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**Title: Expression of dihydropyrimidine dehydrogenase (DPD) and hENT1 predicts survival in pancreatic cancer.**

**Running title:** DPD and hENT1 predict survival in pancreatic cancer.

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**ABSTRACT**

**Background:** Tumour dihydropyrimidine dehydrogenase (DPD) may provide added value to human equilibrative nucleoside transporter-1 (hENT1) in predicting survival following pyrimidine based adjuvant chemotherapy.

**Methods:** DPD and hENT1 immunohistochemistry scoring was undertaken on tumour cores from 238 patients with pancreatic cancer in the ESPAC-3(v2) trial, randomised to postoperative gemcitabine or 5-fluorouracil/folinic acid (5FU/FA).

**Results:** DPD tumour expression was associated with reduced overall survival (hazard ratio, HR=1.73 [95% confidence interval, CI = 1.21-2.49], p=0.003). This was significant in the 5FU/FA arm (HR =2.07 [95% CI=1.22-3.53], p=0.007), but not in the gemcitabine arm (HR=1.47 [0.91-3.37], p=0.119). High hENT1 tumour expression was associated with increased survival in gemcitabine (HR=0.56 [0.38-0.82], p=0.003) but not in 5FU/FA treated patients (HR=1.19 [0.80-1.78], p=0.390). Patients with low hENT1 and high DPD expression had a reduced median [95% CI] survival with 5FU/FA (9.7 [5.3-30.4] versus 29.2 [19.5-41.9] months, p=0.002) but not with gemcitabine (14.0 [9.1-15.7] versus 18.0 [7.6-15.3] months, p=1.000). Interaction of treatment and DPD expression was not significant (*p*=0.303), but interaction of treatment and hENT1 expression was (*p*=0.009).

**Conclusion:** DPD tumour expression was a negative prognostic biomarker. Together with tumour expression of hENT1, DPD tumour expression defined patient subgroups that might benefit from either postoperative 5FU/FA or gemcitabine.

**Key words:** Dihydropyrimidine dehydrogenase (DPD), human equilibrative nucleoside transporter 1 (hENT1), pancreatic cancer, 5-fluorouracil, gemcitabine, randomized trial, adjuvant, predictive, prognostic.

**BACKGROUND**

Pancreatic ductal adenocarcinoma is one of the leading causes of cancer-related death worldwide and will shortly overtake breast cancer as the second leading cause of cancer death in the USA, with limited survival following primary treatment (Siegel *et al*, 2016, Kleeff *et al*, 2016, Rahib *et al*, 2014). Following multicentre studies by the European Study Group for Pancreatic Cancer (ESPAC) and others, it is now clear that adjuvant chemotherapy with either 5-fluorouracil with folinic acid (5FU/FA), gemcitabine monotherapy, or gemcitabine plus capecitabine (a 5FU prodrug) for six months following pancreatic resection increases long term survival (Neoptolemos *et al*, 2001, Neoptolemos *et al*, 2004, Neoptolemos *et al*. 2009, Neoptolemos *et al*, 2010, Oettle *et al*, 2013, Valle *et al*, 2014, Neoptolemos *et al*, 2017). Adjuvant S-1, an orally active drug containing tegafur (another 5FU prodrug), has also improved survival in patients from Japan (Uesaka *et al*, 2016).

Although both 5FU/FA and gemcitabine are efficient at the cohort level, specific individuals may benefit more from either gemcitabine or 5FU/FA. There are currently no established tools to select the optimal treatment for the individual patient with pancreatic cancer. The cellular response to pyrimidine-based chemotherapy is dependent on a series of proteins involved in the trans-membrane uptake and metabolism (Costello *et al*, 2012, Young *et al*, 2008). Our laboratory has previously reported that high protein expression of human equilibrative nucleoside transporter 1 (hENT1) was associated with improved overall survival of patients in the gemcitabine arm of the ESPAC-3(v2) trial, but not in the 5FU/FA arm (Greenhalf *et al*, 2014). These results indicated that other markers should be sought to help predict 5FU activity.

Dihydropyrimidine dehydrogenase (DPD) is an enzyme encoded by the gene *DPYD* located on chromosome *1p22* (Yokota *et al*, 1994), which catabolizes 5FU into dihydrofluorouracil (Longley *et al*, 2003). Metabolites of 5FU interfere with cell function by inhibition of DNA synthesis and repair, RNA transcription and DNA methylation (Longley *et al*, 2003). The main mechanism of 5FU activation is conversion to fluorodeoxyuridine via thymidylate phosphorylase and then conversion to fluorodeoxyuridine monophosphate (FdUMP) by thymidine kinase. FdUMP inhibits thymidylate synthase, which is important for the folate-homocysteine cycle and purine and pyrimidine synthesis. Other key metabolites are fluorouridine triphosphate and fluorodeoxyuridine triphosphate, which are incorporated into RNA and DNA respectively. The rate-limiting step of 5FU catabolism is the conversion of 5FU to dihydrofluorouracil by DPD, which is then converted to fluoro-β-ureidopropionate and subsequently to fluoro-β-alanine (Longley *et al*, 2003). Thus we could hypothesize that low intra-tumoural DPD expression would favor the production of cytotoxic 5FU metabolites and prolong survival. This hypothesis has received some support from small retrospective studies predominantly involving the composite drug S-1 (Kondo *et al*, 2011, Kondo *et al*, 2012, Saif *et al*, 2009, Miyake *et al*, 2007, Nakahara *et al*, 2010, Nakamura *et al*, 2011, Shimoda *et al*, 2015). Gimeracil, a component of S-1, is an inhibitor of DPD that maintains a high concentration of 5FU in blood and tumour tissue (Uesaka *et al*, 2016).

In the present study, the expression of intra-tumoural DPD was analysed in tissue from patients in the ESPAC-3(v2) trial that had been randomised to six months of gemcitabine or 5FU/FA following pancreatic resection. Our primary objective was to test the hypothesis that DPD expression status was a specific marker for 5FU based chemotherapy. Secondary exploratory objectives tested whether DPD expression could add to the predictive value of hENT1 expression in selecting patients for either gemcitabine or 5FU adjuvant therapy (Greenhalf *et al*, 2014).

**MATERIALS AND METHODS**

**Study Design**

The translational ESPAC-T studies received ethics committee approval for the characterization of tumour markers for chemotherapy from the Liverpool (Adult) Research Ethics Committee (07/H1005/87). Good Clinical Practice Standard Operating Procedures (SOPs) were employed to minimize study biases with a full audit trail. The ESPAC-3 trial randomised 551 patients to 5FU/FA and 537 to gemcitabine (Neoptolemos *et al*, 2010, Neoptolemos *et al*, 2014)]. This was originally analysed on an intention-to-treat basis but, for the ESPAC-T study, patients in the treatment arms were selected for inclusion only if treatment was actually received. All patients provided written informed consent. This study was conducted and reported in accordance with the REMARK criteria (McShane *et al*, 2005, Simon *et al*, 2009).

**Tissue Microarray (TMA) Manufacture**

Tissue arrays were manufactured using SOP’s as previously reported (Greenhalf *et al*, 2014). The arrays contained tumour cores from patients included in the ESPAC-3(v2) trial and randomised to 5FU/FA or gemcitabine, or from patients from the ESPAC-1/ESPAC-3(v1) trial randomised to observation only. Cores were taken from tumour regions identified by an experienced pancreatic pathologist (FC) using haematoxylin and eosin (H&E) stained sections. Tissue microarrays were prepared with two cores from each block, with four to eight cores arrayed for each patient. Each of the TMA’s had two cores from each of 88 patients. For all arrays, control cores, comprising three cores each of colon, kidney, liver, normal pancreas, and chronic pancreatitis, were arranged in a fence around the test samples. Each core on each TMA was coded and linked separately to trial identifiers.

**Immunohistochemistry**

TMA blocks were cut in 3 µm sections and placed on Superfrost Ultra Plus® slides (Thermo Fisher Scientific Inc., Waltham, MA, USA). Deparaffinisation and antigen retrieval were performed with the PT-Link® system and pH 9.0 target retrieval buffer (Dako, Glostrup, Denmark). All buffers and reagents were provided in the EnVisionTM kit (Dako): Slides were washed in tris-buffered saline with 0.05% Tween-20 (TBS-T) before peroxidase blocking for 10 minutes. Following TBS-T washes samples were incubated with rabbit-anti-DPD diluted 1:2000, for 60 minutes, followed by incubation with secondary horseradish peroxidase conjugated antibody for 60 minutes. Following repeated TBS-T washes slides were covered in fresh diaminosobenzidine (DAB) working solution for 10 minutes in room temperature. Slides were washed in TBS-T and distilled water and counterstained in Haematoxylin Gills III and dehydrated via a series of ethanol gradients and xylene before being mounted under cover slips.

**Validation and quality assessment of the primary anti-DPD antibody**

The primary antibody (rabbit-anti-DPD, Abcam *Ab 134922*, Abcam, Cambridge, UK) was validated in accordance with ESPAC-T steering committee policy. Western blot and immunocytochemistry on lysates and paraffin embedded naïve as well as anti-DPD siRNA treated cell lines confirmed that the antibody was specific and sensitive for the presence or absence of human DPD (Supplementary Figures 1-3, Online Data). Positive-staining tissue cores (healthy liver) and negative-staining tissue cores (healthy colon) were used as internal controls. Negative control slides underwent identical staining procedures but with the primary antibody replaced by antibody dilution buffer only.

**Scoring**

The DPD expression in tumour cell compartments of all samples were scored by one experienced pancreas pathologist (FC) and one trained assistant (EG) according to a 0-3 point system (0=no staining, 1=weak, 2=moderate, 3=strong staining, with representative images viewed in Supplementary Figures 4A-D). FC and EG were both blinded to patient ID and clinical data. In general the intra-core variability was low, but if staining intensity within the core was not fully consistent, the most commonly observed pattern was scored. This means that if a core contained only one or two cells that were immunopositive, but the predominant pattern was negative (‘0’), then the core in total was scored ‘0’. Any disagreement in scoring of the immunohistochemistry was resolved through discussion and a consensus decision. Each patient was given a single scoring grade equal to the mean of cores, rounded to the nearest integer. Since a score = 3 was found in only three patients in the entire cohort, scores 2-3 were grouped into the high DPD expressing group, and dichotomous comparisons were consequently performed with the low DPD expressing group (scores = 0-1). The previously collected hENT1 scores for the tumours (Greenhalf *et al*, 2014) were added to the data set to investigate a possible relationship with the DPD scores. The DPD and hENT1 scores were not correlated (Pearson correlation = -0.01).

**Statistical considerations**

Survival from the date of randomisation was analysed using Kaplan–Meier curves, with differences between groups assessed using the log rank test (Kaplan *et al*, 1958, Peto *et al*, 1972). Univariable and multivariable analyses, using a backwards elimination method, were carried out using *Cox* proportional hazards (Cox *et al*, 1972). A 2-sided significance level of *P*<0.05 was used throughout. If not otherwise stated, 95% confidence intervals (CI) were presented. To adjust for multiple testing in the combined DPD and hENT1 expression subgroups, Bonferroni correction was performed for these analyses. Analyses were carried out using STATA v14 (StataCorp).

**RESULTS**

**Immunohistochemical staining and scoring**

We stained tissue cores from 303 patients: 272 patients randomised and treated in the chemotherapy arms of the ESPAC-3(2) trial (Neoptolemos *et al*, 2010), and 31 patients randomised to observation in the combined ESPAC-1/ESPAC-3(v1) trials (Neoptolemos *et al*, 2001, Neoptolemos *et al*, 2004, Neoptolemos *et al*, 2009, Neoptolemos *et al*, 2010). Cores from 34 patients from the ESPAC-3(v2) chemotherapy arms and eight patients from the observational arms contained insufficient tissue to score, or only severely damaged tissue. Overall cores from 261 (86.14%) patients were scored including 238 chemotherapy-treated patients, 115 (20.9% originally randomised) given 5FU/FA and 123 (20.9% originally randomised) given gemcitabine plus 23 patients randomised to observation. Demographics, shown in Supplementary Table 1, are similar to those previously reported for the whole trial population (Neoptolemos *et al,* 2010, Greenhalf *et al*, 2014). DPD expression tumour scores in relation to clinical and pathological variables are shown in Supplementary Table 2. Representative images of the different scores and their respective frequencies in the entire population are presented in Supplementary Figure 4.

**Cox regression univariate analyses**

Cox proportional hazards univariate analyses of survival by clinico-pathologic risk factors, DPD tumour expression (low expression, score=0-1; high expression, score=2-3) and hENT1 expression (low/high, cut-off defined by the median H-score) by treatment arm and collectively are shown in Table 1. Significant prognostic factors for the entire chemotherapy treated population (both gemcitabine and 5FU/FA) were resection margin status, WHO performance status, lymph node status, tumour stage, tumour invasion into nearby organs, and DPD expression. High DPD expression was associated with reduced survival (hazard ratio [HR] 1.73, 95% CI 1.21-2.49, *p*=0.003). This difference was significant in the 5FU/FA arm (HR 2.07, 95% CI 1.22-3.53, *p*=0.007), but not in the gemcitabine arm (HR 1.47, 95% CI 0.91-2.37, *p*=0.119). Tumour expression of DPD was not significantly associated with any of the other clinical or pathological factors analysed (Supplementary Table 2). Tumour expression of hENT1 was not prognostic for the whole chemotherapy cohort (HR 0.84, 95% CI 0.63-1.12, *p*=0.230) but was predictive for improved survival with gemcitabine (HR 0.56, 95% CI 0.38-0.82, *p*=0.003) but not for 5FU/FA (HR 1.19, 95% CI 0.80-1.78, *p*=0.390).

**Multivariate analyses of prognostic factors in the respective treatment arms**

A multivariate Cox regression model for prognosis showed that treatment arm was not significant (*p*=0.138), whilst DPD expression was (*p*=0.003), and hENT1 expression was not significant (*p*=0.327). The interaction of treatment arm and DPD expression was not significant (*p*=0.303), but the interaction of treatment arm and hENT1 expression was (*p*=0.009).

Furthermore multivariate analysis revealed that DPD expression status, along resection margin status, WHO performance status, and lymph node involvement were independent prognostic factors in the 5FU/FA treated subgroup but not the gemcitabine treated group (Table 2). High DPD expression was significantly associated with survival in the 5-FU/FA treated group (HR 3.30; 95% CI 1.89-5.77; *p*<0.001) but not in the gemcitabine treated group (HR 1.62; 95% CI 0.97-2.69; *p*=0.065).

**Integrating DPD and hENT1 as predictive biomarkers for adjuvant chemotherapy**

In the combined chemotherapy treated group (5FU/FA and gemcitabine), the median (95% CI) overall survival time was 25.6 (21.2-28.6) months in patients with low DPD tumour expression and 14.3 (10.0-21.1) months in those with high DPD expression (χ2LR,1df =10.4, *p*=0.001, Figure 1). This difference remained statistically significant in the 5-FU/FA arm treated subgroup, where median (95% CI) overall survival was 26.4 (21.8-30.1) months with low DPD tumour expression and 10.0 (5.8-22.6) months in those with high DPD expression (χ2LR, 1 df=9.56, *p*=0.002). Overall median (95% CI) survival in gemcitabine treated patients was not significantly different according to DPD status (24.4 (17.1-28.7) months in those with low DPD tumour expression and 15.7 (13.9-23.6) months in those with high DPD expression (χ2LR, 1 df=2.33, *p*=0.127). The small population of patients randomised to observation was separately analysed. Patients with low DPD expression (*n*=20) had an overall median (95% CI) of 17.5 (6.8-34.3) months compared to 4.6 (3.2-31.6) months in those with high DPD *(n*=3) expression (Figure 1). Due to the low numbers in this subset of patients, no *p* values were calculated and further statistical calculations or subdivisions were not performed.

Patients with high and low hENT1 tumour expression were subdivided according to high and low DPD tumour expression (Supplementary Figure 5). As we have previously reported, high hENT1 expression was associated with more favourable survival in gemcitabine-treated patients (Greenhalf *et al*, 2014). We found no evidence for an additional prognostic value of DPD when added to hENT1 status in gemcitabine-treated patients. The median (95% CI) overall survival of patients treated with gemcitabine with high hENT1 intra-tumoural expression and also with low intra-tumoural DPD expression was 26.3 (17.2-33.0) months compared to 22.3 (9.6-39.5) months in those patients instead with high DPD expression, which was not significantly different (*p*=0.360). The median (95% CI) overall survival of patients treated with gemcitabine with low hENT1 intra-tumoural expression and also low DPD intra-tumoural expression was 18.0 (7.6-15.3) months and 14.0 (9.1-15.7) months for patients with low hENT1 and high DPD intra-tumoral expression (*p*=1.000).

Similarly in patients with high hENT1 intra-tumoural expression treated with 5FU/FA, there was no significant difference between those who also had high or low DPD intra-tumoural expression with a median (95% CI) overall survival of 17.3 (0.6-38) and 26.0 (19.8-30.1) months respectively, (*p*=1.000). However, in patients with low hENT1 expression treated with 5FU/FA, intra-tumoural DPD expression added significant predictive value. Thus, patients with low hENT1 and low DPD tumour expression treated with 5FU/FA had a median (95% CI) overall survival of 29.2 (19.5-41.9) months compared to 9.7 (5.3-30.4) months in those with low hENT1 and high DPD tumour expression (χ2LR=9.28, *p*[raw]=0.002, *p*[post Bonferroni correction]=0.014).

**DISCUSSION**

In the present study, intratumoural DPD expression status was analysed in the ESPAC-3(v2) population of patients with pancreatic adenocarcinoma randomised to postoperative chemotherapy with 5FU/FA or gemcitabine. Given the key role of DPD in the catabolism of 5FU, we hypothesised that low intratumoural expression of DPD would result specifically in increased overall survival in patients treated with 5FU/FU. We found that DPD tumour expression was associated with reduced overall survival. Intra-tumoural DPD expression was also significant in the 5FU/FA arm but not in the gemcitabine arm. As previously shown high hENT1 tumour expression was associated with increased survival in patients treated with gemcitabine but not in those treated with 5FU/FA.

Given the previously reported predictive value of hENT1 tumour expression for adjuvant gemcitabine, we also explored the potentially additional value of DPD tumour expression in those high or low hENT1 intra-tumoural expression subgroups. In patients with high hENT1 tumour expression treated with gemcitabine, either low or high DPD expression showed a favourable median overall survival. Similarly, in 5-FU/FA treated patients with high hENT1 tumour expression no significant difference between high or low DPD tumour expression was observed. This suggests that if hENT1 tumour expression is high, evaluation of DPD tumour expression will not add any useful information, and these patients should generally be recommended for gemcitabine given a more tolerable toxicity profile. Another option, in situations where gemcitabine is unsuitable, would be a 5FU/FA bolus regimen other than the Mayo Clinic schedule or infusion regimen.

In patients with low hENT1 tumour expression treated with gemcitabine, survival was poor irrespective of DPD tumour expression. These data confirm that hENT1 tumour expression is a potentially useful predictive biomarker for improved survival with adjuvant gemcitabine. However, for patients with low hENT1 tumour expression treated with 5FU/FA, evaluation of DPD tumour expression provided additional predictive value. Patients with low DPD tumour expression treated with 5FU/FA survived significantly longer than patients with high DPD tumour expression. This suggests that there is a subgroup of patients with low hENT1 tumour expression and with low DPD tumour expression that derive significant survival benefit from adjuvant 5FU/FA. Conversely, the subgroup of patients with hENT1-low tumour expression and with high DPD tumour expression has a poor survival outcome whether treated with 5FU/FA or gemcitabine. We hypothesize that the additional prognostic information from intra-tumoural DPD expression status could be integrated with the hENT1 expression status to guide the selection of adjuvant chemotherapy regimen. We can conclude the following.

1. Patients with high hENT1 tumour expression are likely to derive a survival benefit from gemcitabine therapy irrespective of DPD tumour expression status. Analysis of DPD expression will not add any useful information.
2. In patients with low hENT1 tumour expression status, gemcitabine is less efficacious. For these patients DPD tumour expression may be analysed for additional prognostic information.
   1. Patients with low hENT1 and low DPD tumour expression have a favourable prognosis with 5FU/FA treatment (median overall survival = 29.2 months).
   2. Patients with high hENT1 and high DPD tumour expression have a poor prognosis whether given 5FU/FA or gemcitabine (9.7 and 14 months median overall survival, respectively). In these patients novel agents or combination regimens may be needed to improve survival.

Earlier studies investigating DPD tumour expression in pancreatic cancer were performed in smaller and/or non-controlled patient populations of Asian origin and involved the use of S-1 and/or combination with gemcitabine or radiotherapy (Uesaka *et al*, 2016, Kondo *et al*, 2011, Kondo *et al*, 2012, Saif *et al*, 2009, Miyake *et al*, 2007, Nakahara *et al*, 2010, Nakamura *et al*, 2011, Shimoda *et al*, 2015). Asian individuals handle the metabolism of fluoropyrimidines differently from Europeans in part due to genotypic differences such as in CYP2A6 (which converts tegafur in S-1 to 5FU, Chuah *et al*, 2011). The present study provides novel evidence as it was performed in a randomised controlled setting in patients who were primarily of European origin, and notably receiving single agent regimens.

Planned biomarker analyses of the ESPAC-4 population (Neoptolemos *et al*, 2017) will assess whether hENT1, DPD and/or other tumour expression biomarker candidates are suitable for the identification of patients particularly benefitting from the gemcitabine plus capecitabine combination regimen. It is plausible that patients with low hENT1 and high DPD tumour expression may be resistant to gemcitabine and 5FU/FA individually and to the gemcitabine/capecitabine combination requiring alternative adjuvant strategies. If this is confirmed by biomarker analysis of the ESPAC4 trial biospecimens, prospective trials of therapies acting independently of hENT1 and DPD would be warranted in this population.

In conclusion, intratumoural DPD expression was a negative prognostic biomarker for patients with pancreatic adenocarcinoma undergoing postoperative chemotherapy. Intratumoural hENT1 expression was confirmed to be a predictive marker for gemcitabine treatment, and the additional prognostic value of DPD tumour expression may be used to estimate the survival in patients with low hENT1 tumour expression, where low DPD tumour expression indicates better prognosis at least for patients treated with 5FU/FA. Patients with low hENT1 and high DPD tumour expression present a particular challenge, and novel agents and/or combination regimens will be needed to improve survival for this subgroup.

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**Conflict of Interest**

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**LEGENDS TO TABLES AND FIGURES**

**Tables**

**Table 1.** Cox proportional hazards univariate analyses of survival by clinical and pathological risk factors, DPD tumour expression (low, score=0-1; high, score=2-3), and hENT1 tumour expression (high versus low defined by median H-score).

**Table 2.** Multivariate analyses for survival of clinical and pathological risk factors and DPD tumour expression in 5-fluorouracil plus folinic acid and gemcitabine treated arms.

**Table 3.** Median overall survival durations in subgroups based on combined hENT1 and DPD tumour expression status.

**Figures**

**Figure 1.** Kaplan-Meier survival curves and median overall survival for DPD-low vs. DPD-high tumour expression in the entire chemotherapy treated population (5FU/FA plus gemcitabine), 5FU/FA treated patients, gemcitabine treated patients, and the small observational (OBS) population.

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**TABLES**

**Table 1.** Cox proportional hazards univariate analyses of survival by clinical and pathological risk factors, DPD tumour expression (low, score=0-1; high, score=2-3), and hENT1 tumour expression (high versus low defined by median H-score).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Univariate Analysis | | | | | |
|  |  | **Hazard Ratio (95% Confidence Interval)** | | | |
|  | **Chemotherapy** | | | **Total** |
| Characteristic |  | **5-fluorouracil /folinic acid** | | **Gemcitabine** |  |
|  |
| Resection margin |  | *n*=115 | | *n*=123 | *n*=238 |
| **Negative** | 1 | | 1 | 1 |
| **Positive** | 2.13 (1.41-3.12) | | 1.12 (0.76-1.66) | 1.52 (1.15-2.01) |
|  | Wald χ2= 12.85, ***p*<0.001** | | Wald χ2= 0.34 *p*=0.558 | Wald χ2= 8.75,  ***p*=0.003** |
| WHO |  | *n*=115 | | *n*=123 | *n*=238 |
| **0** | 1 | | 1 | 1 |
| **1** | 1.62 (1.07-2.47) | | 1.46 (0.95-2.24) | 1.54 (1.14-2.08) |
| **2** | 0.97 (0.43-2.21) | | 1.22 (0.63-2.37) | 1.09 (0.64-1.85) |
|  | Wald χ2= 5.79, *p*=0.055 | | Wald χ2= 3.01, *p*=0.222 | Wald χ2= 8.55,  ***p*=0.014** |
| Lymph Node Status |  | *n*=115 | | *n*=123 | *n*=238 |
| **Negative** | 1 | | 1 | 1 |
| **Positive** | 3.15 (1.76-5.62) | | 1.61 (0.95-2.74) | 2.24 (1.50-3.33) |
|  | Wald χ2= 15.03, ***p*<0.001** | | Wald χ2= 3.08, *p*=0.079 | Wald χ2= 15.82,  ***p*<0.001** |
| Tumour Stage |  | *n*=114 | | *n*=122 | *n*=236 |
| **1/2** | 1 | | 1 | 1 |
| **3/4** | 1.92 (1.19-3.11) | | 1.47 (0.97-2.23) | 1.67 (1.22-2.28) |
|  | Wald χ2= 7.16**, *p*=0.008** | | Wald χ2= 3.34, *p*=0.068 | Wald χ2= 10.13,  ***p*=0.002** |
| Tumour Grade |  | *n*=112 | | *n*=120 | *n*=232 |
| **Well** | 1 | | 1 | 1 |
| **Moderate** | 0.58 (0.36-0.94) | | 0.95 (0.42-2.12) | 0.77 (0.47-1.28) |
| **Poor** | 0.75 (0.39-1.43) | | 1.25 (0.53-2.94) | 1.02 (0.58-1.80) |
|  | Wald χ2= 5.17, *p*=0.075 | | Wald χ2= 1.56, *p*=0.460 | Wald χ2= 3.15  *p*=0.207 |
| Local Invasion |  | | *n*=115 | *n*=122 | *n*=237 |
| **No** | | 1 | 1 | 1 |
| **Yes** | | 1.30 (0.86-1.97) | 1.24 (0.85-1.81) | 1.27 (0.96-1.68) |
|  | | Wald χ2= 1.56, *p*=0.211 | Wald χ2= 1.20, *p*=0.273 | Wald χ2= 5.06  ***p*=0.025** |
| Maximum Tumour diameter |  | | *n*=111 | *n*=118 | *n*=229 |
| **<30mm** | | 1 | 1 | 1 |
| **≥30mm** | | 1.28 (0.84-1.95) | 1.36 (0.91-2.03) | 1.33 (1.00-1.77) |
|  | | Wald χ2= 1.36, *p*=0.244 | Wald χ2= 2.25, *p*=0.134 | Wald χ2= 3.78  *p*=0.052 |
| Diabetes mellitus |  | | *n*=112 | *n*=121 | *n*=233 |
| **No** | | 1 | 1 | 1 |
| **Yes** | | 0.96 (0.54-1.69) | 0.90 (0.55-1.49) | 0.92 (0.64-1.33) |
|  | | Wald χ2= 0.02, *p*=0.875 | Wald χ2= 0.20, *p*=0.653 | Wald χ2= 0.18, *p*=0.673 |
| Gender |  | | *n*=115 | *n*=123 | *n*=238 |
| **Male** | | 1 | 1 | 1 |
| **Female** | | 1.19 (0.78-1.81) | 1.20 (0.80-1.81) | 1.19 (0.89-1.60) |
|  | | Wald χ2= 0.66, *p*=0.418 | Wald χ2= 0.76, *p*=0.383 | Wald χ2= 1.40, *p*=0.237 |
| Age, years |  | | *n*=115 | *n*=123 | *n*=238 |
| **≥64** | | 1 | 1 | 1 |
| **<64** | | 1.33 (0.87-2.02) | 0.89 (0.60-1.33) | 1.07 (0.81-1.42) |
|  | | Wald χ2= 1.74**,** *p*=0.188 | Wald χ2= 0.32, *p*=0.570 | Wald χ2= 0.23, *p*=0.634 |
| Smoking |  | | *n*=108 | *n*=113 | *n*=221 |
| **Never smoker** | | 1 | 1 | 1 |
| **Ex-smoker** | | 0.91 (0.57-1.46) | 1.28 (0.82-1.98) | 1.08 (0.79-1.49) |
| **Current smoker** | | 0.92 (0.52-1.62) | 1.48 (0.77-2.85) | 1.13 (0.74-1.73) |
|  | | Wald χ2= 0.17, *p*=0.920 | Wald χ2= 1.94, *p*=0.380 | Wald χ2= 0.42, *p*=0.810 |
| DPD expression |  | | *n*=115 | *n*=123 | *n*=238 |
| Low | | 1 | 1 | 1 |
| High | | 2.07 (1.22-3.53) | 1.47 (0.91-2.37) | 1.73 (1.21-2.49) |
|  | | Wald χ2= 7.22, ***p*=0.007** | Wald χ2= 2.43**,** *p*=0.119 | Wald χ2= 8.86,  ***p*=0.003** |
| hENT1 expression |  | | *n*=113 | *n*=118 | *n*=231 |
| Low | | 1 | 1 | 1 |
| High | | 1.19 (0.80-1.78) | 0.56 (0.38-0.82) | 0.84 (0.63-1.12) |
|  | | Wald χ2= 0.74, *p*=0.390 | Wald χ2= 8.98, ***p*=0.003** | Wald χ2= 1.44,  *p*=0.230 |

**Table 2.** Multivariate analyses for survival of clinical and pathological risk factors and DPD tumour expression in 5-fluorouracil with folinic acid and gemcitabine treated arms.

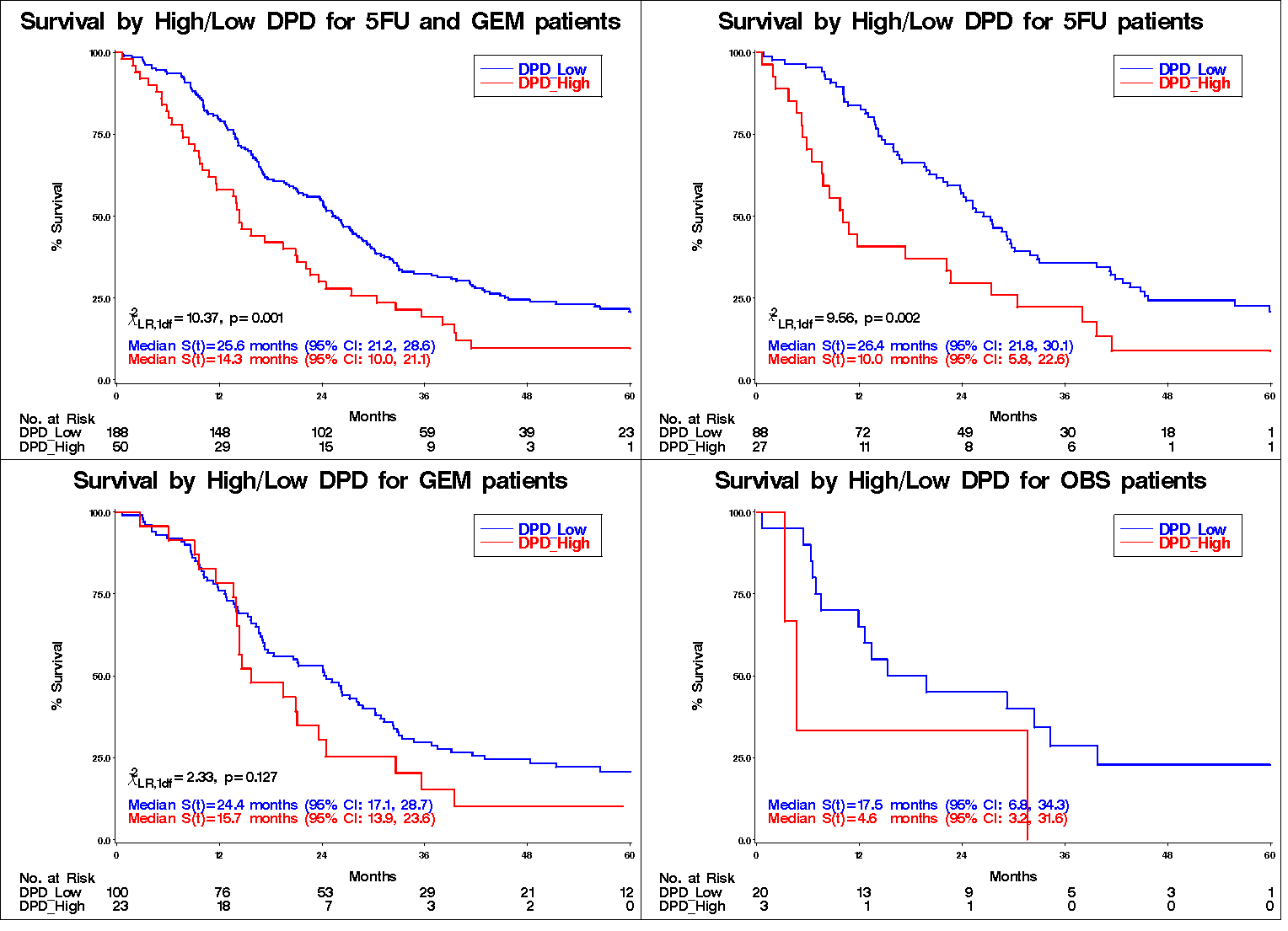
|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Multivariate Analysis | | | | | | | | |
| Variable |  | **5-fluorouracil /folinic acid**  **(*n*=115)** | | | **Gemcitabine**  **(*n*=123)** | | | |
|  | **HR (95% CI)** | **Wald χ2** | ***p*** | **HR (95% CI)** | **Wald χ2** | ***p*** | |
| Country |  |  | 6.55 | **0.050** | **Not included** |  |  | |
| Resection Margin | **Negative** | 1 | 7.75 | **0.005** | 1 | 0.30 | 0.585 | |
|  | **Positive** | 1.95  (1.22-3.11) | 1.12  (0.75-1.67) |
| WHO | **0** | 1 | 8.47 | **0.013** | 1 | 3.38 | 0.184 | |
| **1** | 2.15  (1.28-3.60) | 1.47  (0.95-2.27) |
| **2** | 1.72  (0.76-3.89) | 1.06  (0.53-2.13) |
| Lymph Node Status | **Negative** | 1 | 8.94 | **0.003** | 1 | 3.76 | 0.053 | |
| **Positive** | 2.88  (1.44-5.77) | 1.71  (0.99-2.95) |
| DPD expression | **0/1** | 1 | 17.71 | **<0.001** | 1 | 3.41 | | 0.065 |
|  | **2/3** | 3.30  (1.89-5.77) | 1.62  (0.97-2.69) |

**Table 3.** Median overall survival durations in subgroups based on combined hENT1 and DPD tumour expression status.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Treatment Arm | Subgroup | | Number of patients | Median overall survival  (95% CI)  months | | *P* value (raw) | | *P* value post Bonferroni correction |
| 5-fluorouracil with folinic acid | | **hENT1-high**  **DPD-high** | 9 | | 17.3  (0.6-38.0) | | 0.81 | 1.000 |
| **hENT1-high**  **DPD-low** | 39 | | 26.0  (19.8-30.1) | |
| **hENT1-low**  **DPD-high** | 17 | | 9.7  (5.3-30.4) | | **0.002** | **0.014** |
| **hENT1-low**  **DPD-low** | 47 | | 29.2  (19.5-41.9) | |
| Gemcitabine | | **hENT1-high**  **DPD-high** | 12 | | 22.3  (9.6-39.5) | | 0.060 | 0.360 |
| **hENT1-high**  **DPD-low** | 57 | | 26.3  (17.2-33.0) | |
| **hENT1-low**  **DPD-high** | 11 | | 14.0  (9.1-15.7) | | 0.730 | 1.000 |
| **hENT1-low**  **DPD-low** | 38 | | 18.0  (7.6-15.3) | |

**FIGURES**

**Figure 1.** Kaplan-Meier survival curves and median overall survival for DPD-low vs. DPD-high tumour expression in the entire chemotherapy treated population (5FU/FA plus gemcitabine), 5FU/FA treated patients, gemcitabine treated patients, and the small observational (OBS) population.

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**SUPPLEMENTARY ONLINE DATA**

**SUPPLEMENTARY MATERIALS AND METHODS**

**Summary: Validation and quality assessment of the primary anti-DPD antibody**

The primary antibody (rabbit-anti-DPD, Abcam *Ab 134922*, Abcam, Cambridge, UK) was validated in accordance with ESPAC-T steering committee policy. The quality assessment included: (1) the detection of a band of presumed size on Western blot with lysates from five different pancreatic cancer cell lines (Supplementary Figure 1); (2) siRNA-mediated knockdown of the DPD and the attenuation/disappearance of this band on Western blot (Supplementary Figure 2); and (3) the clear attenuation/disappearance of the staining identified in formalin fixed and paraffin embedded cell pellets of the respective knockdown cell lines (Supplementary Figure 3).

## Western Blot Analysis

Pancreatic cell lines (BxPC-3, CFPAC, MIA PaCa-2, PANC-1 and SUIT-2) were cultured at 37°C with 5.0% CO2 and harvested when 80-90% confluent. Cell pellets were lysed in RIPA lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Igepal CA-630, 0.5% deoxycholate, 0.1% sodium dodechylsulphate [SDS]) and separated by SDS-PAGE according to molecular weight. Samples were loaded at equal concentrations of 20 µg protein/lane. Following transfer of proteins onto a polyvinylidene (PVDF) membrane non-specific proteins were blocked by incubation of the membrane in 5% non-fat milk (Biorad Labs., Hertfordshire, UK) at 4oC overnight before being probed with a rabbit monoclonal anti-DPD antibody (Abcam *ab134922*) at a dilution of 1:1000 for 1 hour at room temperature. Membranes were then washed repeatedly in T-PBS (phosphate-buffered saline with 0.1% Tween-20) for 1 hour followed by incubation with a secondary HRP-conjugated anti-rabbit antibody (Dako) at a dilution of 1:1000 for 1 hour. Subsequent washing in T-PBS was done and the membrane then prepared for chemiluminescence analysis. Membranes were then stripped and reprobed for β-actin to ensure equal protein loading of samples.

Supplementary Figure 1A shows hybridisation of the Abcam antibody to proteins in five different cell lysates. DPD is reported to have a molecular weight of 110 kDa which is consistent with the single band here detected.

**siRNA mediated knock down of DPD in SUIT-2 pancreatic cancer cells**

DPD siRNA knockdown was investigated using SUIT-2 cells and a commercially available pool of siRNA strands targeting DPD mRNA (Dharmacon, GE Healthcare Ltd., Little Chalfont, United Kingdom). SUIT-2 cells were transfected with Lipofectamine 2000® (Life Technologies Ltd., Paisley, United Kingdom) and the respective siRNA pool to a final siRNA concentration of 20 nM. Three different conditions were used as negative control transfections: ‘off-target’ siRNA pool transfected cells [OT], RISC-free siRNA transfected cells [RF], and ‘wild type’ [WT] cells where the siRNA was omitted, respectively. The standard OT and RF siRNA pools were designed and provided by Dharmacon.

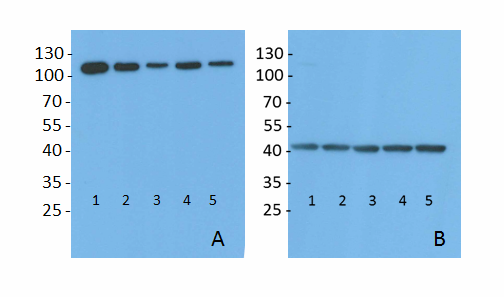
Following 72 hours incubation in 37°C in 5% CO2, cells were split with Trypsin-EDTA (Life Technologies) for five minutes in 37°C. Following a PBS wash cells were centrifuged and divided into two aliquots, of which one was immediately put in formalin for subsequent paraffin embedding, and the other aliquot immediately put in RIPA lysis buffer for subsequent SDS-PAGE electrophoresis and Western blot analysis. The supposed DPD 110 kD band was hardly detectable in the lysate of the DPD knock down treated cells. On the other hand WT, OT, and RF cells all displayed a distinct band of the proper size (Supplementary Figure 2).

## Immunocytochemistry of SUIT-2 cells

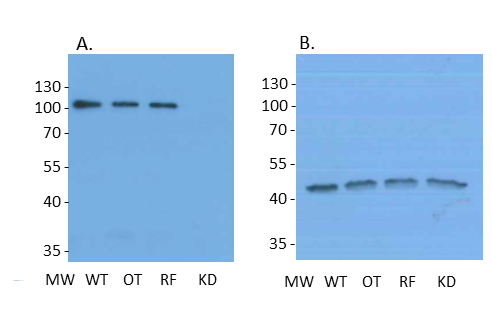
SUIT-2 cell aliquots underwent formalin fixation and agar embedding according to the routines of our laboratory, and were subsequently dehydrated and embedded in paraffin blocks which were cut in 5 µm sections for the following immunocytochemical staining. The sections underwent rehydration and antigen retrieval with the PT-LINK® pH 9.0 buffer system (Dako, Glostrup, Denmark) in 95°C, according to the supplier’s recommendations. Sections then underwent incubation with peroxidase blocker (Dako) for 10 minutes and subsequent TBST (Tris-buffered saline with 0.05% Tween-20) washes followed by the incubation with the anti-DPD antibody at a dilution of 1:2000 for 1 hour in room temperature. Following repeated TBST washes sections were incubated with HRP-conjugated anti-rabbit-antiserum (Dako) and diaminobenzidine (DAB) staining according to supplier’s recommendations. Sections were counterstained with hematoxylin and stepwise dehydrated before mounting. SUIT-2 cells treated with the knock down pool were collected 72-hours post-transfection and displayed in general very weak or absent staining (Supplementary Figure 3A). On the other hand cells treated with control pools displayed a clear cytoplasmic staining with intensity varying among the individual cells (Supplementary Figures 3B-C). There was little or no membranous or nuclear staining observed.

**SUPPLEMENTARY FIGURES**

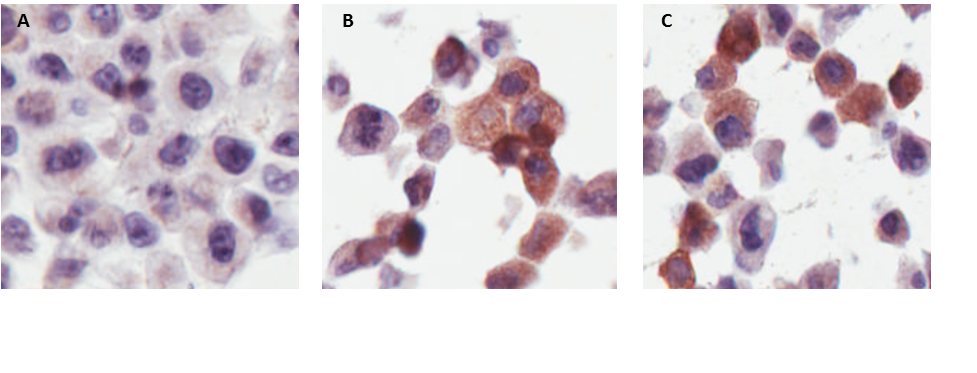
**Supplementary Figure 1.** A) Western blot showing the supposed 110 kDa DPD band in five different cell lines. B) The membrane was stripped and reprobed with anti-β-actin-antibody and HRP-conjugated secondary antibody to ensure equal loading. 1) BxPC-3; 2) CFPAC; 3) MIA PaCa; 4) PANC-1; 5) SUIT-2.



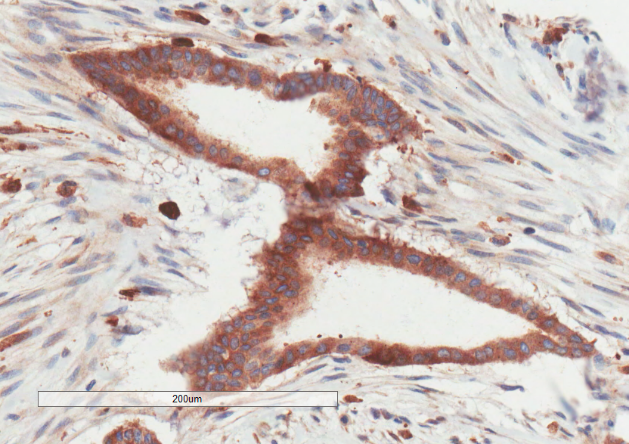
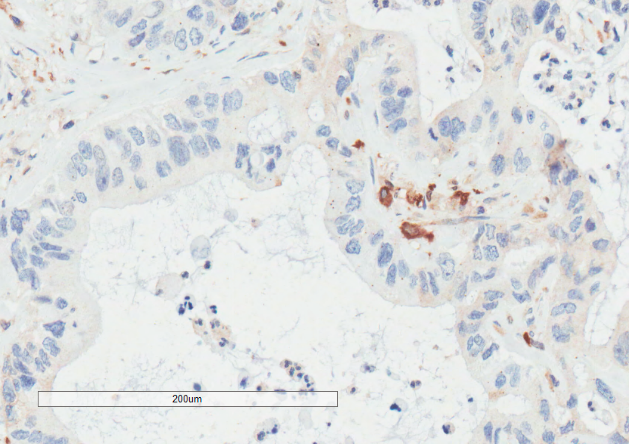
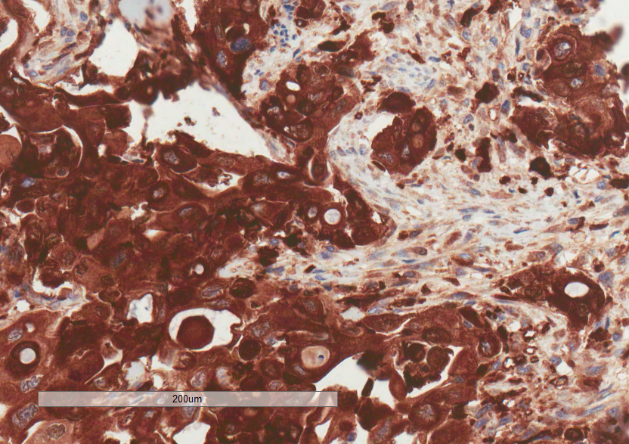
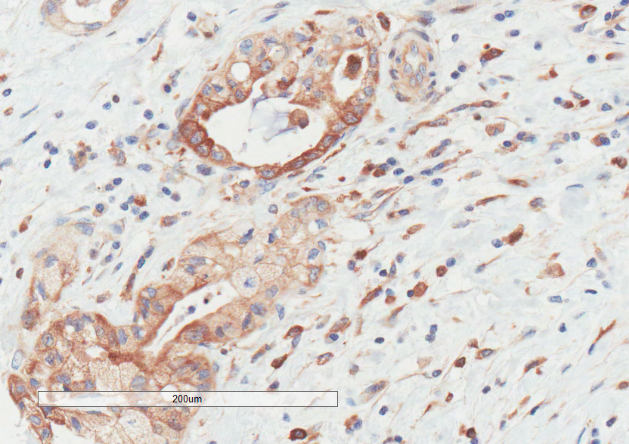
Supplementary Figure 2. A) Western blot depicting the disappearance of the 110 kD DPD band following transfection of an anti-DPD siRNApool into SUIT-2 cells. B) The membrane following stripping and reprobing with anti-β-actin antibody. MW: Molecular weight. WT: no siRNA. OT: Off target siRNA pool. RF: RISC-free siRNA pool. KD: Anti-DPD siRNA pool.



**Supplementary Figure 3.** A) Immunocytochemistry of anti-DPD siRNA treated SUIT-2 cells. B) ‘Off target’ siRNA control. C) RISC-free siRNA control. x200 magnification.

**

**Supplementary Figure 4.** Representative images of DPD immunohistochemical staining. A = DPD-0 (negative, *n*=94). B = DPD-1 (weak, *n*=114). C = DPD-2 (moderate, *n*=50). D = DPD-3 (strong, *n*=3). Arrow indicates a positively staining macrophage (working as internal positive control).



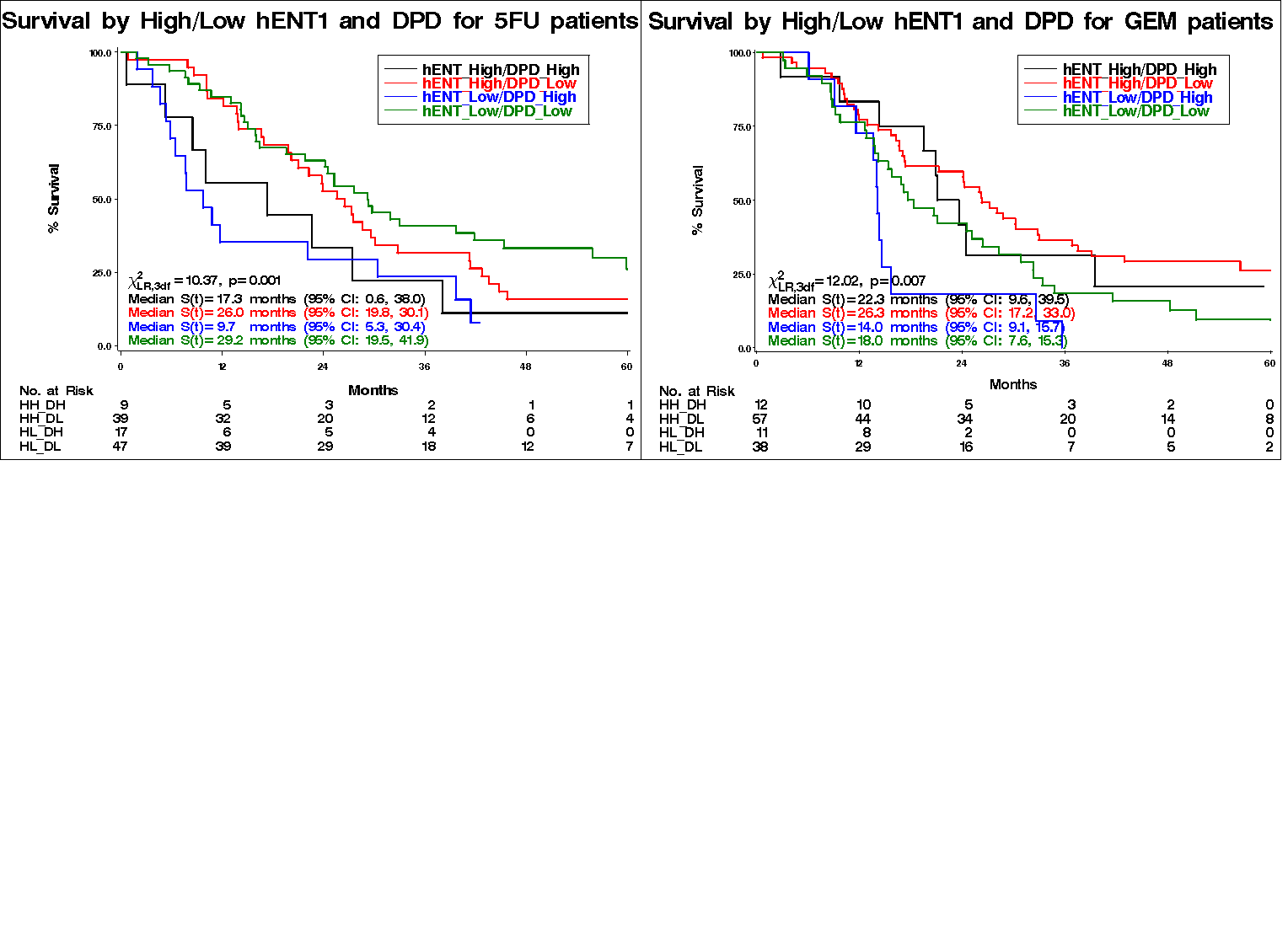
**A**

**B**

**C**

**D**

**Supplementary Figure 5.** Kaplan-Meier survival curves and median overall survival in the 5FU/FA and gemcitabine treated arms, for subgroups defined by the combination of hENT1 and DPD tumour expression status.

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**SUPPLEMENTARY TABLES**

**Supplementary Table 1.** Demographic, surgery and pathology features of the 238 chemotherapy treated patients scored for DPD tumour expression.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Demographics | | |  | | Total |
| Characteristic | | | **5-Fluorouracil/ folinic acid** | **Gemcitabine** |  |
|  | |  | ***n*=115** | ***n*=123** | ***n*=238** |
| Age Median (IQR) years | | | 62 (56-70) | 65 (57-70) | 64 (57-70) |
| Sex | **Female** | | 51 (44%) | 46 (37%) | 97 (41%) |
|  | **Male** | | 64 (56%) | 77 (63%) | 141 (59%) |
| WHO Performance Score | **0** | | 45 (39%) | 42 (34%) | 87 (37%) |
|  | **1** | | 57 (50%) | 67 (54%) | 124 (52%) |
|  | **2** | | 13 (11%) | 14 (11%) | 27 (11%) |
| Diabetes mellitus | **No** | | 89 (79%) | 92 (76%) | 181 (78%) |
|  | **NIDDM** | | 7 (6%) | 8 (7%) | 15 (6%) |
|  | **IDDM** | | 16 (14%) | 21 (17%) | 37 (16%) |
| Smoking status | **Never** | | 45 (42%) | 47 (42%) | 92 (42%) |
|  | **Past** | | 43 (40%) | 50 (44%) | 93 (42%) |
|  | **Present** | | 20 (19%) | 16 (14%) | 36 (16%) |
| Post-operative complications | **No** | | 90 (78%) | 91 (75%) | 181 (76%) |
|  | **Yes** | | 25 (22%) | 31 (25%) | 56 (24%) |
| Hospital stay | **Number** | | 110 | 113 | 223 |
| Median (IQR) days | | | 12 (10-17) | 13 (10-19) | 13 (10-18) |
| Post-Operative CA 19-9 | **Number** | | 89 | 87 | 176 |
| Median (IQR) KU/l | | | 30 (12-100) | 24 (11-54) | 28 (12-81) |
| Surgery to Randomization  Median (IQR) days | | | 49 (38-61) | 50 (39-59) | 49 (38-60) |

**Supplementary Table 2.** Relation between DPD tumour expression scores and clinical and pathological factors in the 238 chemotherapy treated patients.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Characteristic | | Number | DPD mean score | | | | *p*-value |
| **0** | **1** | **2** | **3** |
| Resection Margin | **Negative** | 130 | 44 (34%) | 55 (42%) | 29 (22%) | 2 (2%) | 0.696 |
| **Positive** | 108 | 40 (37%) | 49 (45%) | 18 (17%) | 1 (1%) |
| Lymph Node Status | **Negative** | 44 | 11 (25%) | 26 (59%) | 7 (16%) | 0 (0%) | 0.160 |
| **Positive** | 194 | 73 (38%) | 78 (40%) | 40 (21%) | 3 (2%) |
| Tumour stage | **1** | 14 | 2 (14%) | 8 (57%) | 4 (29%) | 0 (0%) | 0.308 |
| **2** | 51 | 15 (29%) | 28 (55%) | 8 (16%) | 0 (0%) |
| **3** | 164 | 62 (38%) | 64 (39%) | 35 (21%) | 3 (2%) |
| **4** | 7 | 4 (57%) | 3 (43%) | 0 (0%) | 0 (0%) |
| Tumour grade | **Well** | 16 | 5 (31%) | 7 (44%) | 3 (19%) | 1 (6%) | 0.065 |
| **Moderate** | 151 | 58 (38%) | 68 (45%) | 25 (17%) | 0 (0%) |
| **Poor** | 65 | 18 (28%) | 27 (42%) | 18 (28%) | 2 (3%) |
| Local invasion | **No** | 128 | 44 (34%) | 53 (41%) | 30 (23%) | 1 (1%) | 0.431 |
| **Yes** | 109 | 39 (36%) | 51 (47%) | 17 (16%) | 2 (2%) |
| Resection Margin | **R0** | 127 | 44 (34%) | 55 (42%) | 29 (22%) | 2 (2%) | 0.696 |
|  | **R1** | 109 | 41 (38%) | 49 (45%) | 18 (17%) | 1 (1%) |
| Maximum Tumour Diameter | **<30mm** | 103 | 33 (32%) | 49 (48%) | 19 (18%) | 2 (2%) | 0.591 |
| **>30mm** | 126 | 49 (38%) | 51 (40%) | 25 (20%) | 1 (1%) |
| Diabetes mellitus | **No** | 182 | 69 (38%) | 76 (42%) | 34 (19%) | 2 (1%) | 0.408 |
| **Yes** | 51 | 14 (27%) | 26 (51%) | 11  (21%) | 1 (2%) |
| Gender | **Male** | 141 | 52 (37%) | 58 (41%) | 29 (21%) | 2 (1%) | 0.847 |
| **Female** | 97 | 32 (33%) | 46 (47%) | 18 (19%) | 1 (1%) |
| Age (Years) | **<65** | 117 | 39 (33%) | 53 (45%) | 23 (20%) | 2 (2%) | 0.870 |
| **>65** | 121 | 45 (37%) | 51 (42%) | 24 (20%) | 1 (1%) |