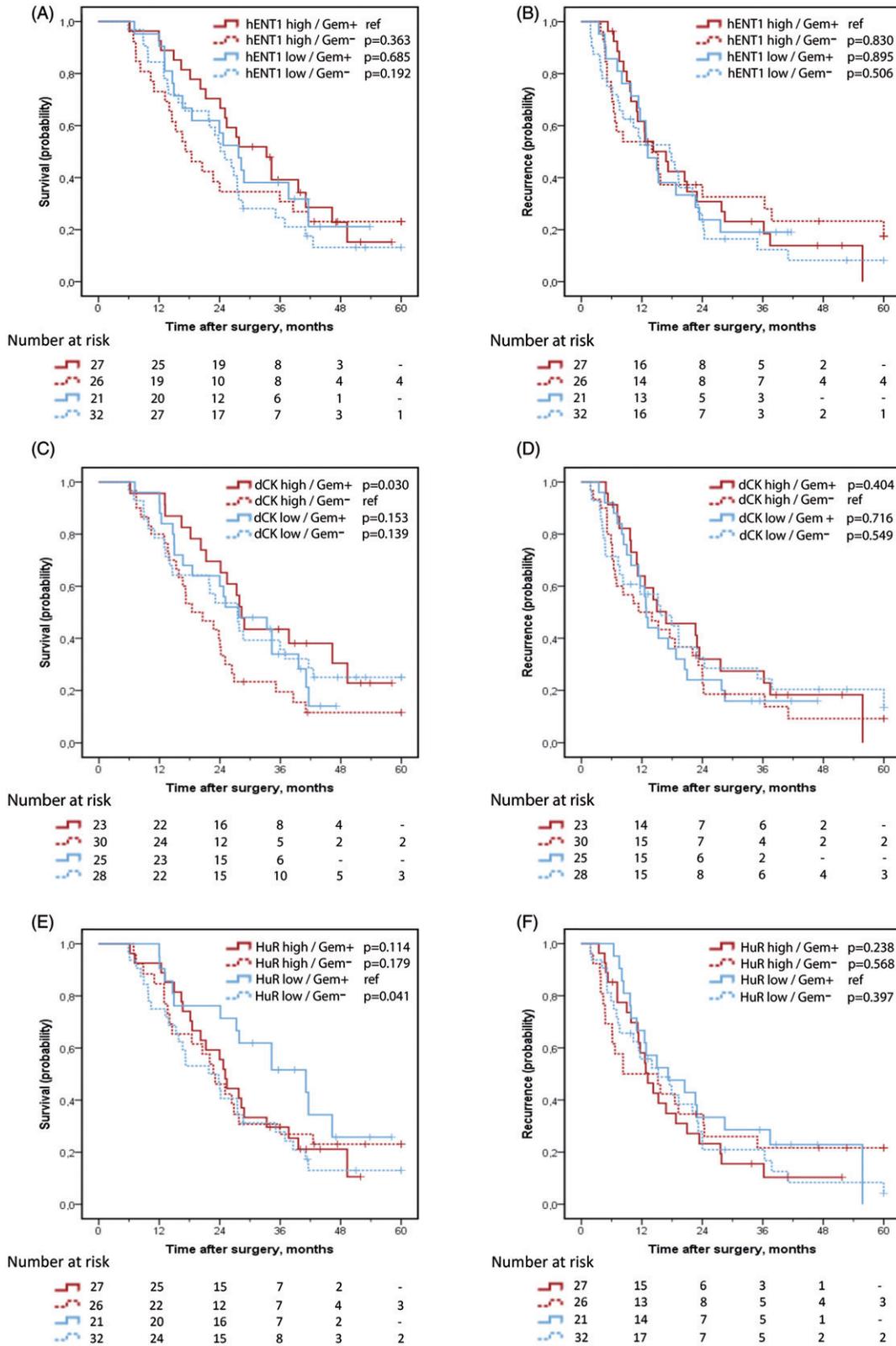
Figure 2. Kaplan–Meier curves of overall survival and recurrence-free survival in pancreatobiliary type tumours stratified by hENT1 (A,B), dCK (C,D) and HuR (E,F) expression and adjuvant gemcitabine.

univariable and multivariable Cox regression analysis for RFS (Table IV) and OS (Supplementary Table III, available online at http://www.informahealthcare.com).

High HuR expression was associated with a significantly longer OS in the entire group of patients with I-type tumours,

and also in patients not receiving adjuvant chemotherapy (Figure 3E and F). These findings were confirmed in univariable analysis for RFS in the entire group (HR 0.41, 95% CI 0.19–0.88) and in patients not receiving adjuvant chemotherapy (HR 0.37, 95% CI 0.15–0.92) (Table IV). Similar results were seen for OS

Table III. Cox proportional hazards analysis of the impact of expression of hENT1, dCK, HuR and HuR cytoplasmic/nuclear ratio on recurrence-free survival in patients with pancreatobiliary type tumours.

	Number (events)	RFS HR (95% CI)		P for interaction
		Unadjusted	Adjusted	
hENT1				
All				
Low	53 (45)	1.00	1.00	
High	53 (43)	0.86 (0.56–1.31)	1.35 (0.83–2.20)	
No gemcitabine				NS
Low	32 (28)	1.00	1.00	
High	26 (20)	0.79 (0.44–1.41)	1.18 (0.62–2.22)	
Gemcitabine				
Low	21 (17)	1.00	1.00	
High	27 (23)	0.96 (0.51–1.81)	1.87 (0.92–3.83)	
dCK				
All				
Low	53 (43)	1.00	1.00	
High	53 (45)	1.03 (0.68–1.57)	1.02 (0.65–1.59)	
No gemcitabine				NS
Low	28 (22)	1.00	1.00	
High	30 (26)	1.19 (0.67–2.11)	0.85 (0.43–1.69)	
Gemcitabine				
Low	25 (21)	1.00	1.00	
High	23 (19)	0.80 (0.42–1.50)	0.97 (0.49–1.94)	
HuR				
All				
Low	53 (45)	1.00	1.00	
High	53 (43)	1.07 (0.70–1.63)	1.30 (0.84–2.00)	
No gemcitabine				NS
Low	32 (28)	1.00	1.00	
High	26 (20)	0.87 (0.49–1.56)	1.00 (0.53–1.87)	
No Gemcitabine				
Low	21 (17)	1.00	1.00	
High	27 (23)	1.47 (0.77–2.79)	1.51 (0.79–2.90)	
HuR C/N ratio				
All				
Low	53 (44)	1.00	1.00	
High	53 (44)	1.04 (0.68–1.58)	1.31 (0.84–2.04)	
No gemcitabine				0.053
Low	30 (27)	1.00	1.00	
High	28 (21)	0.72 (0.40–1.27)	0.78 (0.42–1.46)	
Gemcitabine				
Low	23 (17)	1.00	1.00	
High	25 (23)	1.59 (0.84–3.01)	**2.59 (1.29–5.20)**	

The multivariable model included age (continuous), T-stage (1–2 vs. 3–4), N-Stage, differentiation grade (well-moderate vs. poor), lymphatic invasion, vascular invasion, perineural growth, and in the analysis including all cases also gemcitabine treatment (yes/no). C/N ratio, cytoplasmic/nuclear ratio; NS, non-significant. Bold text indicates significant values.

(Supplementary Table III). Significance was however not retained in multivariable analysis neither for RFS (Table IV) nor OS (Supplementary Table III). A high HuR C/N ratio was also significantly associated with a longer OS and borderline significantly associated with a prolonged RFS, which was confirmed in univariable analysis for RFS (HR 0.47, 95% CI 0.22–1.02) (Table IV) and OS (Supplementary Table III). The associations were significant in multivariable analysis for OS (Supplementary Table III), but not for RFS (Table IV).

Potential predictive value of hENT1, dCK and HuR expression

In patients with PB-type tumours receiving adjuvant gemcitabine, a high HuR C/N ratio was significantly associated with a reduced OS in univariable analysis (HR 2.07, 95% CI 1.03–4.17), with a significant interaction ($p_{interaction}$=0.028) (Supplementary Table II). For RFS, there was a borderline significant treatment interaction ($p_{interaction}$=0.053) (Table III).

There was no significant treatment interaction for hENT1, dCK or cytoplasmic HuR expression with regard to RFS or OS (Table III and Supplementary Table II).

In I-type tumours, high dCK expression was significantly associated with a prolonged RFS in patients receiving adjuvant chemotherapy (univariable HR 0.09, 95% CI 0.01–0.73), with a significant treatment interaction ($p_{interaction}$=0.023) (Table IV). Cox regression and interaction analysis could not be performed for dCK with regard to OS, as there were no fatalities among the nine patients with high dCK expression having received adjuvant chemotherapy.

The prognostic value of hENT1, HuR, or HuR C/N in I-type tumours did not differ by adjuvant treatment, neither for RFS (Table IV) nor OS (Supplementary Table III).

Discussion

In the group of PB-type tumours, including pancreatic cancer, our results do not support previous results in large cohorts on the predictive value of high hENT1 expression [2,4]. Our results on high dCK expression in relation to gemcitabine response are however in line with previous findings [2]. Our results do not confirm the previously described association between HuR and dCK expression described in 116 cases of pancreatic cancer [9] and in cell lines [13]. Moreover, our results regarding the predictive value of HuR, with a better survival in patients having received gemcitabine with tumours displaying low expression of HuR or a low C/N ratio, differ from previous reports on two smaller series of gemcitabine-treated patients with pancreatic cancer (n=32 and n=24, respectively), where high HuR expression was found to be associated with a prolonged survival [13,14]. Our results are however plausible, as HuR increases proteins related to proliferation, angiogenesis and evasion of apoptosis, thus promoting a more malignant phenotype [10,11]. The findings of an association between high HuR or a high HuR C/N ratio and a poorer prognosis also harmonise with a majority of reports on HuR in different tumour types, where a high cytoplasmic expression of HuR or a high C/N ratio were found to confer a worse prognosis [12].

In the group of I-type periampullary adenocarcinomas, expression of dCK was found to be potentially predictive of response to adjuvant chemotherapy, which has, to the best of our knowledge, not been described before. Although several of these patients had received adjuvant gemcitabine, there are indications that dCK also increases sensitivity to 5-FU [9].

Our findings on HuR in I-type tumours are more surprising, with high expression being significantly associated with a better prognosis, regardless of treatment. I-type periampullary tumours are often assumed to behave similarly to colorectal or gastric cancer, but our results on HuR differ from previous reports on these tumour types [15,21], and also deviate from the concept of HuR being a positive regulator of malignant behaviour in other tumour types [12]. In the herein investigated tumours, perineural growth was less common in I-type tumours with high HuR expression, which is in line with its beneficial impact on survival. Whether the distribution of perineural growth in the groups of high or low HuR is coincidental or biologically related to levels of HuR cannot be
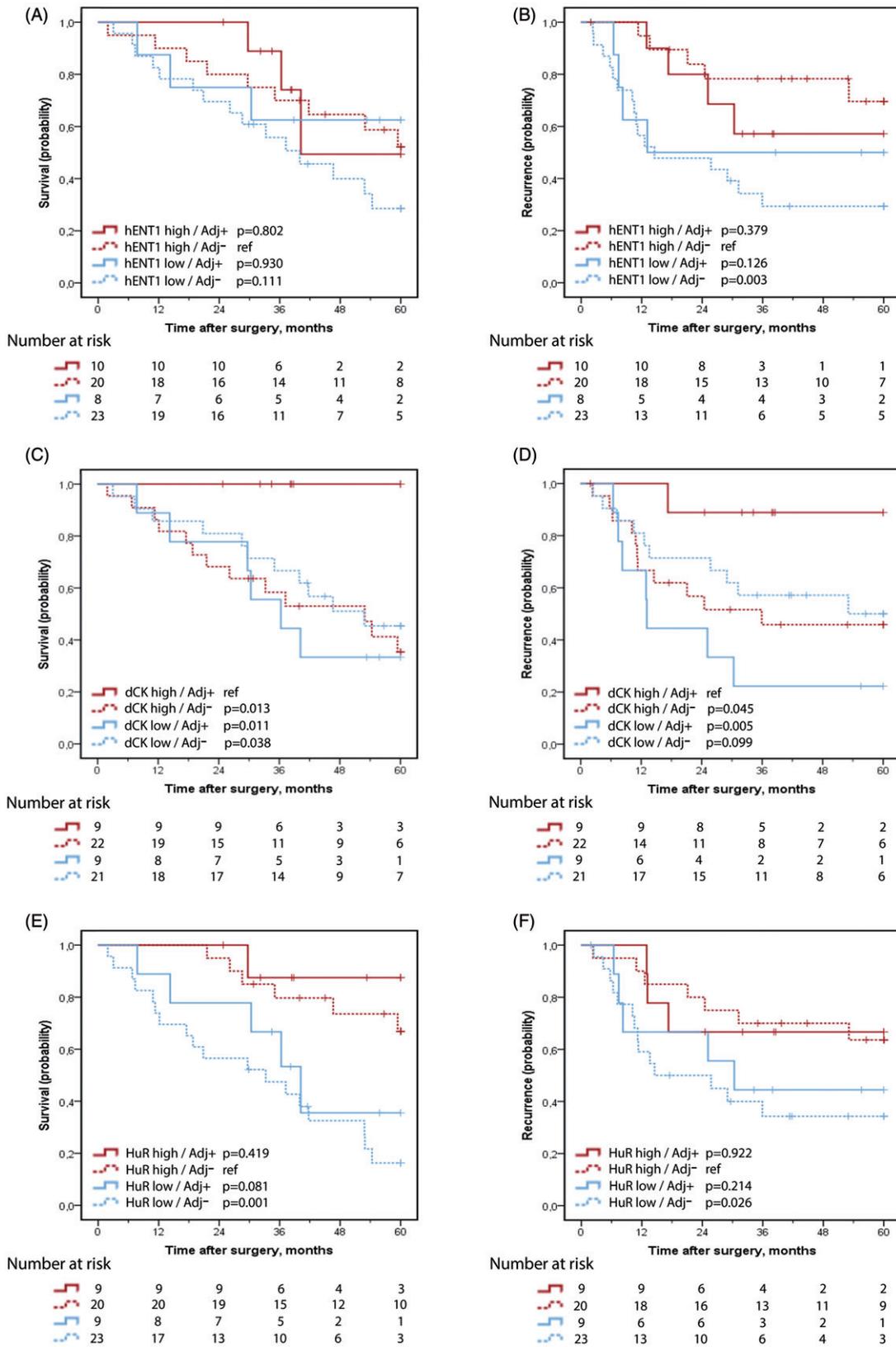
Figure 3. Kaplan–Meier curves of overall survival and recurrence-free survival in intestinal type tumours, stratified by hENT1 (A,B), dCK (C,D) and HuR (E,F) expression and adjuvant chemotherapy.

determined based on the results from this study, but the non-significant hazard ratio for HuR in multivariable analysis indicates that its associations with other parameters may explain its prognostic effect in I-type tumours.

High hENT1 expression was more common in I-type tumours of duodenal origin than of ampullary origin, which could explain its association with a more favourable prognosis. A borderline significant association between a longer RFS

Table IV. Cox proportional hazards analysis of the impact of expression of hENT1, dCK, HuR and HuR cytoplasmic/nuclear ratio on recurrence-free survival in patients with intestinal type tumours.

	Number (events)	RFS HR (95% CI)		p for interaction
		Unadjusted	Adjusted	
hENT1				
All				
Low	31 (20)	1.00	1.00	
High	30 (9)	**0.33 (0.15–0.72)**	**0.24 (0.10–0.59)**	
No adjuvant				NS
Low	23 (16)	1.00	1.00	
High	20 (5)	**0.24 (0.09–0.67)**	**0.07 (0.02–0.28)**	
Adjuvant				
Low	8 (4)	1.00		
High	10 (4)	0.59 (0.15–2.39)	†	
dCK				
All				
Low	30 (17)	1.00	1.00	
High	31 (12)	0.68 (0.33–1.43)	0.82 (0.38–1.76)	
No adjuvant				*0.023*
Low	21 (10)	1.00	1.00	
High	22 (11)	1.26 (0.53–2.97)	1.56 (0.61–4.02)	
Adjuvant				
Low	9 (7)	1.00		
High	9 (1)	**0.09 (0.01–0.73)**	†	
HuR				
All				
Low	32 (19)	1.00	1.00	
High	29 (10)	**0.41 (0.19–0.88)**	0.47 (0.21–1.04)	
No adjuvant				NS
Low	23 (14)	1.00	1.00	
High	20 (7)	**0.37 (0.15–0.92)**	0.46 (0.16–1.32)	
Adjuvant				
Low	9 (5)	1.00		
High	9 (3)	0.52 (0.12–2.21)	†	
HuR C/N ratio				
All				
Low	32 (19)	1.00	1.00	
High	29 (10)	0.47 (0.22–1.02)	0.44 (0.19–1.02)	
No adjuvant				NS
Low	24 (15)	1.00	1.00	
High	19 (6)	**0.39 (0.15–1.00)**	**0.14 (0.03–0.62)**	
Adjuvant				
Low	8 (4)	1.00		
High	10 (4)	0.80 (0.20–3.23)	†	

The multivariable model included age (continuous), T-stage (1–2 vs 3–4), N-Stage, differentiation grade (well-moderate vs. poor), lymphatic invasion, vascular invasion, perineural growth, and in the analysis including all cases also adjuvant treatment (yes/no). Dagger (†) indicates that multivariable analysis was not performed due to few cases and events. C/N ratio, cytoplasmic/nuclear ratio; NS, non-significant. Bold text indicates significant values.

and high hENT1 was however retained in subgroup analysis of I-type cases of ampullary origin. Invasion of lymphatic vessels was less common in cases displaying high hENT1 expression, but the significant association between high hENT1 and RFS was retained in multivariable analysis, adjusting for growth in lymphatic vessels. These findings are in contrast with previous reports on ampullary and gastric cancer, where hENT1 expression was demonstrated to be associated with a shorter survival [6,22].

The associations between HuR and hENT1 in I-type tumours and clinicopathological parameters also illustrate the risk for type I errors when the number of correlation tests are many, and should thus be evaluated with caution.

The hENT1 antibody used in the present study has been validated in a study by Poplin et al. [23] against a different, not commercially available, antibody (10D7G2) used, e.g. in the studies by Farrell et al. and Maréchal et al. [2,3]. To this end, tumour samples from the RTOG [3] study were independently stained and analysed with the SP120 antibody on newly constructed TMAs, with concordant results [23]. Of note, the aim of the study by Poplin et al. was to evaluate hENT1 expression prospectively in order to compare the efficacy of gemcitabine with CO-101, a lipid-drug conjugate of gemcitabine. According to the results, based on analyses of metastatic lesions, CO-101 was not demonstrated to be superior to gemcitabine in patients with low tumour-specific hENT1 expression and hENT1 expression did not predict survival within the gemcitabine arm [23].

We are not aware of any previous studies comparing the expression of the herein investigated biomarkers in primary tumours and paired lymph node metastases. Our results demonstrate a significantly increased expression from primary tumour to metastasis of both dCK and hENT1 in PB-type tumours. The potential mechanistic basis for this observation remains unclear, but may however have implications in the clinical setting, i.e. that biomarker assessment in metastatic components may be sufficient when the primary tumour is not available for analysis, i.e. in the palliative setting.

The cohort used in this study is well characterised regarding clinicopathological parameters, and follow-up, and adjuvant chemotherapy has only been given to approximately half of the patients, which enables a fairly good assessment of both prognostic and potentially predictive biomarkers even in the retrospective setting. Limitations due to the size of the cohort are mostly seen in I-type tumours, in particular when stratifying both for biomarker expression and adjuvant treatment. Still, similar results regarding the predictive effect of dCK as described by others in pancreatic cancer was seen in both PB- and I-type tumours.

In conclusion, the results from the present study demonstrate that hENT1 expression is a favourable prognostic factor in patients with I-type, but not in PB-type tumours, and not potentially response predictive in neither morphological subtype. Moreover, a high cytoplasmic/nuclear HuR ratio was found to be a negative prognostic factor in patients with PB-type tumours receiving adjuvant gemcitabine, and high dCK expression was found to be a positive prognostic factor in patients with I-type tumours receiving any adjuvant treatment. The finding regarding dCK expression in I-type tumours is novel and of potential clinical relevance, and therefore merits further study, preferably in tumours from randomised, prospective trials. These findings also highlight the importance of taking morphological subtype into consideration in biomarker studies related to periampullary cancer.

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Supplementary material available online
Supplementary Figures 1 and 2, Tables 1–3