1 **Human Equilibrative Nucleoside Transporter-1 (hENT1) Expression in**

2 **Endoscopic Ultrasonography-Guided Fine-Needle Aspiration Biopsy Samples is a**

3 **Strong Predictor of Clinical Response and Survival in the Patients With**

4 **Pancreatic Ductal Adenocarcinoma Undergoing Gemcitabine-Based**

5 **Chemoradiotherapy**

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11   **Running title:** hENT1 Expression by EUS-FNAB is Prognostic Marker

1   **Objectives:** To clarify whether pretreatment human equilibrative nucleoside transporter

2   (hENT1) expressions in endoscopic ultrasonography-guided fine-needle aspiration

3   biopsy (EUS-FNAB) specimens obtained from resectable, borderline resectable, and

4   locally advanced unresectable pancreatic ductal adenocarcinoma (PDAC) are

5   concordant with those in the resected specimen after gemcitabine-based

6   chemoradiotherapy (Gem-CRT), and to validate the utility of hENT1 expression using

7   EUS-FNAB samples as a prognostic marker.

8   **Methods:** We evaluated the relationship between hENT1 expressions assessed by

9   immunohistochemical staining and clinical outcomes in the 51 of 76 PDAC patients

10   who were diagnosed by EUS-FNAB and received preoperative Gem-CRT.

11   **Results:** The concordance rate of hENT1 expressions was 89.2% ($K = 0.681$). Median

12   survival time (month) in the 51 whole patients and 37 with resection was significantly

13   longer in hENT1 positive than in negative: 25.0 and 30.0 vs. 9.0 and 9.0, respectively.

14   A multivariate analysis confirmed that hENT1 expression was an independent

15   prognostic factor in both whole patients and those with resection. Regardless of T3 and

1   T4, hENT1-positive patients with resection had significantly better prognosis than

2   negative patients, whose prognosis was similar to those without resection.

3   **Conclusions:** The assessment of hENT1 expression using EUS-FNAB samples prior to

4   Gem-CRT provides important information on PDAC patients who can benefit from

5   curative-intent resection.

6

7   **Key words:** EUS-FNAB, hENT1, chemoradiotherapy, gemcitabine, pancreatic ductal

8   adenocarcinoma

9

1   **INTRODUCTION**

2   Gemcitabine (Gem) therapy has been the standard treatment for pancreatic ductal

3   adenocarcinoma (PDAC) since Burris et al. [1] reported that Gem offered better overall

4   survival (OS) than fluorouracil. However, its efficacy is limited; only 15% of patients

5   with recurrent and metastatic PDAC [2] and up to 30% in general [3] can be expected to

6   respond to treatment. Because Gem is strongly hydrophilic, passive diffusion through

7   hydrophobic cellular membranes is slow. Efficient permeation of Gem into cells

8   requires specialized integral membrane transporter proteins to cross plasma membranes

9   [4]. Among these transporters, the major mediators of Gem uptake into human cells are

10   the human equilibrative nucleoside transporter 1 (hENT1) and, to a lesser degree, the

11   human concentrative nucleoside transporter 3 (hCNT3) [5-7].

12   The hENT1 has been reported as an important predictive marker of Gem-based

13   therapy [8]. In vitro studies indicated that hENT1 gene expression was positively

14   associated with Gem-chemosensitivity [9]. High hENT1 expression in resected

15   specimen was also reported to be associated with increased OS in PDAC patients who

16   received postoperative Gem-based chemotherapy [10-17]. These studies indicate that

8

1 hENT1 expression is important in predicting the survival of PDAC patients in the

2 adjuvant setting. However, there have been a few reports describing the impact of

3 hENT1 expression on the outcome after preoperative Gem-based chemoradiotherapy

4 (Gem-CRT) in PDAC patients. Our previous study showed that hENT1 expression was

5 an independent predictor of OS after neoadjuvant Gem-CRT in the patients with Union

6 Internationale Contrele Cancer (UICC) T3–T4 [18]. We also reported that positive

7 expression of hENT1 in the resected specimen was the significant prognostic factor

8 especially for the treatment of locally unresectable (LUR) PDAC defined by the

9 National Comprehensive Cancer Network (NCCN) guidelines (2010) [19, 20].

10 Based on these results, pretreatment/preoperative evaluation of hENT1 expression

11 in PDAC tissue can be beneficial in predicting the efficacy of Gem-based therapy

12 before initial treatment. The specimens obtained by endoscopic ultrasound-guided

13 fine-needle aspiration biopsy (EUS-FNAB) might be suitable for evaluating hENT1

14 expression; however, the immunohistochemical (IHC) analysis of hENT1 expression in

15 the pancreatic tumor tissue taken by EUS-FNAB has not been established. There have

16 been several studies which examined gene expression including hENT1 in pre-treated

tissue biopsy samples obtained by EUS-FNAB in the patients with unresectable PDAC [21-23]. Based on genetic analysis of EUS-FNAB tissue samples, it is suggested that hENT1 mRNA expression levels might be biomarkers for predicting and monitoring Gem sensitivity in patients with unresectable PDAC [23]. The examination of total RNA isolated from EUS-FNAB tissue samples without micro-dissection has a risk of contaminating cells which could lead to false results. In contrast, IHC analysis using EUS-FNAB samples can examine cancer-specific expression of hENT1. However, there have been no previous reports performing IHC analysis of hENT1 expression in the pretreatment tissue taken by EUS-FNAB and comparing to post-treatment resected specimens of PDAC. One of the reasons why such studies were rare is the difficulty in obtaining sufficient quantity of cancer cells for IHC analysis, because the materials aspirated for analysis are often bloody and contain contamination from gastrointestinal tract epithelium [24-27]. Recently, Yamao et al. [24] has revealed that EUS-FNAB with rapid on-site evaluation (ROSE) provides more accurate diagnosis than EUS-FNAB without it, because a cytopathologist ensures that the samples taken by EUS-FNAB are adequate for assessment. Because sampling rate of PDAC tissue in our institute has

1   been high owing to introduction of ROSE, we could retrospectively evaluate the stored

2   cell block specimens for the IHC analysis of hENT1 expression.

3   The aim of our study was to clarify whether pretreatment hENT1 expressions in

4   the EUS-FNAB specimens are concordant with those in the resected specimen after

5   Gem-CRT, and to validate the utility of hENT1 expression using EUS-FNAB samples

6   as a prognostic marker in the locally advanced PDAC patients who underwent

7   Gem-CRT.

8

9   **Patients and methods**

10   Between February 2005 and November 2011, we had enrolled 117 patients for our

11   Gem-CRT protocol reported previously [18, 19], who were cytologically or

12   histologically diagnosed as PDAC and having UICC-T3 and -T4 tumors determined by

13   using 64-slice multi-detector computed tomography (MDCT). CT was performed

14   according to a defined pancreas protocol as 4-phasic contrast-enhanced MDCT with

15   thin slices at intervals of 1 mm. Patients were excluded when they showed evident

16   distant metastatic lesions at the time of enrollment. They all gave their written informed

1 consent for inclusion in the study. These patients were also retrospectively reclassified

2 into the three respectability groups: resectable (R), borderline resectable (BR), or locally

3 unresectable (LUR), according to the NCCN guidelines (2010) [20] .

4   Out of the 117 patients, 76 were diagnosed as PDAC by cytology and/or histology

5 using EUS-FNAB specimen (Fig. 1). Among 76 cases 94.7% (72/76) were diagnosed

6 by cytology, 81.6% (62/76) were diagnosed by histology, 100% (76/76) were diagnosed

7 by either of two methods. We retrospectively reviewed the formalin embedded

8 specimens obtained by EUS-FNAB for these 76 patients, and the adequate amount of

9 histological specimens required for the examination of hENT1 expression could be

10 found in 52 patients (68.4%), all of which could have IHC staining successfully

11 performed. Among these 52 patients, hENT1 positive was found in 34 (65.4%), of

12 whom 29 (85.3%) could receive resection and 5 (14.7%) could not, while hENT1

13 negative was found in 18 (34.6%), of whom one was excluded due to refused of

14 treatment, 8 (47.0%) could receive resection and 9 (53.0%) could not.

15   We evaluated the relation between hENT1 expressions and clinical courses in these

16 51 patients. The study measured intratumoral hENT1 expression, concordance rates of

1   hENT1 expressions of EUS-FNAB specimens with those of resected tumors, and

2   survival analysis based on hENT1 expression of EUS-FNAB specimen.

3

4   **EUS-FNAB procedure**

5   EUS was performed using a linear array endoscope (GF-UCP240; Olympus

6   Medical Systems Co., Ltd, Tokyo, Japan), connected to a processor with a color

7   Doppler function (SSD-α10; Hitachi-Aloka Medical., Ltd, Tokyo, Japan). After the

8   tumor was identified using B mode imaging, we confirmed the absence of vessels in the

9   target area with the color Doppler mode. After we punctured an aspiration needle into

10   the tumor under ultrasonographic guidance, the stylet was pulled out and the specimen

11   was aspirated with a 20 ml syringe, then the needle moved back and forth several times

12   within the tumor. Negative pressure was released before the needle was removed from

13   the tumor. A cytologist immediately examined the specimen with ROSE using rapid

14   stain (Diff-Quik stain; International Reagents, Kobe, Japan) to verify that sufficient

15   sample was obtained. When a tentative diagnosis of malignancy could be made by the

16   on-site evaluation, we finished the EUS-FNAB procedure. If not, we performed an

1   additional one to two punctures to obtain the diagnosis. The specimen from each

2   EUS-FNAB pass was fixed in alcohol and then stained using the Papanicolaou

3   multichromatic procedure. The remaining material was fixed in 10% formalin and then

4   embedded in paraffin for the cell block analysis to obtain histological diagnosis

5   (hematoxylin and eosin; H&E).

6

7   **IHC analysis and evaluation of hENT1 expression**

8   After cytological and/or histological diagnosis of PDAC had been confirmed, we

9   retrospectively evaluated 76 stored cell block specimens for the IHC analysis of hENT1

10   expression: IHC staining was able to be performed successfully on 52 specimens, while

11   the remaining 24 failed. The causes of failure were as follows: blood clot alone in 4,

12   normal pancreatic tissue in 9, and an insufficient quantity of malignant cells in 11. For

13   the hENT1 IHC analysis, we used only cell block samples, neither core biopsy samples

14   nor cytologic smear.

15   The cell blocks were sliced into 2-μm paraffin sections. The 2-μm sections were

16   used for the assessment of intratumoral hENT1 expressions with immunohistochemistry

1   as well as being stained with hematoxylin and eosin (HE). Immunostaining procedure

2   was done using the labeled streptavidin-biotin peroxidase complex method with the

3   Benchmark XT auto-immunostaining system (Ventana Japan, Tokyo, Japan). The

4   antigen retrieval step was carried out at 90°C, 30 min, and then the sections were

5   incubated in rabbit-derived anti-hENT1 polyclonal antibody (Medical and Biological

6   Laboratories Co., Ltd, Nagoya, Japan). The sections were labeled with an automated

7   immunostaining system with I-View detection kit. Immunostained sections were lightly

8   counterstained with Mayer's hematoxylin.

9   The resected specimens were fixed in a formalin solution, sliced into 5-mm

10   sections and embedded in paraffin blocks. A 3-μm section was obtained from each

11   block and stained with H&E. The sections were routinely examined for pathological

12   differentiation, and resection margin status. The histological response of Gem-CRT was

13   evaluated according to Evans' histopathological criteria [28]. According to the result of

14   H&E staining, the most appropriate one section which contained tumor cells rich

15   enough for immunostaining was stained to assess intratumoral hENT1 expression in the

16   same manner as the EUS-FNAB samples.

1   Two pathologists (T.S., K.U.) who were blinded to the clinical characteristics of

2   the patients assessed EUS-FNAB samples and resected specimens. Scoring for hENT1

3   immunostaining was done on the basis of the relative intensities of staining of the

4   cancer cells, with reference to the normally strong hENT1 staining of cytoplasm within

5   the lymphocytes in the EUS-FNAB samples and of cell membranes within the islets of

6   Langerhans cells in the resected specimen as internal controls, respectively. The degree

7   of hENT1 expression in the resected specimen was determined by the intensity as well

8   as extent of positive staining according to our previous study [18]. A revised scoring

9   system expressing the degree of hENT1 expression in the EUS-FNAB samples was

10   devised based on our previous study; the scoring system is represented as follows: a

11   score ranging from 0 to 3 was assigned based on the intensity of staining, where 0 = no

12   staining, 1 = weakly positive, 2 = moderately positive (same intensity as internal

13   control), and 3 = strongly positive. The degree of hENT1 expression was defined as

14   high (neoplastic cells with score 3 accounting for more than 50% of the total tumor

15   cells), low (neoplastic cells with score 0 or 1 accounting for more than 50% of the total

16   tumor cells), and intermediate (all other neoplastic cells). We defined high and

16

1   intermediate staining as hENT1 positive, and low staining as hENT1 negative in both

2   EUS-FNAB samples and resected specimens (Fig. 2).

3

4   **Treatment protocol**

5   The treatment protocol of Gem-CRT was described by our previous reports [18,

6   19]. Briefly, the total radiation dose was 45 Gy, delivered in 25 fractions (5

7   fractions/week), and the patients were administered an infusion of Gem at a dose of 800

8   mg/m^2 on days 1, 8, 22, and 29 for one cycle. The patients underwent reassessment at 4

9   to 6 weeks after the completion of Gem-CRT; when we determined that curative-intent

10   resection was possible, they were scheduled to undergo pancreatectomy. At the time of

11   reassessment, especially in the case of LUR patients, we determined that curative-intent

12   resection was possible when the following findings on MDCT were observed: no

13   stenosis or change of shape in the celiac trunk and SMA as well as the absence of

14   metastatic lesions in other distant organs. Even after we decided that the tumor was

15   inoperable, we continued chemotherapy mainly using Gem. Pancreaticoduodenectomy

16   (PD) or distal pancreatectomy (DP) was performed as previously described [18, 19].

1   From 6 weeks after resection, we planned to start the postoperative chemotherapy

2   regimen, consisting of Gem at a dose of 800 mg/m^2 biweekly for at least 6 months.

3   After pancreatectomy, all patients were evaluated as follows: physical examination

4   every month; laboratory tests including CEA serum levels and CA19-9 levels every 2 or

5   3 months; and MDCT every 3 months within 2 years, and thereafter every 6 months [18,

6   19].

7

8   **Analysis of factors contributing to survival**

9   We analyzed various clinicopathological factors in the whole patients and those

10   with resection in order to clarify the significant prognostic factors, including (1)

11   pre-treatment factors such as tumor location, tumor size before Gem-CRT, UICC-T

12   classification, respectability according to NCCN guideline 2010, and hENT1 expression

13   of EUS-FNAB samples; (2) post-treatment clinical factors, such as response to

14   Gem-CRT evaluated according to the Response Evaluation Criteria in Solid Tumors

15   (RECIST) [29] , reduction rate in serum carbohydrate antigen (CA) 19-9 level as

1   previously described [19], presence of distant metastasis after Gem-CRT, and hENT1

2   expression of resected specimen.

3

4   **Statistical analyses**

5   The results for continuous variables were expressed as mean or median. For the

6   clinicopathological features of the patients, P values were calculated by χ^2 test or

7   Fisher's exact test, as appropriate. In the whole patients, the date of the initial treatment

8   was chosen as the starting point for the measurement of survival time. The day of final

9   follow-up was December 31, 2013, and there was no loss of follow-up. Survival time

10   was calculated using the Kaplan-Meier method and was compared between the groups

11   using the Wilcoxon's test. The factors affecting survival time were analyzed using the

12   multivariate Cox proportional hazard model. Individual variables with a significance of

13   P<0.05 in the univariate Cox proportional hazard model were selected for inclusion into

14   the multivariate analysis. In the multivariate analysis, variables with a significance of

15   P<0.05 were selected. For all statistical tests, a P value less than 0.05 was considered

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1 statistically significant. All statistical analyses were performed using SPSS version 20

2 (IBM Inc., Chicago, IL) software.

3

4 **Results**

5 **Immunostaining and patient background**

6   Patient characteristics are summarized in Table 1. Comparing to the whole patients

7 and those with resection, the resection rate according to tumor location, UICC-T

8 classification, resectability classification, and hENT1 expression in EUS-FNAB

9 samples differed significantly: head vs. body / tail (85.3% vs. 47.1%; P=0.004), T3 vs.

10 T4 (89.2% vs. 52.2%; P=0.003), resectable vs. borderline resectable vs. locally

11 unresectable (40.0% vs. 89.3% vs. 55.6%; P=0.01), and hENT1 positive vs. hENT1

12 negative (85.3% vs. 47.1%; P=0.004). The positive rate of hENT1 expression in

13 EUS-FNAB samples was 66.7% in the whole 51 patients and 78.4% in the 37 patients

14 with resection.

15   We examined the homology of hENT1 expression between pretreatment samples

16 obtained by EUS-FNAB and resected specimens after Gem-CRT in 37 resected

1   specimens (Table 2). As the status of hENT1 expression in the resected specimens was

2   determined as control, sensitivity, specificity, positive predictive value, negative

3   predictive value, and accuracy of EUS-FNAB samples were 93.1%, 75.0%, 93.1%,

4   75.0%, and 89.2%, respectively. Therefore, the rate of concordance between

5   EUS-FNAB samples and resected specimens was 89.2% ($K = 0.681$). We examined the

6   characteristics of the 4 patients in whom hENT1 expression differed between

7   EUS-FNAB samples and resected specimen (Table 3). In cases 1 and 2, hENT1

8   expression was found to be negative (low) in the EUS-FNAB samples, while positive

9   (intermediate) in the resected specimen. On the other hand, in cases 3 and 4, it was

10   positive (intermediate) in the EUS-FNAB sample, while negative (low) in the resected

11   specimen. When we compared the intensity scores of hENT1 staining between the

12   EUS-FNAB samples and the resected specimen (control) as shown in Table 3, the

13   intensity scores in the resected specimen in all of 4 cases contained more than two kinds

14   of intensity with various dominant area, and those in the EUS-FNAB samples contained

15   one or more scores of the resected specimen with dominant area which was different

16   from the resected specimen. These findings suggested that the discrepancy between

3   **Patient characteristics and effect of gem-CRT according to hENT1 expression**

4   Pre-treatment clinical factors and the clinical response after Gem-CRT in the

5   whole patients and those with resection are summarized in Table 4. Pre-treatment

6   clinical factors in the whole patients as well as in those with resection did not differ

7   between hENT1 expression positive and negative. As for RECIST after Gem-CRT in

8   the whole patients, the percentage of the patients with partial response (PR) and stable

9   disease (SD) was significantly higher in hENT1 positive than in negative: 82.4% vs.

10   52.9% (P=0.047). Distant metastasis after Gem-CRT occurred significantly less

11   frequently in hENT1 positive than in negative: 11.8% vs. 47.1% (P=0.005). The

12   incidence of the patients with CA19-9 reduction rate of 50% or more was significantly

13   higher in hENT1 positive than in negative: 64.7% vs. 24.5% (P=0.006). In the patients

14   with resection, the incidence of patients with CA19-9 reduction rate of 50% or more

15   was significantly higher in hENT1 positive than in negative: 75.9% vs. 37.5% (P=0.04),

16   whereas other factors did not differ between the two groups.

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2 **Univariable and multivariable analysis for prognostic factors**

3   In the whole patients, UICC-T classification (P = 0.002), hENT1 expression of

4 EUS-FNAB samples (P < 0.001), response of Gem-CRT (P < 0.001), CA19-9 reduction

5 rate (P = 0.001), and distant metastasis after Gem-CRT (P < 0.001) were found to be

6 significant, in the univariate model; however, in the multivariate model, only hENT1

7 expression and UICC-T classification were found to be significant independent

8 prognosis factors (Table 5). In the patients who underwent resection, UICC-T

9 classification (P = 0.015), hENT1 expression of EUS-FNAB samples (P < 0.001) and

10 hENT1 expression of resected specimen (P < 0.001) were found to be statistically

11 significant in the univariable analyses; however, once again, in the multivariate model,

12 only hENT1 expression and UICC-T classification were found to be significant (Table

13 5).

14   In the 51 whole patients and 37 with resection, survival rates were significantly

15 higher in hENT1 positive than in hENT1 negative as shown in Figure 3. Furthermore,

16 we compared survival curves according to hENT1 expression in T3 (Fig. 4a, b) and T4

1   patients (Fig. 5a, b). In T3 patients, the survival rates were significantly higher in

2   hENT1 positive than in hENT1 negative in the whole patients and in those with

3   resection. In T4 patients, the survival rates did not significantly differ between hENT1

4   positive and negative in the whole patients, while in the patients with resection the

5   survival rates were significantly higher in hENT1 positive than in negative.

6   Interestingly, survival curves in the patients without resection (14 patients in Fig. 3b, 3

7   in Fig. 4b, and 11 in Fig.5b) were very similar to those of hENT1 negative with

8   resection (8 in Fig. 3b, 6 in Fig .4b, 2 in Fig. 5b).

9

10   **Discussion**

11   The hENT1 expression assessed immnunohistochemically in the resected specimen

12   has been proven to be a significant prognostic marker of PDAC patients undergoing

13   Gem-based adjuvant therapy [10-18], although the assessment method for grading of

14   expression and reference cells (Langerhans cells or lymphocytes) differed among the

15   studies. In our previous study on the 55 patients using Langerhans cells as a reference

16   [18], staining intensity and extension of stained tumor cells (I-E) were graded as high

1   (n=14, 25.5%), intermediate (n=25, 45.5%), and low (n=16, 29.0%). High and

2   intermediate were defined as positive (71.0%) and low was done as negative (29.0%),

3   and survival rate was significantly higher in the hENT1 positive group than in the

4   negative group. Using lymphocytes as a reference, Farrell et al. [10] reported that I-E

5   was categorized as high (n=34, 37.4%), low (n=39, 42.8%), and no staining (n=18,

6   19.8%), in which greater than 50% of cells showed no staining, and that survival rate

7   was significantly higher in the hENT1 high/low than in the no staining. Using

8   Langerhans cells as a reference, Nakagawa et al. [16] also reported that I-E was graded

9   high (n=78, 71.6%) and low (n=31, 28.4%), and that survival rate was significantly

10   higher in the hENT1 high than in the low. Therefore, the proportion of hENT1

11   expression was similar among these previous three studies, although the assessment

12   method based on I-E for grading of expression slightly differed. In contrast, Kawada et

13   al. [30] revealed that hENT1 expression in the resected specimens was not associated

14   with prognosis in the patients who underwent resection after preoperative Gem-CRT

15   and immediately received postoperative liver perfusion chemotherapy using continuous

16   infusion of 5-fluorouracil (for 28 days) into the hepatic artery and portal vein through a

1   catheter inserted during the surgical procedure. They suggested that 5-FU liver

2   perfusion had a negative impact on the role of hENT1 expression in prognosis.

3   If pretreatment evaluation of hENT1 expression in PDAC specimen obtained by

4   EUS-FNAB becomes possible without difficulty, it is very useful to predict the efficacy

5   of Gem-based therapy. The IHC analysis of hENT1 expression in the EUS-FNAB

6   specimens has not been established and thus we first examined whether pretreatment

7   hENT1 expressions in the EUS-FNAB specimens were concordant with those in the

8   resected specimen after Gem-CRT. As a result, the rate of concordance between them

9   was 89.2%, which is higher than the previous two reports concerning the other IHC

10   studies: 86.5% in the study on SMAD4 protein and 73.9% in the study on ZIP4 [31, 32].

11   The reason why the concordance rate in the three studies including ours did not reach

12   100% is unclear.

13   However we could identify the features of the 4 patients in whom hENT1 expression

14   differed between EUS-FNAB samples and resected specimen by comparing the

15   intensity scores and its area of hENT1 staining between the EUS-FNAB samples and

16   the resected specimen: the intensity of staining and its area in the resected specimen

varied widely, indicating the existence of tumor heterogeneity in these 4 cases. A recent study on the evaluation of Ki-67 index in pancreatic neuroendocrine tumors also demonstrated intratumoral heterogeneity by comparing its index in EUS-FNAB specimens and resected specimens as the criterion standard: concordance rate remained 74.0% using the mean Ki-67 index [33]. It is however interesting to note that Gem-CRT did not appear to change the preoperative/postoperative correlation of hENT1 staining.

Using EUS-FNAB specimens, our hENT1 IHC analysis could be successfully performed in 68.4% (52/76) among the cases diagnosed cytologically and/or histologically as PDAC, under the situations that the remaining materials followed by cytologic/histologic diagnosis were used and that some of adequate samples might be already consumed before the IHC analysis. These results suggested that EUS-FNAB specimens obtained from PDAC were appropriate for IHC analysis of hENT1. In the method similar to ours which used the remaining samples after diagnosis to evaluate SMAD4 protein, only 44.4% (52/117) could be analyzed [31]. It is therefore considered that the success rate of IHC analysis using EUS-FNAB samples obtained from PDAC specimens remains not so high. Concerning the reason why the success rate remains low,

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1   Navina S, et al. [34] recently evaluated the adequacy of EUS-FNAB samples of

2   pancreatic masses for theranostic studies by assessing cellularity of cytology material.

3   They retrospectively evaluated 169 EUS-FNAB specimens with positive diagnoses of

4   solid epithelial pancreatic neoplasms (adenocarcinoma: 88%) for smear and cell block

5   cellularity. Cellularity of cell blocks was scored on a scale of 1 to 4 (score 1 for fewer

6   than 50 lesional cells, score 2 for 50 to 100, score 3 for 100 to 200, and score 4 for more

7   than 200), and scores of 3 or 4 were deemed adequate for ancillary studies such as IHC

8   analysis. As a result, only 12.4% of the positive cases had a cell block cellularity score

9   that was adequate for theranostic studies. This score was not associated with ROSE,

10   needle gauge, or number of passes. Tumor size and fibrosis score of resected tumors

11   correlated with cellularity, but only larger size in pancreatic neuroendocrine tumors was

12   significantly associated with adequacy. Furthermore, 75 PDAC cases were

13   prospectively evaluated for cellularity score: score 0 in 39%, score 1 to 2 in 49%, and

14   score 3 in 12%. Taking this cellularity score 1 to 3 of 61% and our result of yield 68.4%

15   for hENT1 IHC analysis together, the cellularity score 1 or more might be enough for

16   hENT1   IHC   analysis.   Consequently,   to   enhance   the   clinical   utility   of

1 pretreatment/preoperative IHC hENT1 examination, we have to develop a novel method

2 to improve tumor cell yield, including modified cytologic techniques and new needle

3 designs.

4 Serum levels of CA19-9 have been accepted as a measure of pancreatic cancer

5 burden and the role of CA19-9 has been recently underscored for the evaluation of

6 patients with pretreatment/preoperative therapy before planned surgical resection. Our

7 previous two studies, which evaluated the clinical response after Gem-CRT for PDAC

8 according to the hENT1 expression in the resected specimen, revealed that hENT1

9 positive group had significantly higher reduction rate of CA19-9 than hENT1 negative,

10 although RECIST did not differ between the two groups [18, 19]. In our present study

11 using pretreatment/preoperative EUS-FNAB samples in the whole patients, incidence of

12 the patients with CA19-9 reduction rate of 50% or more was significantly higher in

13 hENT1 positive than in negative, and percentage of the patients with PR and SD in

14 RECIST after Gem-CRT was significantly higher in hENT1 positive than in negative.

15 In the patients with resection, however, RECIST did not differ between the two groups.

16 Concerning the reason why RECIST results differed between the whole patients and

1 those with resection, 47.1% (8/17) of hENT1 negative showed PD after Gem-CRT and

2 all of them could not receive pancreatectomy, while only 18.6% (6/34) of hENT1

3 positive showed PD and one of them could receive pancreatectomy. It was therefore

4 considered that hENT1 expression was not associated with RECIST in the patients with

5 resection. Other than our studies comparing the clinical response between hENT1

6 positive and negative, Poplin et al. [35] evaluated clinical response using RECIST and

7 survival in metastatic PDAC patients, and hENT1 status had no influence on RECIST

8 and survival (MST): the percentage of PR/CR was 15.5% (9/58) and MST was 5.2

9 months in hENT1 high, whereas 26.3% (30/118) and 6.1 months in hENT1 low. They

10 considered that the role of hENT1 was less important in metastatic disease than after

11 surgery with a presumed micrometastatic state. In contrast, our study included the

12 locally advanced (T3/T4) PDAC patients without distant metastasis at the time of

13 enrollment, and at the time of reassessment (about 2-3 months after enrollment) distant

14 metastasis became apparent in 23.5% (12/51) of the patients: hENT1 positive (n=4) and

15 negative (n=8). These 12 patients died within 12 months regardless of hENT1

16 expression.

1 As for Gem-based pretreatment studies on hENT1 expression in PDAC, to the best

2 of our knowledge, there have been four studies including our study: the clinical

3 outcomes of patients undergoing Gem-CRT could be predicted by IHC analysis of

4 hENT1 in EUS-FNAB samples obtained from T3/T4 (R/BR/LUR) PDAC. The one

5 study, which evaluated mRNA expression levels of hENT1 using EUS-FNAB

6 specimens obtained from stage III/IV inoperable (LUR and metastatic) PDAC patients,

7 did not show that its expression levels influenced survival [23]. The remaining two

8 studies on IHC hENT1 evaluation in PDAC, of which one used biopsy specimens of

9 metastatic lesions [34] and the other used biopsy specimens from the primary and

10 metastatic lesions in stage III/IV inoperable (LUR and metastatic) patients [36], did not

11 demonstrate any significant differences in prognosis between the high and low hENT1

12 subgroups either. The reason for conflicting results between our study and the other

13 three probably is that the other three studies included only inoperable patients who had

14 basically poor prognosis in itself, while ours included the locally advanced (T3/T4)

15 PDAC patients without distant metastasis. It is considered that tumor progression

16 influences the role of hENT1 expression in clinical response as well as prognosis in

1 PDAC patients, and we therefore compared survival curves according to hENT1

2 expression in T3 (R/BR) and T4 (BR/LUR) patients. In T3, prognosis was significantly

3 better in hENT1 positive in the whole patients and in those with resection. In T4, it did

4 not significantly differ between hENT1 positive and negative in the whole patients,

5 while it was significantly better in hENT1 positive in those with resection. These results

6 indicate that the role of hENT1 expression in Gem-based treatment become less

7 important as tumor progresses.

8   Our treatment protocol of Gem-CRT for locally advanced (T3/T4) PDAC patients

9 was conducted for aiming to achieve curative-intent resection after reassessment, even

10 though it was determined initially locally unresectable. Therefore, we have to clarify the

11 significance of preoperative/pretreatment assessment of hENT1 expression using

12 EUS-FNAB specimens based on our results: its assessment identifies PDAC patients

13 who can benefit from curative-intent resection followed by Gem-based adjuvant therapy.

14 Regardless of T3- and T4-tumors, hENT1-positive patients who underwent

15 curative-intent resection had significantly better prognosis compared to

16 hENT1-negative patients with resection, whose prognosis was similar to those without

1   resection. To improve the prognosis in hENT1-negative patients, a novel regimen other

2   than Gem-based treatment needs to be further investigated.

3   In conclusion, pretreatment hENT1 expressions in the EUS-FNAB specimens are

4   concordant with those in the resected specimen after Gem-CRT, and its assessment

5   prior to Gem-CRT provides us the important information on the PDAC patients who

6   can benefit from curative-intent resection followed by Gem-based adjuvant therapy.

7

8

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11

1   Figure Legends

2   Figure 1. Flow diagram of the study participants.

3   PDAC: Pancreatic ductal adenocarcinoma. Gem-CRT: gemcitabine-based

4   chemoradiation therapy.

5   ERCP: endoscopic retrograde chorangiopancreatography.

6   EUS-FNAB: endoscopic ultrasonography-guided fine-needle aspiration.

7   hENT1: human equilibrative nucleoside transporter 1.

8

9   Figure 2. Immunohistochemical staining of PDAC for hENT1.

10   a. EUS-FNAB sample showing high hENT1 expression relative to internal control

11   (lymphocyte), "hENT1 positive".

12   b. EUS-FNAB sample showing low hENT1 expression, "hENT1 negative".

13   c. Resected specimen showing high hENT1 expression relative to internal control (islet

14   cells), "hENT1 positive".

15   d. Resected specimen showing low hENT1 expression, "hENT1 negative".

16

1   Figure 3. Cumulative survival curves according to hENT1 expression.

2   a. Whole patients comparing hENT1 positive (n=34) and negative (n=17).

3   b. Patients with resection comparing hENT1 positive (n=29) and negative (n=8), and

4   those without resection (n=14).

5   *: P < 0.001 vs. hENT1 negative. #: P < 0.001 hENT1 negative with resection.

6

7   Figure 4. Cumulative survival curves in T3 patients according to hENT1 expression.

8   a. Whole T3 patients comparing hENT1 positive (n=20) and negative (n=8).

9   b. T3 patients with resection comparing hENT1 positive (n=19) and negative (n=6), and

10   those without resection (n=3).

11   *: P < 0.001 vs. hENT1 negative. #: P < 0.001 hENT1 negative with resection.

12

13   Figure 5. Cumulative survival curves in T4 patients according to hENT1 expression.

14   a. Whole T4 patients comparing hENT1 positive (n=14) and negative (n=9).

15   b. T4 patients with resection comparing hENT1 positive (n=10) and negative (n=2), and

16   those without resection (n=11).

1   *: P = 0.126 vs. hENT1 negative. #: P < 0.001 hENT1 negative with resection.

Table 1

Characteristic	Whole patients N = 51		Patients with resection N = 37		Resection rate (%)	
Age, mean ± SD	66.4 ± 9.6		66.1 ± 8.8		-	
Sex						
Male	29	(56.9%)	22	(59.5%)	75.9%	
Female	22	(43.1%)	15	(40.5%)	68.2%	
Tumor location						
Head	34	(66.7%)	29	(78.4%)	85.3%	]*
Body / Tail	17	(33.3%)	8	(21.6%)	47.1%	
UICC-T classification						
T3	28	(54.9%)	25	(67.6%)	89.2%	]*
T4	23	(45.1%)	12	(32.4%)	52.2%	
Resectability classification						
Resectable (R)	5	(9.8%)	2	(5.4%)	40.0%	
Borderline resectable (BR)	28	(54.9%)	25	(67.6%)	89.3%	]*
Locally unresectable (LUR)	18	(35.3%)	10	(27.0%)	55.6%	
hENT1 expression in EUS-FNAB samples						
positive	34	(66.7%)	29	(78.4%)	85.3%	]*
negative	17	(33.3%)	8	(21.6%)	47.1%	

Table 1. Background characteristics of the patients.

UICC: International Union for Cancer Control.

*: $P < 0.05$: χ^2 test

Table 2

	FNAB sample positive	FNAB sample negative
Resected specimen positive	27	2
Resected specimennegative	2	6

Table 2. Homology of hENT1 expression between pretreatment samples obtained by EUS-FNAB and resected specimens after Gem-CRT (K=0.681).

sensitivity: 93.1%, specificity: 75.0%, positive predictive value: 93.1%, negative predictive value: 75.0%, and accuracy: 89.2%.

Table 3

Table 3. The characteristics of the 4 patients in whom hENT1 expression differed between EUS-FNAB samples and resected specimen.

	Age	Sex	hENT1 expression of EUS-FNAB sample		hENT1 expression of resected specimen		Tumor Size (mm)	UICC	Resecta-bility	Tumor location	Pre-treatment CA19-9	Post-treatment CA19-9	Response	Histology	Evans grade	Survival time (month)
			Judge	Score	Judge	Score										
1	49	M	Negative	Score: 1 Low	Positive	Score: 2>1>3 Intermediate	28	T3	BR	Head	458.0	249.3	SD	Well diff.	IIa	9
2	63	M	Negative	Score: 0>1 Low	Positive	Score: 2>1>0 Intermediate	22	T3	BR	Head	10093.0	940.9	SD	Well diff.	IIa	24
3	77	M	Positive	Score: 2>1 Intermediate	Negative	Score: 1>2 Low	32	T3	BR	Head	316.5	122.7	SD	Well diff.	IIa	16
4	65	M	Positive	Score: 2>1 Intermediate	Negative	Score: 1>2>0 Low	46	T4	UR	Head	1.0	1.0	SD	Moderate diff.	IIa	12

A score ranging from 0 to 3 for the intensity of hENT1 staining is shown in decreasing order according to the dominant area like intensity: 2>1>3 (intermediate), which means that score 2 (moderately positive) occupied the most predominant area followed by score 1 (weakly positive) and score 3 (strongly positive). BR: borderline resectable. UR: unresectable. SD: stable disease.

Table 4

Values	Whole patients			Patients with resection		
	hENT1 positive	hENT1 negative	P value	hENT1 positive	hENT1 negative	P value
	n=34	n=17		n=29	n=8	
Pre-treatment clinical factors						
Age (years), mean ± SD	67.3 ± 8.5	64.7 ± 11.6	0.357	66.0 ± 8.1	66.4 ± 11.7	0.917
Sex (Male / Female)	18 / 16	11 / 6	0.552	17 /12	5 / 3	0.221
Tumor size before gem-CRT (cm), mean ± SD	32.5 ± 9.3	33.5 ± 13.0	0.747	31.8 ± 9.2	30.5 ± 7.5	0.724
UICC-T classification			0.553			0.612
T3	20	8		19	6	
T4	14	9		10	2	
Resectability			0.373			0.722
Resectable (R)	3	2		2	0	
Borderline resectable (BR)	21	7		19	6	
Locally unresectable (LUR)	10	8		8	2	
Clinical response after Gem-CRT						
Response of Gem-CRT (RECIST)			**0.047**			0.450
Complete response (CR)	0	0		0	0	
Partial response (PR)	4 (11.8%)	0		4 (13.8%)	0	
Stable disease (SD)	24 (70.6%)	9 (52.9%)		24 (82.8%)	8 (100%)	
Progressive disease (PD)	6 (17.6%)	8 (47.1%)		1 (4.4%)	0	
Distant metastasis after Gem-CRT	4 (11.8%)	8 (47.1%)	**0.005**	0	0	-
CA19-9 levels, median						
Pre-CA19-9 (U/ml)	313.15	218.6	0.839	309.9	202.75	0.928
Post-CA19-9 (U/ml)	82.4	249.3	0.100	40.4	134.5	0.346
Degree of reduction rate in CA19-9			**0.006**			**0.040**
50% or more	22 (64.7%)	4 (23.5%)		22 (75.9%)	3 (37.5%)	
Less than 50%	12 (35.3%)	13 (76.5%)		7 (24.1%)	5 (62.5%)	

Table 4. Patient characteristics and effect of Gem-CRT according to hENT1 expression.

Table 5

	Whole patients				Patients with resection			
	Univariate analysis		Multivariate analysis (Stepwise method: Wald)		Univariate analysis		Multivariate analysis (Stepwise method: Wald)	
	HR (95%CI)	P value	HR (95%CI)	P value	HR (95%CI)	P value	HR (95%CI)	P value
Age								
<65	1				1			
≧65	1.030 (0.558 – 1.900)	0.925			0.933 (0.440 – 1.976)	0.856		
Sex								
male	1				1			
female	0.982 (0.534 – 1.806)	0.954			0.943 (0.445 – 2.002)	0.879		
Tumor location								
head	1				1			
body/tail	1.782 (0.949 – 3.345)	0.072			0.909 (0.384 – 2.149)	0.828		
Tumor size before Gem-CRT (cm)								
<3.0 cm	1				1			
≧3.0 cm	1.117 (0.597 – 2.089)	0.730			1.166 (0.542 – 2.507)	0.695		
UICC-T classification								
T3	1		1		1		1	
T4	**2.812 (1.483 – 5.331)**	**0.002**	**2.325 (1.206 – 4.482)**	**0.012**	**2.629 (1.211 – 5.707)**	**0.015**	**3.862 (1.690 – 8.826)**	**0.001**
Resectability								
BR / LUR	1				1			
R	0.956 (0.320 – 2.855)	0.936			0.396 (0.050 – 3.144)	0.396		
hENT1 expression of EUS-FNAB samples								
Positive	1		1		1		1	
Negative	**4.061 (2.045 – 8.066)**	**<0.001**	**3.380 (1.688 – 6.768)**	**0.001**	**6.192 (2.439 – 15.715)**	**<0.001**	**9.613 (3.476 – 26.586)**	**<0.001**
Response of Gem-CRT (RECIST)								
PD	1				1			
PR / SD	**0.283 (0.144 – 0.555)**	**<0.001**			1.100 (0.148 – 8.171)	0.926		
Reduction rate in serum CA19-9 level								
≧50%	1				1			
<50%	**2.954 (1.574 – 5.545)**	**0.001**			2.137 (0.978 – 4.670)	0.057		
Distant metastasis after Gem-CRT								
Metastasis	1				-			
non Metastasis	**0.229 (0.110 – 0.477)**	**<0.001**			-			
hENT1 expression of resected specimen								
Positive	-				1			
Negative	-	-			**7.791 (2.887 – 21.022)**	**<0.001**		

Table 5. Univariate and multivariate analysis of factors affecting Cox proportional hazard model.
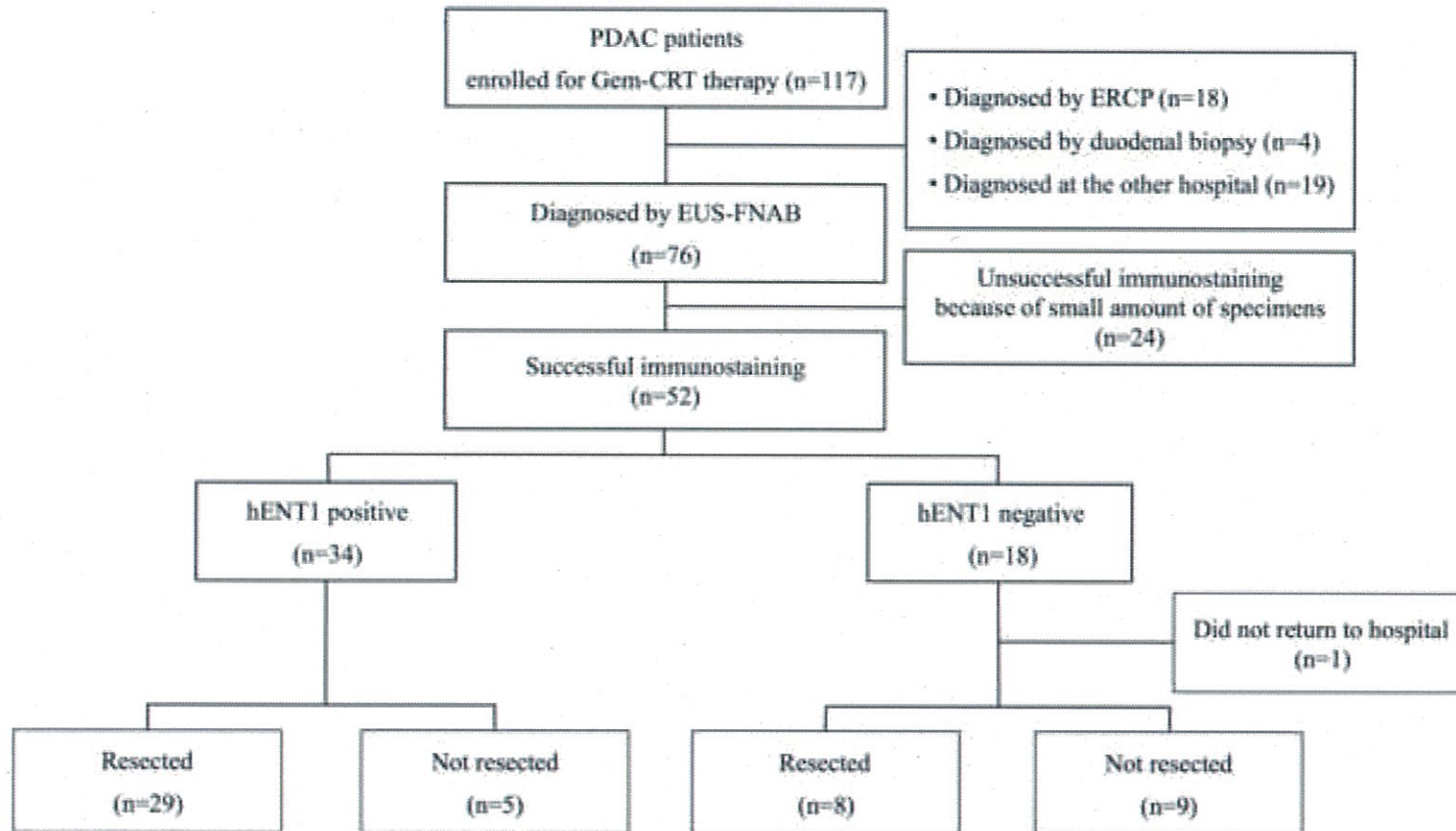
Figure 1

Figure 1. Flow diagram of the study participants.
PDAC: Pancreatic ductal adenocarcinoma. Gem-CRT: gemcitabine-based chemoradiation therapy.
ERCP: endoscopic retrograde chorangiopancreatography.
EUS-FNAB: endoscopic ultrasonography-guided fine-needle aspiration. hENT1: human equilibrative nucleoside transporter 1
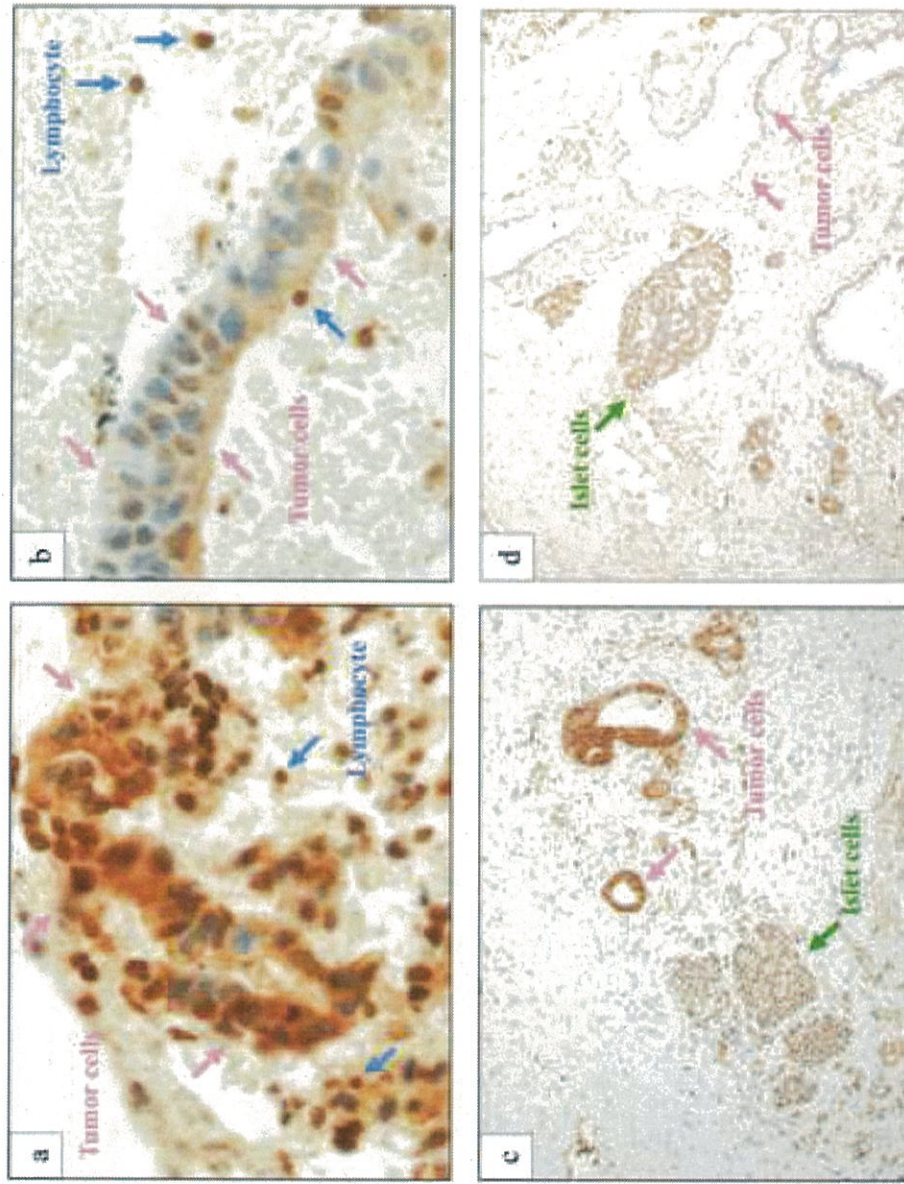
Figure 2

Figure 2. Immunohistochemical staining of PDAC for hENT1.
a. EUS-FNAB sample showing high hENT1 expression relative to internal control (lymphocyte), "hENT1 positive". b. EUS-FNAB sample showing low hENT1 expression, "hENT1 negative". c. Resected specimen showing high hENT1 expression relative to internal control (islet cells), "hENT1 positive". d. Resected specimen showing low hENT1 expression, "hENT1 negative".

Figure 3

a. Whole patients

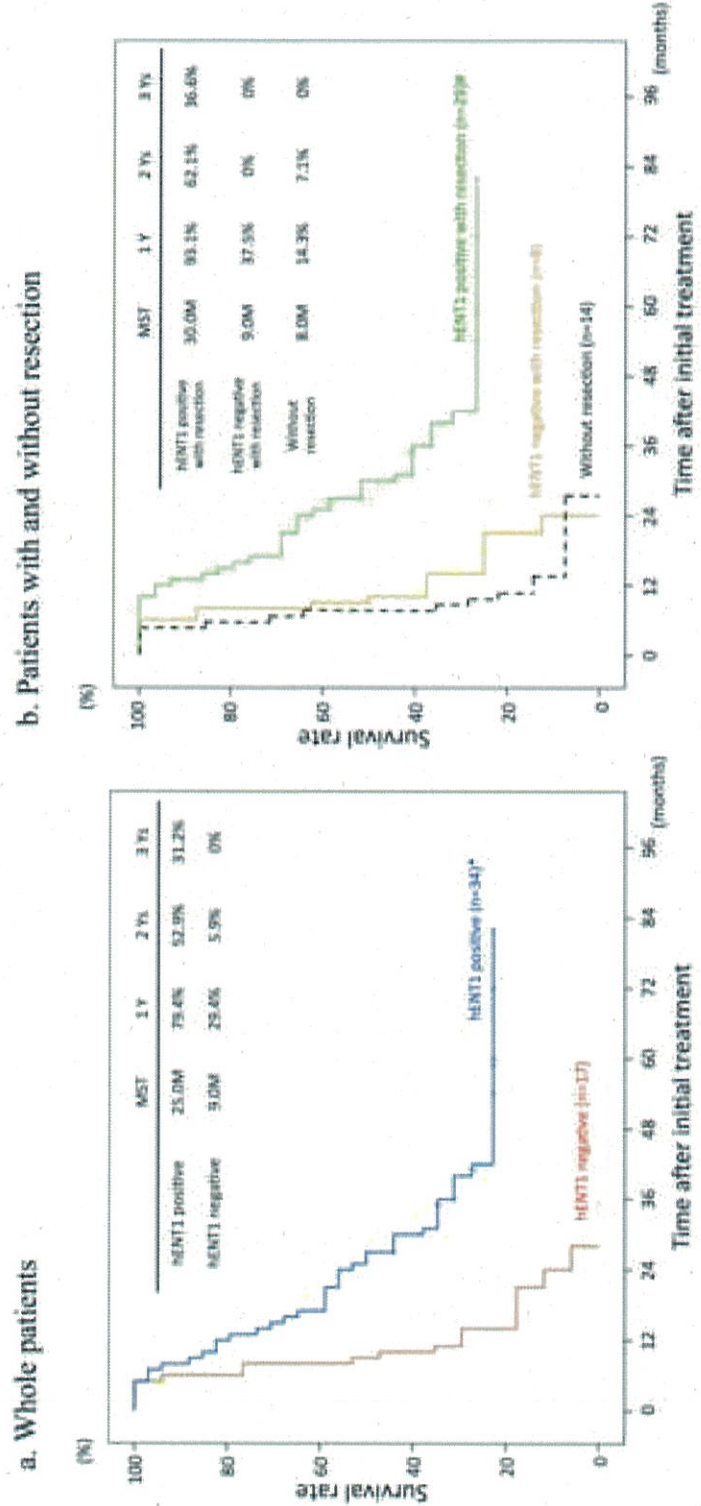b. Patients with and without resection

Figure 3. Cumulative survival curves according to hENT1 expression. a. Whole patients comparing hENT1 positive (n=34) and negative (n=17). b. Patients with resection comparing hENT1 positive (n=29) and negative (n=8), and those without resection (n=14). *: P < 0.001 vs. hENT1 negative. #: P < 0.001 hENT1 negative with resection.
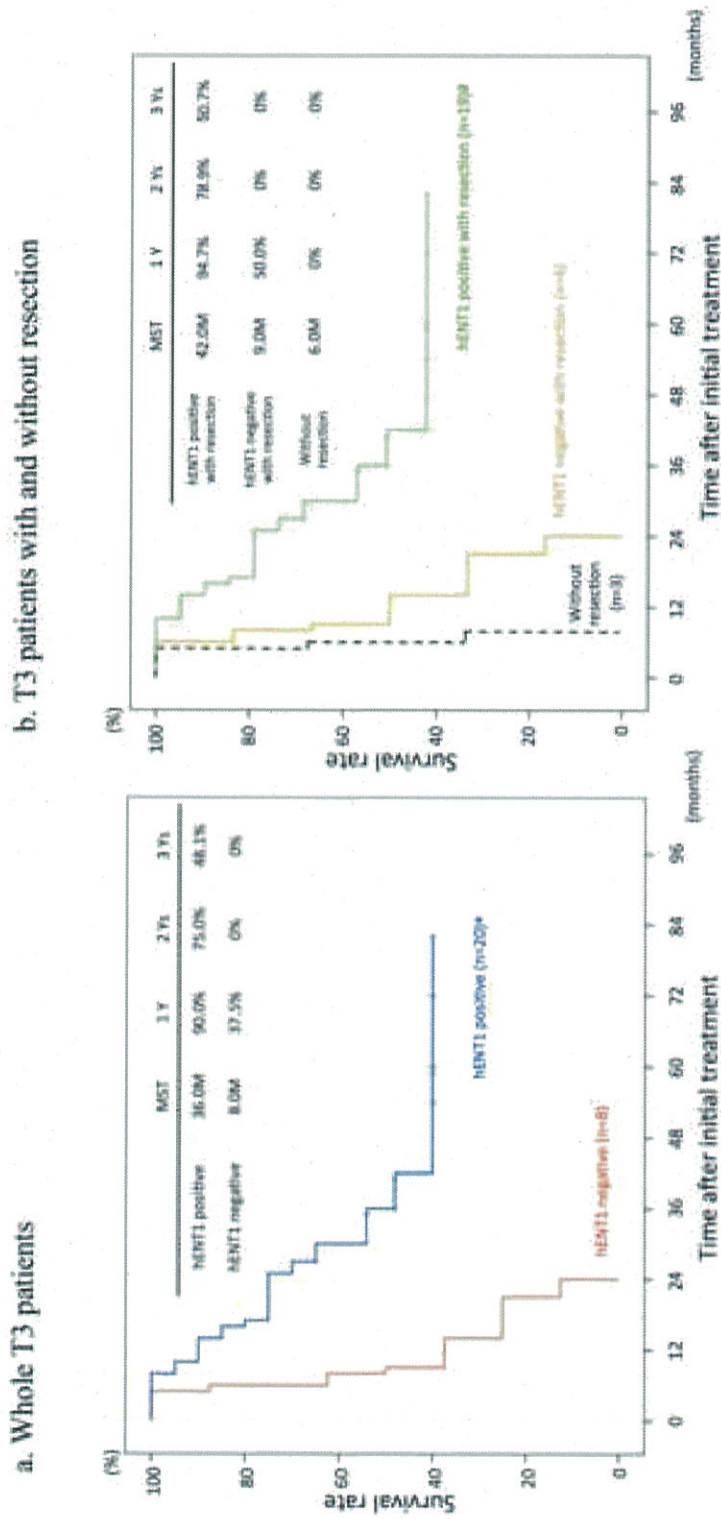
Figure 4

a. Whole T3 patients

b. T3 patients with and without resection

Figure 4. Cumulative survival curves in T3 patients according to hENT1 expression. a. Whole T3 patients positive (n=20) and negative (n=8). b. T3 patients with resection comparing hENT1 positive (n=19) and negative (n=6), and those without resection (n=3). *: P < 0.001 vs. hENT1 negative. #: P < 0.001 hENT1 negative with resection.

Figure 5

a. Whole T4 patients
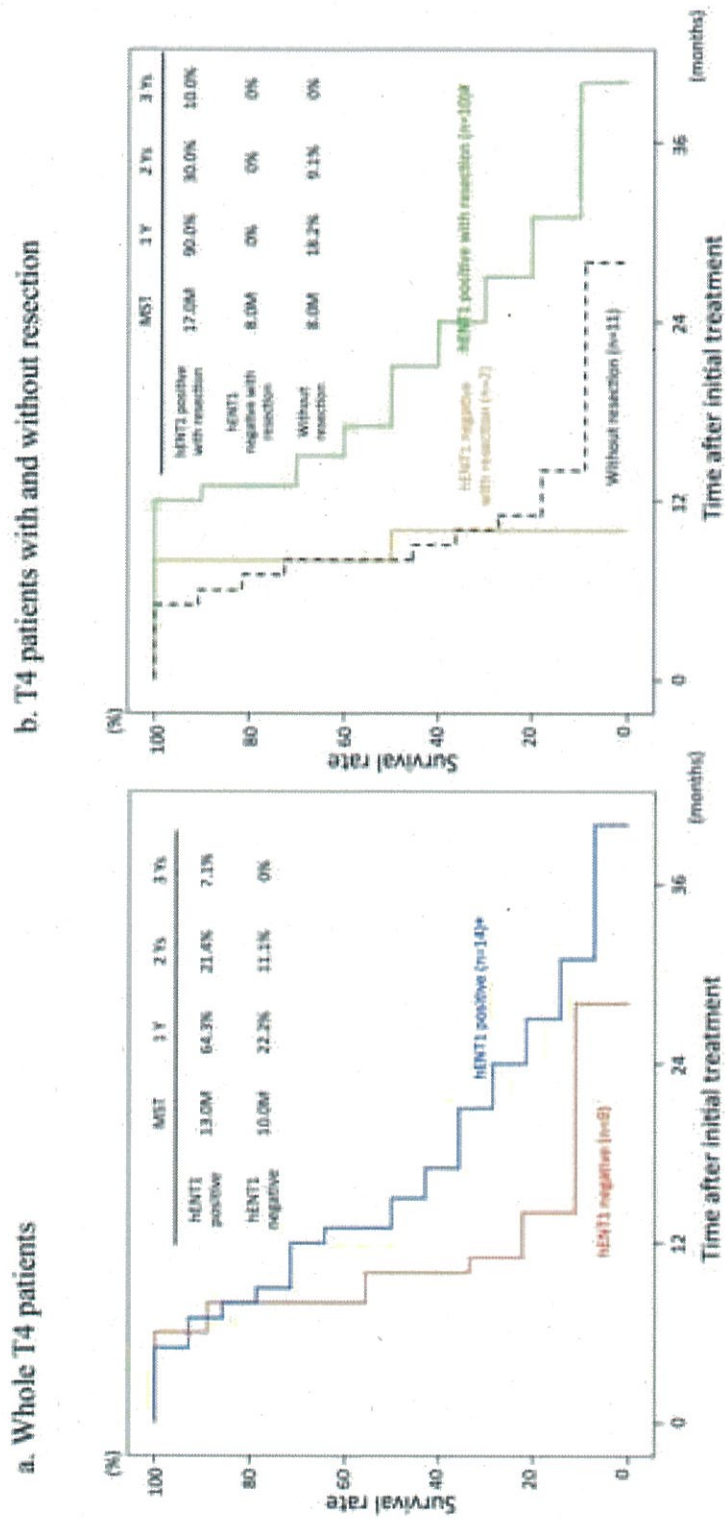

b. T4 patients with and without resection

Figure 5, Cumulative survival curves in T4 patients according to hENT1 expression. a. Whole T4 patients comparing hENT1 positive (n=14) and negative (n=9). b. T4 patients with resection comparing hENT1 positive (n=10) and negative (n=2), and those without resection (n=11). *: P = 0.126 vs. hENT1 negative. #: P < 0.001 hENT1 negative with resection.