A predictive analysis of the SP120 and 10D7G2 antibodies for human equilibrative nucleoside transporter 1 (hENT1) in pancreatic ductal adenocarcinoma treated with adjuvant gemcitabine

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Abstract

Expression of human equilibrative nucleoside transporter 1 (hENT1) in pancreatic ductal adenocarcinoma (PDAC) has been postulated to be a marker of sensitivity to gemcitabine. However, heterogeneity in the studies attempting to quantify hENT1 expression in patients with PDAC treated with gemcitabine has yielded inconclusive results that impede the adoption of hENT1 expression as a predictive biomarker. Tissue microarrays consisting of PDAC specimens from 227 patients acquired between 1987 and 2013 annotated with treatment and outcome information were subjected to staining with two antibodies for hENT1 (10D7G2 and SP120) on a single automated platform and scored by two independent pathologists blinded to treatment and outcome. The resultant scores were subjected to individual predictive disease-specific survival analysis and to unsupervised hierarchical clustering to generate a multi-marker classification. Tumour cell staining prevalence using either SP120 or 10D7G2 was predictive of gemcitabine sensitivity (p = 0.02; p = 0.01). When combined, three groups emerged, classified as SP120Low_10D7G2Low, SP120Low_10D7G2High, and SP120High_10D7G2High, in which adjuvant gemcitabine conferred median survival differences of 0.2, 0.8, and 1.5 (p = 0.76, p = 0.06, p = 0.01) years, respectively. These results were largely replicated in multivariable analysis with the P value for the SP120Low_10D7G2High cluster achieving statistical significance (p = 0.03). These data suggest that either antibody for hENT1 can be used to predict gemcitabine sensitivity in resected PDAC. However, using both antibodies adds valuable information that enables the stratification of patients who can expect to have a good, intermediate, and poor response to adjuvant gemcitabine.

Keywords: pancreatic ductal adenocarcinoma; gemcitabine; predictive biomarker; hENT1

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No conflicts of interest were declared.

Introduction

Despite a decade of research since the first definitive clinical trials [1,2] demonstrating the advantage of adjuvant gemcitabine over observation in resected pancreatic ductal adenocarcinoma (PDAC), no validated predictive biomarker exists to triage patients into treatment groups that would receive gemcitabine-based regimens and those who should be given other treatment options. Previous studies have demonstrated that the expression of SLC29A1 –
Solute Carrier Family 29 (Equilibrative Nucleoside Transporter), Member 1 (hENT1), a membrane bound protein that facilitates the passive transport of nucleosides across the plasma membrane, confers sensitivity to gemcitabine in several cancer types including mantle cell lymphoma [3], breast cancer [4], non-small cell lung cancer [5], and PDAC [6]. However, the predictive ability of hENT1 expression in PDAC treated with gemcitabine has shown mixed results in the literature. While some studies have examined this effect in patients with resectable disease [7–12] others have evaluated it in those with metastatic disease [13,14]. In addition, there are competing immunohistochemical antibodies, both monoclonal, one murine derived (10D7G2) and one rabbit derived (SP120), which were applied on different staining platforms and assessed by differing scoring techniques, yielding non-uniform binarization cut-points. To add further questionability to the results, the statistical analyses presented do not represent a widely accepted approach to determine whether a biomarker is truly predictive. The previous studies to date have chosen to examine cases that are hENT1High compared to hENT1Low in a particular treatment group. While there are studies that have demonstrated a positive survival difference for patients with hENT1High tumours who received gemcitabine, this approach discounts the fact that there may be an inherent survival difference between hENT1High and hENT1Low patients. Therefore, we aimed to examine treated and untreated cases within hENT1 categories as was recently recommended by Ballman [15]. Results from the eight previous studies to assess the predictive ability of hENT1 immunohistochemistry in PDAC are summarized in Table 1.

Table 1. Summary of the studies conducted on the relationship between hENT1 expression and gemcitabine sensitivity in PDAC

| Authors          | Year | Cohort type     | Antibody       | Statistical approach                        | Finding                           |
|------------------|------|-----------------|----------------|--------------------------------------------|-----------------------------------|
| Maréchal et al   | 2009 | Resected (N = 45) | 10D7G2         | Prognostic                                | Improved DFS and OS               |
| Farrell et al    | 2009 | Resected (N = 91) | 10D7G2         | Prognostic                                | Improved DFS                      |
| Maréchal et al   | 2012 | Resected (N = 434) | 10D7G2         | Prognostic with a mixed control arm        | Improved OS                       |
| Poplin et al     | 2013 | Metastatic (N = 177) | SP120         | Prognostic                                | Improved OS                       |
| Greenhalh et al  | 2013 | Resected (N = 204) | 10D7G2         | Prognostic over 5FU control               | Improved OS                       |
| Ormanns et al    | 2014 | Metastatic (N = 169) | SP120         | Prognostic over capcitabine + erlotinib   | No differences in OS              |
| Sinn et al       | 2015 | Resected (N = 156) | SP120          | Prognostic                                | No differences in DFS or OS       |
| Svrcek et al     | 2015 | Resected (N = 294) | SP120 and 10D7G2 | Prognostic                                | SP120 – No differences in OS 10D7G2 – Improved OS |

DFS, disease-free survival; OS, overall survival.

Materials and methods

Ethical approval

This study was subjected for ethical review and approved by the University of British Columbia Clinical Research Ethics Board (Approval Number: H12–03484).

Patients and samples

A retrospective cohort of all patients who underwent a Whipple procedure for PDAC between 1987 and 2013 was identified and assembled through the electronic medical records in the Vancouver Coastal Health Region. After confirming that clinicopathological, treatment, and outcome variables were available, a set of tissue microarrays (TMAs) were constructed of the patients that met eligibility. The TMAs were constructed using duplicate 0.6 mm cores from diagnostically confirmed PDAC. Sampled areas were assessed as having >70% epithelial tumour component.

Staining and scoring

Both SP120 and 10D7G2 antibodies were optimized using a small TMA consisting of various normal tissues that included kidney, thyroid, tonsil, and pancreas. Observed staining patterns were confirmed with those found on the Human Protein Atlas. Optimization of SP120 was based on the manufacturer’s recommended protocol on the BenchMark Ultra

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A range of titrations was performed with the equivalent assay on Ventana’s research platform and one of the study pathologists (DG) chose the optimal dilution. Final staining conditions for SP120 on the Discovery Ultra instrument are 64 min of CC1 (Tris-based buffer, Ventana), followed by 32 min of primary antibody incubation at 37 °C at 1:25 and detection with UltraMap DAB anti-Rb kit (biotin-free DAB detection system, Ventana). Using previously known staining conditions for 10D7G2 [10], a range of titrations were performed using the most sensitive detection kit for the Discovery platform and the optimal dilution was chosen by the study pathologist (DG) who determined the SP120 titration. Final staining conditions for 10D7G2 on the Discovery Ultra instrument were 64 min of CC1 with 2 h at room temperature for primary incubation at 1:2 and detection with the HQ-HRP system (Ventana).

The TMAs were sectioned at 4 μm and the staining was performed as outlined above. The study pathologists (DG and BTC), blinded to treatment and outcome, independently scored the slides using an H-Score motif. The percentage of positive cells was determined by the identification of epithelial tumour cells and a subjective determination of the proportion that are positively stained. The intensity of the positively staining cells was determined after a general review of the staining pattern for each hENT1 antibody and are defined as: faint staining yielding a score of 1, moderate staining a score of 2, and strong staining a score of 3. The product of this semi-quantitative assessment yields H-scores that range between 0 and 300. The highest H-score derived from each core in the duplicate core series was considered the final score for that case.

Statistics
To determine if the SP120 and 10D7G2 are predictive of gemcitabine sensitivity, the median H-Score for each antibody was used to determine the binarization cut-point for high and low expressers. The resultant groups for each antibody were subjected to Kaplan-Meier survival analysis with adjuvant gemcitabine and post-surgical observation occupying the two arms of the analysis. Survival time was defined as the difference between the date of last follow-up or death and the date of surgery in years. Patients were censored if they were: alive regardless of disease progression status or dead of other causes including treatment-related toxicities and inter-current diseases.

Hierarchical clustering, utilizing the centroid method, was used to categorize the patients into discrete clusters based on the percentage of cells staining positive and the H-Score method for both the 10D7G2 and SP120 antibodies. The maximum number of clusters was determined by an a priori decision that no cluster could contain fewer than 30 patients. To demonstrate the internal consistency of the clustering procedure, comparisons of high and low expressing cases that populate disparate clusters were compared for each antibody using the Wilcoxon Rank Sum Test. Heterogeneity for clinico-pathological variables across the resultant clusters was assessed with the likelihood ratio $\chi^2$ test, or a 1-way ANOVA after ensuring for normality and equal variances. To investigate the efficacy of adjuvant gemcitabine, the resultant clusters were subjected to univariable disease-specific survival (DSS) analysis using the Kaplan-Meier method as outlined previously.

The prognostic effect for each clinico-pathological variable was performed with the Cox Proportional Hazards Model with DSS as the outcome measure.
Multivariable analysis was also performed with the Cox Proportional Hazards Model and utilized a multi-criterion variable elimination procedure for each cluster where the covariates: age at surgery, sex, histological grade, lymphovascular invasion, perineural invasion, regional lymph node status, resection status, and adjuvant gemcitabine were included in the model. Variables were removed in a backwards elimination fashion based on having the highest effect likelihood ratio $P$ value down to a critical level of < 0.05. After each variable was removed, the Bayesian Information Criterion (BIC) was calculated to ensure that, with each iteration, the BIC decreased, which indicates a more robust model. In the event that the BIC increased with the removal of a variable, this was used as stopping rule for the variable elimination procedure even if the $P$ value was > 0.05.

**Figure 2.** Examination of disease-specific survival (DSS) for each hENT1 antibody. The cut-point between high and low was based on the median H-Score (20 and 90) for the SP120 and 10D7G2 antibodies, respectively. The high hENT1 cases as defined by the 10D7G2 antibody have increased sensitivity to adjuvant gemcitabine which translates into a 1.29 year median DSS difference compared to the high hENT1 cases as defined by the SP120 antibody with a 0.83 year median survival difference.
An inter-observer study utilizing the scores derived from the two independent pathologists was performed utilizing the clustering methods outlined above. Assessments of DSS for both sets of clusters were made to determine if the findings were replicated across both readers. All analyses were computed with

Figure 3. Hierarchical clustering, using the centroid method, of percent positive cells with both hENT1 antibodies yielded three separate clusters generally classified as SP120\textsubscript{Low}, 10D7G2\textsubscript{High}, SP120\textsubscript{High}, 10D7G2\textsubscript{High}, and SP120\textsubscript{Low}, 10D7G2\textsubscript{Low}. 

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JMP v13.1 (SAS Institute, Cary NC, USA) and a \( P \) value < 0.05 was considered statistically significant.

**Results**

From an initial cohort of 277 resected PDACs, 50 were excluded due to hENT1 assay failure, missing clinico-pathological data, or treatment with 5-fluorouracil, which yielded a final analyzable cohort of 227 (Figure 1 and supplementary material, Table S1).

The median H-Score for the SP120 and 10D7G2 antibodies was 20 and 90, respectively, and these values were used to binarize the cohorts into hENT1High and hENT1Low groups. The predictive analysis for these two antibodies suggests that both are useful in predicting sensitivity to adjuvant gemcitabine (Figure 2). Adjuvant chemotherapy had a significant improvement on DSS in both the SP120High and 10D7G2High groups with 0.83 (\( p = 0.03 \)) and 1.29 (\( p = 0.01 \)) year median survival improvements over post-surgical observation, respectively. However, adjuvant chemotherapy had minimal impact on the SP120Low and 10D7G2Low groups where the survival differences were 0.35 (\( p = 0.14 \)) and 0.41 (\( p = 0.16 \)) years, respectively.

The hierarchical clustering procedure was performed on the H-Score data and yielded a clustering pattern that did not satisfy our *a priori* rule that no cluster could have fewer than 30 members (supplementary material, Figure S1). Consequently, we chose to perform the hierarchical clustering procedure on the percentage of positive cells, which yielded three clusters with \( Ns \) of 70, 91, and 66 patients (Figure 3). Representative images of the

![Figure 4. Representative images of hENT1 immunohistochemistry for the SP120 and 10D7G2 antibodies from each cluster derived from the hierarchical clustering procedure using percent positive cells from each case.](image_url)
staining observed for both antibodies in the three clusters are shown in Figure 4. The three clusters are generally defined as: Cluster 1 (SP120Low_10D7G2High), Cluster 2 (SP120High_10D7G2High), and Cluster 3 (SP120Low_10D7G2Low). Figure 5 depicts an analysis to demonstrate the rigour of the classification of the resultant scores and indicates that the 10D7G2High tumours that exist in Clusters 1 and 2 are not statistically different in terms of percentage of positive cells \((p = 0.62)\) and, similarly, the SP120Low tumours that are found in Clusters 1 and 3 are also statistically indifferent in terms of percentage of positive cells \((p = 0.40)\). An assessment for heterogeneity of clinico-pathological variables across the three clusters revealed that the clustering procedure did not significantly bias the composition of cases in the cohort (Table 2).

Univariable DSS analyses to ascertain the predictive effect of hENT1 expression between patients treated with adjuvant gemcitabine and those subjected to post-surgical observation only demonstrated that, for Cluster 1 (SP120Low_10D7G2High), adjuvant gemcitabine yielded a statistically insignificant benefit with a difference in median survival of 0.81 years \((p = 0.06)\). Cluster 2 (SP120High_10D7G2High) demonstrated the largest survival difference for patients who received adjuvant gemcitabine with a median survival difference of 1.46 years \((p = 0.01)\). In Cluster 3 (SP120Low_10D7G2Low), patients who received adjuvant gemcitabine had a 0.17 year median survival benefit \((p = 0.76;\) Figure 6).

Multivariable DSS analysis on all three clusters to ascertain tumoural sensitivity to adjuvant gemcitabine showed that the borderline significance found in Cluster 1 in the univariable approach became statistically significant in the context of the addition of regional lymph node status and lymphovascular invasion with a risk ratio of 0.52 and 95% confidence interval of 0.28–0.94. In Cluster 2, gemcitabine

Table 2. Assessment of heterogeneity for clinico-pathological variables across the groups derived from the hierarchical clustering procedure

| Variable              | Level | Cluster 1 \(N = 70\) | Cluster 2 \(N = 91\) | Cluster 3 \(N = 66\) | \(P\) value |
|-----------------------|-------|-----------------------|-----------------------|-----------------------|-------------|
| Age                   | Mean (95% CI) | 67.9 (65.5–70.2) | 66.2 (64.2–68.3) | 64.5 (62.1–66.9) | 0.13        |
| Sex                   | Male  | 39 (55.7%)           | 46 (50.6%)           | 40 (60.6%)           | 0.45        |
|                       | Female | 31 (44.3%)           | 45 (49.4%)           | 26 (39.4%)           |             |
| Grade                 | 1     | 0 (0.0%)             | 1 (1.1%)             | 0 (0.0%)             | 0.07*       |
|                       | 2     | 49 (70.0%)           | 72 (79.1%)           | 42 (63.6%)           |             |
|                       | 3     | 21 (30.0%)           | 18 (19.8%)           | 24 (36.4%)           |             |
| Lymphovascular invasion | Present | 35 (50.0%)           | 53 (58.2%)           | 40 (60.6%)           | 0.41        |
|                       | Absent | 35 (50.0%)           | 38 (41.8%)           | 26 (39.4%)           |             |
| Perineural invasion   | Present | 64 (91.4%)           | 79 (86.8%)           | 64 (97.0%)           | 0.06        |
|                       | Absent | 6 (8.6%)             | 12 (13.2%)           | 2 (3.0%)             |             |
| pN-stage              | N0    | 19 (27.1%)           | 28 (30.8%)           | 14 (21.2%)           | 0.40        |
|                       | N1    | 51 (72.9%)           | 63 (69.2%)           | 52 (78.8%)           |             |
|                       | N2    | 51 (72.9%)           | 69 (75.6%)           | 53 (80.3%)           |             |
| Adjuvant gemcitabine  | Yes   | 19 (27.1%)           | 22 (24.2%)           | 13 (19.7%)           | 0.59        |
|                       | No    | 51 (72.9%)           | 69 (75.6%)           | 53 (80.3%)           |             |
| Resection status      | R0    | 51 (72.9%)           | 72 (79.1%)           | 50 (75.8%)           | 0.65        |
|                       | R1    | 19 (27.1%)           | 19 (20.9%)           | 16 (24.2%)           |             |

*In the comparison of histological grade, a single grade 1 case was excluded.
The inter-observer validation study demonstrated a high level of cluster reproducibility between the assessors with 79, 60, and 74% agreement between raters for Clusters 1, 2, and 3, respectively. Most importantly, the DSS differences derived for the second pathologist were very similar to the first pathologist with adjuvant gemcitabine conferring a 0.42 year survival advantage for Cluster 1 ($p = 0.06$), a 1.99 year survival advantage for Cluster 2 ($p = 0.004$), and a 0.25 year survival advantage for Cluster 3 ($p = 0.50$).

**Discussion**

The use of gemcitabine-based therapies has been adopted as the standard of care for resected PDAC since the first Phase III trials indicated superior efficacy over post-surgical observation [1,2]. Both of these trials indicated a modest median survival benefit of 0.47 and 0.54, respectively. The lack of a clinically validated predictive biomarker for gemcitabine has led to the utilization of a general approach to the application of chemotherapy rather than an approach based on precision oncology which, unfortunately, has not yielded improved outcomes for the general PDAC population [16]. As new non-gemcitabine regimens are being approved for PDAC, the risk of inappropriate treatment is increasing and predictive biomarkers are desperately needed to address this emerging issue. Drug permeability is one of the key factors to consider when differential response is observed in the clinic and since gemcitabine is in a larger class of compounds known as nucleosides, it follows that hENT1 was identified as a potential predictive biomarker as early as 1999 [17].

The previous studies of hENT1 in PDAC have chosen to examine the prognostic effect of hENT1 expression in cases that were subjected to post-surgical observation or other treatment regimens including gemcitabine. While this approach does have the ability to detect large effects that may indicate the predictive potential of a biomarker, this approach discounts the possibility that hENT1 expression, in the absence of treatment, may have an effect on prognosis. We performed an exploratory analysis of the patients subjected to post-surgical observation in our cohort and confirmed that both the 10D7G2 and SP120 antibodies had a borderline significance on prognosis with low expressers showing improved DSS compared to those cases with higher expression levels (Figure 7). Consequently, we believe that our approach to the analysis of hENT1

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**Figure 6.** Disease-specific survival for the three clusters derived from the hierarchical clustering procedure. The median survival differences were 0.81, 1.46, and 0.20 years for Clusters 1–3, respectively.
as a predictive biomarker is justified and is supported by the literature [15].

In this study, we have demonstrated differential expression between the 10D7G2 and SP120 antibodies with the former showing much higher rates of expression which replicates the results found by Svrcek et al [12]. The amino acid residue sequence for the 10D7G2 antibody is known to correspond to the intracellular loop between transmembrane segments 6 and 7 [18]. However, the sequence for the SP120 antibody is proprietary, and we are therefore unable to explain this differential expression. However, it can be seen from Figure 4 that the 10D7G2 antibody has a more diffuse staining pattern than is found for SP120 and it is possible that the SP120 antibody more accurately identifies the membranous localization for hENT1 where it would have increased activity in nucleoside transport.

Nevertheless, this study illustrates that hENT1 expression, as quantified by either the 10D7G2 or SP120 antibodies, is a predictive biomarker for gemcitabine sensitivity in resected PDAC and that use of both antibodies yields three groups of patients that demonstrate a gradient of substantial to intermediate to no sensitivity to adjuvant gemcitabine. Previous studies have suggested that patients whose tumours have high hENT1 expression with the 10D7G2 antibody have a greater sensitivity to gemcitabine compared to SP120. However, as shown in Cluster 1, when the SP120 antibody is used in combination with 10D7G2, over 40% of cases that were classified as hENT1High with the 10D7G2 antibody have only a slight sensitivity to adjuvant gemcitabine which failed to reach statistical significance on univariable analysis. However, in the context of other prognostic variables: (pN-Stage and lymphovascular invasion), the use of adjuvant chemotherapy achieved statistical significance in multivariable analysis. In contrast, when both antibodies are expressed at a high level, the sensitivity to adjuvant gemcitabine is readily apparent and is enhanced in multivariable DSS analysis (Table 3). This finding suggests that the SP120 antibody is a more specific predictive biomarker for gemcitabine sensitivity in resected PDAC. Of note, cases that had the lowest expression levels for both antibodies had essentially no sensitivity to adjuvant

### Table 3. Univariable and multivariable disease-specific survival analysis for each cluster derived from the hierarchical clustering procedure

| Variable                  | Comparisons       | Univariable RR (95% CI) | Univariable P value | Multivariable RR (95% CI) | Multivariable P value |
|---------------------------|-------------------|-------------------------|---------------------|---------------------------|-----------------------|
| **Cluster 1 (N = 70)**    |                   |                         |                     |                           |                       |
| Adjuvant chemotherapy     | Gem versus Obs    | 0.56 (0.29–1.00)        | 0.05                | 0.52 (0.28–0.94)          | 0.03                  |
| Sex                       | Male versus Female| 1.85 (1.05–3.36)        | 0.03                |                           |                       |
| Age (over entire range)   | Mean (95% CI)     | 2.10 (0.59–7.31)        | 0.25                |                           |                       |
| Histological grade        | 2 versus 3        | 0.56 (0.32–1.02)        | 0.06                |                           |                       |
| pN-stage                  | N0 versus N1      | 0.39 (0.20–0.74)        | 0.003               | 0.46 (0.22–0.90)          | 0.02                  |
| LVI                        | Absent versus Present | 0.34 (0.10–0.91)   | 0.03                |                           |                       |
| Resection status          | R1 versus R0      | 0.46 (0.26–0.82)        | 0.008               | 0.55 (0.30–0.98)          | 0.04                  |
| **Cluster 2 (N = 91)**    |                   |                         |                     |                           |                       |
| Adjuvant chemotherapy     | Gem versus Obs    | 0.49 (0.27–0.83)        | 0.01                | 0.36 (0.19–0.63)          | 0.0002                |
| Sex                       | Male versus Female| 1.08 (0.69–1.70)        | 0.72                |                           |                       |
| Age (over entire range)   | Mean (95% CI)     | 0.92 (0.29–3.05)        | 0.89                |                           |                       |
| Histological grade        | 1 versus 2        | 0.67 (0.04–3.06)        | 0.18                | 0.68 (0.04–3.30)          | 0.03                  |
|                            | 1 versus 3        | 0.38 (0.02–1.91)        |                     | 0.28 (0.01–1.55)          |                       |
|                            | 2 versus 3        | 0.57 (0.03–1.06)        |                     | 0.41 (0.23–0.79)          |                       |
| pN-stage                  | N0 versus N1      | 0.57 (0.34–0.91)        | 0.02                | 0.46 (0.27–0.77)          | 0.003                 |
| LVI                        | Absent versus Present | 0.42 (0.20–0.81)   | 0.008               | 0.39 (0.18–0.76)          | 0.004                 |
| Resection status          | R1 versus R0      | 0.52 (0.32–0.82)        | 0.005               |                           |                       |
| **Cluster 3 (N = 66)**    |                   |                         |                     |                           |                       |
| Adjuvant chemotherapy     | Gem versus Obs    | 0.90 (0.42–1.74)        | 0.76                |                           |                       |
| Sex                       | Male versus Female| 1.30 (0.74–2.36)        | 0.37                |                           |                       |
| Age (over entire range)   | Mean (95% CI)     | 1.56 (0.34–7.62)        | 0.57                |                           |                       |
| Histological grade        | 2 versus 3        | 0.85 (0.48–1.55)        | 0.59                |                           |                       |
| pN-stage                  | N0 versus N1      | 0.68 (0.31–1.35)        | 0.28                |                           |                       |
| LVI                        | Absent versus Present | 1.17 (0.19–3.83)   | 0.83                |                           |                       |
| Resection status          | R1 versus R0      | 1.05 (0.59–1.84)        | 0.86                |                           |                       |

Missing multivariable statistics reflect lack of criteria for variable entry into the model-building procedure. 
RR, relative risk; LVI, lymphovascular invasion; PNI, perineural invasion.
gemcitabine suggesting that this immunophenotype should be considered for other non-gemcitabine options. While the results of this study are fairly clear for the SP120\textsubscript{High}×10D7G2\textsubscript{High} and SP120\textsubscript{Low}×10D7G2\textsubscript{Low} groups, there is some ambiguity when it comes to the SP120\textsubscript{Low}×10D7G2\textsubscript{High} group. It could be argued that there is a benefit to treating this group with adjuvant gemcitabine. However, given the relatively small sample size of this study, it is not possible to provide any treatment guidance for this immunophenotype other than these patients may receive some benefit from a gemcitabine-based regimen. We are of the opinion that the SP120\textsubscript{Low}×10D7G2\textsubscript{High} group may represent a scenario where patient performance status and shared decision making could affect the treatment decision for these patients. While the approach of using two antibodies for the same protein may seem redundant, in this instance the use of only SP120 may lead to an under-treatment scenario as it would lump those with an intermediate sensitivity to gemcitabine into those with a minimal response. Conversely, using only 10D7G2 may lead to an overtreatment scenario which may serve to preclude patients from non-gemcitabine-based therapies which may harbour a greater benefit compared to gemcitabine-based regimens.

This study has several limitations that include a non-randomized and unbalanced treatment allocation with a temporal bias towards more recent cases receiving adjuvant gemcitabine. These deficiencies are common to most retrospective studies that span a time period of treatment adoption. However, adjuvant gemcitabine was not differentially applied based on any clinico-pathological variables with the exception of age at diagnosis, where the patients who received adjuvant gemcitabine were on average 3.3 years younger than those subjected to post-surgical observation ($p = 0.03$). We also concede that, due to the relatively small number of cases that received adjuvant gemcitabine, this may have led to a potential Type II error that we observed in the SP120\textsubscript{Low}×10D7G2\textsubscript{High} group. Consequently, the findings of this study need to be externally validated in cohorts derived from randomized controlled trials.

However, from a methodological standpoint, this is the first study to demonstrate that both antibodies can be successfully optimized and run on the Ventana platform, which demonstrates external validity and, using two independent pathologists who were blinded to treatment and outcome, we have also demonstrated the inter-observer reproducibility of our study. By abandoning the H-Score methodology, and focusing on the percentage of cells staining positive for hENT1, we have removed a large portion of the subjectivity from the assessment of this antibody, which should lead to improved reproducibility across readers. This approach is analogous to the widely accepted scoring method of Ki67 in breast cancer [19]. From a statistical analysis perspective, we have adhered to the recommended guidelines for the determination of a predictive biomarker [15], which is also unique to this study compared to the existing body of literature on hENT1 immunohistochemistry in PDAC. Unlike the previous studies in the literature that have focused on how high levels of hENT1 expression are a marker of improved prognosis in cases that have received adjuvant gemcitabine, the study of this cohort demonstrated how both

![Figure 7](image_url)

Figure 7. To determine if the expression of hENT1 with either antibody had a prognostic effect, recursive partitioning was used to determine the maximal difference in disease-specific survival in cases that were subjected to post-surgical observation only. The resultant survival analyses based on the cut-point derived from the recursive partitioning procedure indicates that cases with higher levels of hENT1 expression tend to have inferior survival.
antibodies independently predict sensitivity to gemcitabine in the adjuvant setting and can be used in combination to provide more detailed information on which patients are likely to experience a good, intermediate, and poor response to adjuvant gemcitabine. This new methodological platform offers the opportunity for other groups to reexamine their previously studied cohorts derived from clinical trials towards building a consensus on the predictive ability of hENT1 in resected PDAC. We feel that once a uniform approach to the analyses is in place, the appropriate systematic review can be conducted to generate the evidence required for clinical adoption. Given the emergence of the non-gemcitabine based option, FOLFIRINOX, in the metastatic setting [20], and currently under investigation in the adjuvant setting [21], further development of predictive biomarkers for gemcitabine sensitivity is of immediate clinical relevance.

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Author contributions statement

SEK, MR, JMK, DJR, DFS: conceived the study and composed the manuscript; BTC, DG: served as the two independent pathologists who assessed hENT1 expression for both antibodies; RDP, SS, HW: abstracted the treatment and outcome data from the BC Cancer Agency archives; CC: performed all immunohistochemistry optimization and staining procedures; JRM: provided the 10D7G2 antibody and advised on its staining and interpretation. All authors have read, reviewed, and approve of this manuscript in its present form.

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**SUPPLEMENTARY MATERIAL ONLINE**

**Figure S1.** The hierarchical clustering procedure using H-Score instead of percent positive cells. The colour of each case corresponds to the cluster derived using the percent positive cells found in Figure 2 and clearly demonstrates imprecision between clusters 1 & 2. In addition, the first cluster identified using the H-Score would have violated our *a priori* rule of a cluster having no less than 30 members.

**Table S1.** Raw data file