Low hENT1 expression indicates poor prognosis in gemcitabine-treated pancreatic cancer patients

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Research article

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Abstract

Background: Pancreatic cancer (PC) is still a lethal disease and has a poor prognosis, gemcitabine-based chemotherapy is now the standard regimen in the treatment of pancreatic cancer. Gemcitabine resistance is an important obstacle for effective treatment of patients and improvement in patients' overall survival (OS) and disease free survival (DFS). Results: Current research shows that human equilibrative nucleoside transporter 1 (hENT1) is related to gemcitabine chemoresistance for it mediates drug entry into cancer cells, and has an aberrant expression level in tumor tissues. But now discrepancies still exist between different research groups and studies about the expression level and prognostic value of hENT1 in PC patients, and the exactly underlying mechanism through which hENT1 transfer GEM into tumor cells remains unclear. Conclusion: hENT1 was low expressed in tumor tissues and this decreased expression level indicated a worse outcome (including shortened OS and DFS) in patients who received gemcitabine treatment postoperatively.

Background

Pancreatic cancer (PC) is a highly malignant disease with a 5-year survival rate of only 9%[1]. Radical surgical resection is the most effective way for pancreatic cancer treatment and patients who have no contraindication stoic evidence should all be treated with complementary chemotherapy after operation[2]. Gemcitabine is the first-line adjuvant chemotherapy drug for postoperative PC patients but the therapeutic effect is different among different patients for the existence of primary and acquired chemoresistance[3]. Given this, finding a novel effective biomarker to identify patients those who are sensitive to gemcitabine and to predict the outcome of patients is imperative.

Human equilibrative nucleoside transporter 1 (hENT1), which was reported to facilitate cross-membrane transport of nucleosides and nucleoside-derived drugs, plays an important role in cancer chemotherapy[4]. Most notably, an elevated hENT1 expression is regarded as a diagnostic and therapeutic biomarker for PC patients treated with gemcitabine[5 – 7]. High hENT1 expression was also associated with better survival in other kinds of cancer patients who received gemcitabine treatment such as cholangiocarcinoma[8,9], leiomyosarcoma and angiosarcoma[10]. But some studies reached an opposite conclusion that hENT1 expression level had no relation with patients' outcome[11,12]. Thus the prognostic value of hENT1 in PC patients still needs to be further verified by large sample size studies.

Here we performed immunohistochemistry (IHC) to assess hENT1 expression levels in 359 tumor tissues and adjacent normal tissues from surgical specimens of PC patients, and analyzed the relationships between hENT1 expression level and several clinicopathological features. We found that tumor tissues tended to have a relative low hENT1 expression level and this low expression level was correlated with tumor differentiation degree rather than other clinical parameters. In addition, we evaluated the prognostic value of hENT1 and drew the conclusion that a low hENT1 expression level is an independent risk factor for gemcitabine-treated patients.

Methods

Tissue sample collection

We collected the surgical pathological tissue of pancreatic cancer patients who received radical surgery in our hospital from September 2004 to December 2014 continuously, the eligible criteria for patients included having R0 surgical resection, postoperative pathology diagnosis were pancreatic ductal adenocarcinoma, having no gemcitabine-based neoadjuvant chemotherapy and/or radiotherapy before surgery and surgical specimens were suitable for immunohistochemistry. In addition, these patients started adjuvant chemotherapy within 8 weeks after surgery with a regular follow-up monitoring CA-199/CT/B-ultrasonography and their clinical and pathological data were complete. We excluded patients who had severe basic diseases or had serious complications during perioperative period which could affect survival analysis results, and those who had poor compliance and cannot being followed regularly. We finally had 375 samples for the next-step analysis. This study was approved by the Ethics Committee of Beijing Union Medical College Hospital.
375 pancreatic cancer tissues and paired adjacent non-tumor tissues were employed for the construction of 4 tissue microarrays (TMAs) using routine methods, namely TMA1, TMA2, TMA3, TMA4. The quality of 4 TMAs was reexamined by HE staining and in accordance with the design requirements. We did immunohistochemistry analysis to test hENT1 expression and excluded 16 samples because the tissue sections detached from the slides. The hENT1 antibody was used to measure hENT1 expression in TMAs by IHC staining according to standard protocols. TMAs were firstly blocked by hydrogen peroxide and then incubated with an anti-hENT1 antibody (1:200, Anti-SLC29A1 polyclonal antibody produced in rabbit from Sigma-Alorich Company in America). Subsequently, DAB (diaminobezidin) was used for coloration and hematoxylin was used for counterstaining. All immunohistochemistry results were determined by two independent pathologists on a double-blind basis. The staining intensities were graded as 0 (negative), 1 (low), 2 (medium), or 3 (high) while the staining extent was scored from 0 to 100%. And intensity score × percentage score × 100 made up the final IHC staining score together, namely composite expression score (CES), which ranged from 0 to 300. The average CES value of 359 tumor tissues 80.5 was defined as the optimal cutoff value. CES 80.5 represents a high hENT1 expression and CES ≤ 80.5 indicates a low hENT1 expression.

Statistical analyses were performed using SPSS 22.0 (SPSS Inc., Chicago, IL, USA), the diagrams were sketched by Prism software (GraphPad, La Jolla, CA, USA). Pearson's chi-square test was performed for categorical data; continuous variables were analyzed by the Student's t test and ANOVA analyses to evaluate the associations between hENT1 expression levels and clinicopathological features of patients. The clinical end points of patients were calculated using Kaplan-Meier analysis and differences compared by log-rank test. Cox regression (proportional hazards model) was applied to determine the prognostic values of multivariate factors on patients' overall survival (OS) and disease free survival (DFS). Hazard ratios (HRs) obtained by COX regression analyses are reported as relative risks with corresponding 95% confidence intervals (CIs). At last, p value of < 0.05 was considered statistically significant.

Results

hENT1 expression is down-regulated in pancreatic cancer tissues

The IHC results of 359 samples showed that the hENT1 protein was mainly localized in the membranes and/or the cytoplasm of cancer cells as previous literature described (Fig. 1a) [4]. The average CES of hENT1 expression in tumor tissues was 80.5 and average CES in non-tumor tissues was 89.5. The hENT1 protein expression in tumor tissues was much lower than in normal tissues (Fig. 1b, 80.5 ± 8.8 versus 89.5 ± 8.9, p = 0.005). Subsequently, we use the average CES 80.5 as the cut-off value to divide 359 patients into hENT1 high expression group (n = 165) and low expression group (n = 194).

According to the grouping result, we then compared clinical pathological parameters between high and low hENT1 expression groups. The expression level of hENT1 was only related to the tumor differentiation degree, the patients in high hENT1 expression group tended to have highly differentiated tumors (Table 1). As to other pathological features such as gender, age, CA-199 level, tumor site, TNM stage, vascular and neural infiltration, they were not correlated with the expression level of hENT1.

Table 1

Relations between hENT1 expression and clinical characteristics

characteristics	Patients		hENT1 expression		
	n	%	High	Low	P value
All patients	359	100	165	194	-
Gender					0.165
Male Female	204 155	56.8 43.2	87 78	117 77	
Age					0.432
< 65 >=65	239 120	66.6 33.4	106 59	133 61	
Diabetes					0.758
Yes No	48 308	13.4 85.8	21 144	27 164	
Chronic Pancreatitis					1
Yes No	1 358	0.3 99.7	0 165	1 193	
CA19-9,u/ml					1
> 467 < 467	73 228	24.3 75.7	34 108	39 120	
Pathological differentiation degree					0.032
High Low	116 243	32.3 67.7	63 102	53 141	
Capsular invasion					0.458
Yes No	342 17	95.3 4.7	159 6	183 11	
Vascular infiltration					0.167
□ □	28 331	7.8 92.2	9 156	19 175	
Neural infiltration					0.525
Yes No	165 194	46.0 54.0	79 86	86 108	
T classification					0.653

characteristics	Patients		hENT1 expression		
	n	%	High	Low	P value
T1/2 T3/4	21 338	5.8 94.2	11 154	10 184	
N classification					0.336
N0 N1/2	153 206	42.6 57.4	75 90	78 116	
M classification					1
M0 M1	336 23	93.6 6.4	154 11	182 12	
TNM stage					0.574
I/II III/IV	328 31	91.4 8.6	149 16	179 15	

Low hENT1 expression is correlated with a poor prognosis of gemcitabine-treated patients

To explore the relationship between the level of hENT1 and the prognosis of pancreatic cancer patients, firstly we analyzed the relationship between hENT1 expression level and disease free survival (DFS)/overall survival (OS) in all patients by Kaplan-Meier method and found a low hENT1 expression level indicated a significant poor outcome of PC patients, including shortened DFS(Fig. 2a, 21.6 ± 2.8 months versus 36.9 ± 4.0 months, p□0.001) and OS(Fig. 2b, 33.6 ± 3.9 versus 39.6 ± 3.9, p = 0.004). Meanwhile, the result revealed that the level of CA19-9, the M stage, the TNM stage of patients and whether the tumor invaded into the pancreas capsule are also related to DFS when analyzed respectively. The DFS period of patients who had CA19-9 value ≤ 467 u/ml (the average value of all patients) were longer than those patients who had CA19-9 value□467 u/ml preoperatively (Fig. 3f, 37.9 ± 4.1 versus 22.9 ± 4.0, p = 0.04). Similarly, patients in stage □/□ of TNM stage had longer DFS compared with stage □/□ patients (Fig. 3a, 31.0 ± 3.1 versus 12.4 ± 1.9, p = 0.016); as for M stage, patients in M0 stage had longer DFS than patients in M1 stage (Fig. 3c, 30.7 ± 3.0 versus 11.8 ± 2.2, p = 0.031); and patients with tumors not invading the capsule had better DFS than those with tumor invasion into the capsule (Fig. 3e, 30.8 ± 3.0 versus 12.6 ± 2.3, p = 0.053). What's more, the M stage of patients and the TNM stage also had significant correlation with patients' overall survival (OS). M0 stage patients had longer OS than M1 stage patients (Fig. 3d, 39.7 ± 3.4 versus 16.2 ± 1.9, p = 0.026); patients in stage □/□/of TNM stage had better OS than those in stage □/□ (Fig. 3b, 40.2 ± 3.4 versus 15.4 ± 1.7,p = 0.002).

For further exploring the connection between hENT1 level and gemcitabine treatment efficacy, we analyzed the relationship between hENT1 expression and DFS/OS in two separated groups (patients in one group received gemcitabine treatment after surgery while the other group didn't receive chemotherapy). The findings suggested that the results were much more remarkable in the gemcitabine

subgroup, a high hENT1 expression level was related to longer DFS (Fig. 4a, 35.7 ± 4.0 versus 20.6 ± 2.7; $p < 0.0001$). Similarly, patients with high hENT1 expression in gemcitabine-treated group showed longer overall survival (OS) compared with low expression group (Fig. 4b, 39.4 ± 4.0 versus 31.5 ± 3.9, $p = 0.001$). In contrast, no significant difference of DFS and OS was found in non-gemcitabine treated group between the expression level of hENT1 and the prognosis of patients (Fig. 4c, d, $p = 0.413$ and $p = 0.152$).

A low hENT1 expression level is an independent indicator for poor prognosis of PC patients

Through the survival analysis, we found that the M stage, the TNM stage, whether or not have tumor capsular invasion, preoperative CA19-9 value and hENT1 expression level were connected with prognosis of pancreatic cancer patients. Therefore, we put these factors into multivariate analysis by COX regression to further evaluate their prognostic value in PC patients. The results showed that a low hENT1 expression level in tumor tissues was an independent risk factor for PC recurrence or metastasis, cause it was connected with shorter DFS in patients with pancreatic cancer (HR 0.53; 95% CI: 0.39–0.72; p□0.001). In addition, an advanced TNM stage (HR 2.68; 95% CI: 1.09–6.58; P = 0.031) and a low expression level of hENT1 (HR 0.60; 95%CI: 0.43–0.82; p = 0.001) were independent risk factors for overall survival of PC (Table 2). Besides, we also put these factors into multivariate analysis in the separate gemcitabine-treated group and have produced similar findings, advanced TNM stage predicted worse OS (HR 2.90; 95%CI: 1.06–7.92; p = 0.038); low hENT1 expression level predicted worse OS (HR 0.60; 95%CI: 0.43–0.82; p = 0.002) and DFS (HR 0.56; 95%CI: 0.41–0.77; p□0.001). In conclusion, low hENT1 expression level is an independent risk factor for patients' shortened DFS and OS.

Table 2

In the general population, the correlations between several parameters and patients' long-term prognosis.

Characteristics	OS (overall survival)			DFS (disease free survival)		
	Hazard ratio (HR)	95% CI (confidence interval)	P value	Hazard Ratio (HR)	95% CI (confidence Interval)	P value
M classification	0.65	0.22–1.91	0.433	0.91	0.32–2.64	0.867
TNM stage	2.68	1.09–6.58	0.031	1.88	0.77–4.59	0.169
Capsular invasion	0.84	0.37–1.92	0.682	0.74	0.34–1.59	0.440
CA19-9	1.19	0.85–1.67	0.309	1.36	0.99–1.86	0.057
hENT1	0.60	0.43–0.82	0.001	0.53	0.39–0.72	0.001

Table 3
In the gemcitabine-treated population, the correlations between several parameters and patients' long-term prognosis.

Characteristics	OS (overall survival)			DFS (disease free survival)		
	Hazard ratio (HR)	95% CI (confidence interval)	P value	Hazard Ratio (HR)	95% CI (confidence Interval)	P value
M classification	1.03	0.32–3.36	0.963	0.98	0.30–3.19	0.976
TNM stage	2.90	1.06–7.92	0.038	2.31	0.85–6.28	0.100
Capsular invasion	0.87	0.38–1.98	0.734	0.76	0.35–1.64	0.485
CA19-9	1.16	0.82–1.65	0.395	1.32	0.95–1.83	0.097
hENT1	0.60	0.43–0.82	0.002	0.56	0.41–0.77	0.001

Discussion

At present, pancreatic cancer is the fifth and fourth cancer-related cause of death respectively in China and America[1,13], and the incidence and mortality of pancreatic cancer are still on the rise. As yet, surgical treatment is still the most effective and thorough therapeutic method for PC patients and combined chemotherapy based on gemcitabine is still the standard protocol for postoperative treatment. But the inherent and acquired resistance to gemcitabine are stunting its effective therapeutic effect hence increasing the sensitivity of PC patients to gemcitabine is a hoping direction to improve the survival of PC patients, especially those with advanced cancers.

Human equilibrative nucleoside transporter 1 (hENT1) mediates both influx and efflux of nucleotide or nucleoside drugs across the membrane dependent on drug concentration. The hENT1 protein expression level in cancer cell membrane is believed to influence the accumulation of nucleoside anticancer drugs in cancer cells, and is one of the hoping directions to fully understand the resistance mechanism of cancer cells to chemotherapy drugs and improve the effect of chemotherapy. As to pancreatic cancer, gemcitabine is the main treatment method and has been proved to enter into tumor cells mainly through transportation by hENT1 to undergo a series of metabolic transformations then play its anti-cancer effect. But the expression and the prognostic value of hENT1 still have discrepancies between different studies. For example, Bird et al. did a meta-analysis containing 770 patients (405 hENT1-negative, 365 hENT1-positive) and came to the conclusion that high hENT1 expression was significantly associated

with prolonged DFS (HR 0.58, 95% CI: 0.42 to 0.79) and OS (HR 0.52, 95% CI: 0.38 to 0.72). This result existed in patients receiving adjuvant gemcitabine but not those having fluoropyrimidine-based adjuvant therapy[5]. Similarly, a systematic review made by Stina et al. found that patients with high expression of hENT1 had significantly longer OS in all included studies that evaluated this outcome measurement[6]. Oppositely, Poplin et al. detected no difference in OS in the low hENT1 subgroup or overall, with hazard ratios (HRs) of 0.994 (95% CI: 0.746 to 1.326) and 1.072 (95% CI: 0.856 to 1.344) respectively, which suggested hENT1 expression did not predict gemcitabine-treated patients' outcome[11]. This discrepancy may be on account of the diversity of sample size and study characteristics, for some were retrospective studies but some were prospective case-control studies. What's more, the lack of an unified and clear cut-off value to define high/low hENT1 expression level must be taken into consideration.

Hence we did a large-scale IHC study containing 359 paired PC tissues and normal tissues to confirm the prognostic value of hENT1. Our TMA analysis revealed that hENT1 protein had a low expression level in PC tumor tissues compared with adjacent normal tissues. Among a separated group in which patients received gemcitabine treatment after surgery, those who had low hENT1 expression level tend to have worse outcomes (shortened DFS and OS) than those with high hENT1 expression. The results indicated that hENT1 expression level might be a diagnostic marker and serve as a factor monitoring the recurrence and metastasis risk for PC patients who received radical surgery.

Conclusion

In the future, by evaluating the hENT1 expression level, we can distinguish those who are sensitive to gemcitabine to promote individualized treatment for PC patients and predict the prognosis of patients who received gemcitabine chemotherapy postoperatively. But we still have limitation for we did study retrospectively by analyzing the tissue sample and clinical data of PC patients, so we hope to verify our research results by large prospective studies in the future.

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Abbreviations

PC Pancreatic cancer

hENT1 human equilibrative nucleoside transporter 1

IHC immunohistochemistry

TMAs tissue microarrays

Declarations

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Novelty & Impact Statements

This work further investigates the expression and prognostic features of human equilibrative nucleoside transporter 1 (hENT1) in pancreatic cancer patients extensively. Given the current situation that gemcitabine is the preferred postoperative chemotherapy drug, using hENT1 as an index to distinguish those who are sensitive to gemcitabine and predict the long-term prognosis of patients is very promising.

Conflicts of Interests:

None declared.

Ethics approval and consent to participate

All participants provided written informed consent. Patient data were de-identified and anonymized before analysis. The study on human data collection was approved by the Ethics Committee of the

Peking Union hospital.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Author Contributions

JX and FZ collected the data and wrote the draft, GY, YW, JQ, and YL created the figures and table. LWH revised the manuscript, figures and tables. LY, LZ provided guidance for language expression. TZ and YZ made suggestions for revision. All authors approved the final manuscript.

Consent for publication

Not applicable.