**High expression of intra-tumoral dihydropyrimidine dehydrogenase is a negative prognostic factor in patients treated with adjuvant 5-fluorouracil based chemotherapy following resection of pancreatic adenocarcinoma**

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**Conflict of Interest**

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**ABSTRACT**

**Purpose:** Adjuvant chemotherapy with gemcitabine or 5-fluorouracil/folinic acid prolongs survival following resection of pancreatic cancer. Dihydropyrimidine dehydrogenase (DPD) is a key pyrimidine metabolizing enzyme and a potential response marker for 5-fluorouracil treatment.

**Methods:** DPD immunohistochemical staining was performed on tumors from 272 patients with pancreatic cancer in the ESPAC-3(v2) adjuvant trial, randomized to either gemcitabine or 5-fluorouracil/folinic acid, and 31 patients from the ESPAC-3(v1)/ESPAC-1 trials randomized to observation.

**Results:** Cores from 261 patients (86%) were suitable for scoring (semi-quantitative 0-3 point scale).There was a lower overall survival (OS) for patients with high DPD (2-3) tumor scores versus low DPD (0-1) scores (HR 1.74, 95% CI 1.24-2.44, *p*=0.002). This difference was significant in the 5-fluorouracil/folinic acid arm (HR 2.07, 95% CI 1.29-3.33, *p*=0.003) but not in the gemcitabine arm (HR 1.47, 95% CI 0.89-2.41, *p*=0.129). Multivariate analysis showed that high DPD tumor expression was an independent prognostic marker in the 5-fluorouracil/folinic acid subgroup (HR 3.30, 95% CI 1.89-5.77, *p*<0.001). The median (95% CI) OS in the 5-fluorouracil/folinic acid arm was 26.4 (21.8-30.1) months in the low DPD subgroup and 10.0 (5.8-22.6) months in the high DPD subgroup (*p*=0.002). The corresponding median (95% CI) OS in gemcitabine-treated patients was 24.4 (17.1-28.7) months and 15.7 (13.9-23.6, *p*=0.127) months, respectively. In patients with low hENT1 treated with 5-fluorouracil those with high DPD expressing tumors had a median OS of 9.7 months versus 29.2 months for those low DPD expression (*p*=0.014). In gemcitabine-treated patients hENT1 status was the dominant predictive marker.

**Conclusion:**DPD expression was an independent treatment predictive marker for benefit for postoperative 5-fluorouracil/folinic acid and, combined with hENT1 expression, is a promising approach for personalized adjuvant chemotherapy.

**BACKGROUND**

Ductal adenocarcinoma of pancreas 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 treatment1–3.

Following multicenter studies by the European Study Group for Pancreatic Cancer (ESPAC) and others, it is now clear that either 5-fluorouracil with folinic acid or gemcitabine-based chemotherapy for six months following pancreatic resection increases median and long term survival4–10.

More recently adjuvant S-1, an orally active drug containing tegafur (a prodrug of 5-fluorouracil) and the metabolism modifiers gimeracil and oteracil potassium, has also improved survival in a study from Japan11.

Although both 5-fluorouracil with folinic acid and gemcitabine are efficient at the population level, specific individuals may benefit more from gemcitabine than 5-fluorouracil or vice versa. There are currently no established tools to select the optimal treatment for the individual patient.

The cellular response to pyrimidine-based chemotherapeutics is dependent on a series of proteins involved in the trans-membrane uptake and metabolism of pyrimidines12, 13. Our laboratory has previously reported that high protein expression of human equilibrative nucleotide transporter 1 (hENT1) was associated with improved overall survival in the gemcitabine arm of the ESPAC-3(v2) trial population, but not in the 5-fluorouracil with folinic acid arm14. These data indicated that other markers should be sought to help predict 5-fluorouracil activity.

Dihydropyrimidine dehydrogenase (DPD) is an enzyme encoded by the gene *DPYD* located on chromosome *1p22*15, that converts 5-fluorouracil into dihydrofluorouracil as part of a clearance pathway16. Earlier studies on smaller retrospective materials indicate that the intra-tumoral expression of DPD may be associated with clinical response to pyrimidine-based therapies in pancreatic cancer17–23. Gimeracil, a component of S-1, is an inhibitor of DPD; it helps maintain a high concentration of 5-fluorouracil in blood and tumor tissue11.

In the present study, the expression of intra-tumoral DPD was analyzed in tissue from patients in the ESPAC-3(v2) trial and participants in the ESPAC-1 and ESPAC-3(v1) trials who were randomized to observation only. Since hENT1 is predictive for gemcitabine activity, we assessed the predictive value of DPD in hENT1-high and -low subsets.

**MATERIALS AND METHODS**

**Study Design**

The translational ESPAC-T studies received ethics committee approval for the characterization of tumor 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. Both the ESPAC-1 and ESPAC-3(v2) studies4–6, 10 were originally analyzed 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 criteria24, 25.

**Tissue Microarray (TMA) Manufacture**

Tissue arrays were manufactured using SOPs as previously reported14. The treatment arrays contained cores from patients entirely from ESPAC-3(v2) randomized to 5-fluorouracil with folinic acid or gemcitabine. The observation array contained cores from both ESPAC-1 and ESPAC-3(v1) because of the paucity of samples from patients randomized to observation. Cores were taken from tumor regions identified by an experienced pancreatic pathologist (FC) using hematoxylin and eosin-stained sections. Tissue microarrays were prepared with two cores from each block, with four to eight cores arrayed for each patient. Each of the treatment ESPAC-3(v2) arrays had two cores from each of 88 patients, whereas the observation array had four cores from each of 31 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). Deparaffinization 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 being treated with peroxidase blocker for 10 minutes. Following TBS-T washes samples were sequentially 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 fresh 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. A full validation protocol is provided (Supplementary). In brief, 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).

Positively-staining control tissues (liver) as well as tissue samples that had been confirmed not to stain (healthy colon) were used as internal controls. Negative control slides underwent identical procedure but with primary antibody replaced by antibody dilution buffer only.

**Scoring**

The tumor 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) both being blinded to patient ID and clinical data. If staining intensity within the core was not consistent, the most commonly observed pattern was scored. Any disagreement 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.

**Statistical considerations**

Survival from date of randomization was analyzed using Kaplan–Meier curves, with differences between groups assessed using the log rank test26, 27.

Univariate and multivariate analyses were carried out using *Cox* proportional hazards28. A backward elimination method was used to select variables in the multivariate analysis. Country was included as a random factor in the multivariate analyses but was estimated as zero for the gemcitabine arm. A 2-sided significance level of *P* < 0.05 was used throughout.

Earlier work on pancreatic cancer suggested a skewed distribution of DPD immunostaining grades, with a majority of tumors displaying no or low staining intensity18. Presuming a low/high group ratio of 3:1, 90 events were needed for the log rank test to have approximate power of 80% for detecting a hazard ratio (HR) of 2.0 with significance level *α* = 0.05.

All analyses were carried out using SAS version 9.3 software (SAS Institute, Cary, NC).

**RESULTS**

We stained tissue cores from 272 patients randomized and treated in the chemotherapy arms of the ESPAC-3(2) trial6 and 31 patients randomized to the observational arms of the ESPAC-1 and ESPAC-3(v1) trials4, 5, 10.

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 patients (86%) were scored including 238 chemotherapy-treated patients (115 given 5-fluorouracil with folinic acid and 123 given gemcitabine plus 23 patients randomized to observation). Representative images of the different scores and their frequencies are presented in Figure 1, and a more detailed description of the distribution of the scoring grades in various subgroups is shown in Supplementary Table 1.

**Clinical and pathological characteristics and survival according to DPD tumor expression**

Cox proportional hazards univariate analyses of survival by clinical and pathological risk factors and DPD tumor expression (low, score=0-1; high, score=2-3) by treatment arm and collectively are shown in Table 1. Significant prognostic factors were resection margin status, WHO performance status, lymph node status, tumor stage, tumor invasion into nearby organs, maximum tumor size, and DPD expression. The tumor expression of DPD was not significantly associated with any of the other clinical or pathological factors analyzed (Supplementary Table 2).

In multivariate analysis, resection margin status, WHO performance status, lymph node status, and DPD expression were all independent prognostic factors in the 5-fluorouracil with folinic acid treated arm but not in the gemcitabine treatment arm (Table 2). In the 5-fluorouracil with folinic acid arm, low DPD expression favored improved survival with an HR 3.30 (95% CI 1.89-5.77, Wald χ2 = 17.71, *p* < 0.001) while in the gemcitabine treated arm this association was much weaker and was not statistically significant (HR 1.62, 95% CI 0.97-2.69, Wald χ2 = 3.41, *p* = 0.065, Table 2).

Overall survival was compared among the different subgroups (Figure 2). In the combined chemotherapy treated populations (5-fluorouracil with folinic acid and gemcitabine), the hazard ratio (HR) for survival in patients with high DPD versus low DPD-expressing tumors in the overall chemotherapy treated group was 1.74 (95% CI 1.24-2.44, *p* = 0.002). In the 5-fluorouracil with folinic acid arm and gemcitabine arm treated subgroups, the corresponding HRs were 2.07 (1.29-3.33, *p* = 0.003) and 1.47 (0.89-2.41, *p* = 0.129) respectively. In the additional small population of observed only patients, the corresponding HR was 1.88 (*p*-value not given because of the small patient numbers). In the combined chemotherapy treated populations (5-fluorouracil with folinic acid and gemcitabine), the median (inter-quartile range, IQR) overall survival times were 25.6 (21.2-28.6) months in patients with low DPD tumor expression and 14.3 (10.0-21.1) months in those with high DPD expression (χ2LR,1df = 10.4, *p* = 0.001). These differences remained statistically significant in the 5-fluorouracil with folinic acid arm treated subgroup, where median overall survival was 26.4 (21.8-30.1) months with low DPD tumor expression and 10.0 (5.8-22.6) months in those with high DPD expression (χ2LR, 1 df = 9.56, *p* = 0.002). The corresponding median (IQR) overall survival in gemcitabine treated patients was 24.4 (17.1-28.7) months in those with low DPD tumor expression and 15.7 (13.9-23.6) months in those with high DPD expression (χ2LR, 1 df = 2.33, *p* = 0.127). In patients who were randomized not to receive and did not receive any treatment, the median (IQR) overall survival was 17.5 (6.8-34.3) months in those with low DPD tumor expression and 4.6 (3.2-31.6) months in those with high DPD expression (*p*-value not given because of the small patient numbers [*n*=3 in DPD-high subgroup]).

**Interaction between DPD and hENT1 expression**

Previously reported hENT1 IHC scoring data14 performed with the 10D7G2 murine monoclonal antibody and with exactly the same TMAs were co-analyzed with the DPD scoring. No statistically significant interaction between DPD and hENT1 status was evident based on a Cox proportional hazards model (data not shown). The Pearson correlation coefficient between DPD and the log of hENT1 scores was -0.05 (*n* = 230, *p* = 0.430). Cox proportional hazard regression analysis on hENT1 expression status on all patients available for DPD scoring confirmed previous results14 and revealed that hENT1 expression is a marker for benefit from gemcitabine- but not 5-fluorouracil-based chemotherapy. The hazard ratio for high hENT1 tumor expression versus low hENT1-expression in the 5-fluorouracil with folinic acid arm was 1.22 (χ2 = 0.85, *p* = 0.356) and for the gemcitabine arm this was 0.56 (χ2 = 8.14, *p* = 0.004).

Patients with high and low hENT1 tumor expression were further subdivided according to high and low DPD tumor expression (Table 3 and Figure 3). In patients treated with 5-fluorouracil with folinic acid, those with low hENT1 and low DPD tumor expression had a median overall survival of 29.2 (19.5-41.9) months compared to those with low hENT1 and high DPD tumor expression with a median overall survival of 9.7 (5.3-30.4) months (χ2LR = 9.28, *p* [raw] = 0.002, *p* [post Bonferroni correction] = 0.014). In the gemcitabine-treated population, survival was predominantly influenced by hENT1 status rather than DPD tumor expression levels. There was no significant difference in survival according to DPD tumor expression in gemcitabine-treated populations with either high hENT1 or low hENT1 tumor expression (Table 3 and Figure 3).

**DISCUSSION**

This study of patients from randomized controlled trials, has found a detrimental effect on survival in patients with pancreatic tumors that have high DPD expression treated with post-operative 5-fluorouracil with folinic acid. The difference in median overall survival time between patients with low and high intra-tumoral DPD expression was approximately 16 months (26.4 vs 10.0 months, Figure 2) while there was no significant difference in the gemcitabine-treated patients. The prognostic value of DPD tumor status in patients treated with 5-fluorouracil with folinic acid was also shown to be an independent factor in the multivariate analysis. Taken together, these findings support the hypothesis that DPD tumor expression is a negative prognostic factor for patients with pancreatic cancer treated with post-operative 5-fluorouracil with folinic acid.

Previous work in Asian populations with pancreatic cancer indicated that DPD expression may discriminate between good and poor responders to chemotherapy and/or chemoradiotherapy. In a study from Japan, the survival of 72 patients that had had S-1-based adjuvant chemotherapy was much better for patients with low DPD tumor expression and no bearing on survival in 34 patients that had not had S-1-based therapy17. In the same study, intra-tumoral expression of thymidylate synthase or orotate phosphoribosyltransferase had no bearing on treatment related survival. In another study from Japan, the overall survival of 86 patients who had received post-operative gemcitabine plus S-1 following pancreatic cancer resection was independently associated with tumor size, lymph node metastasis, DPD expression, and hENT1 expression18. Results by Shimoda et al23 from a study of 57 patients indicated that low intra-tumoral DPD mRNA levels predicted good response for S-1 but not gemcitabine, and Saif19 reported the thymidine phosphorylase/DPD mRNA ratio to be a potential prognostic marker in 35 patients with locally advanced disease treated with capecitabine plus radiotherapy. These results from Japanese populations were not immediately transferable to non-Asian populations as Europeans experience more toxicity with S-1 at equivalent doses because of differences in metabolism29. Thus, the results of the current study of patients from the ESPAC-3 trial, in which nearly all patients are of Caucasian background, are especially important. These findings support the potential application of DPD tumor expression as a predictive marker for 5-fluorouracil-based therapies in pancreatic cancer. Patients with high-DPD-expressing tumors should be discouraged from treatment with 5-fluorouracil-based regimens.

As a single predictive biomarker, immunohistochemistry for hENT1 can inform the decision for gemcitabine-based chemotherapy, with low hENT1 tumor expression indicating that gemcitabine is not suitable14. On the other hand, hENT1 expression as a single marker is not predictive for outcomes after 5-fluorouracil-based chemotherapy14.Combining both markers showed that the longest median survival (29.2 months) was achieved in patients given 5-fluorouracil with folinic acid with low hENT1 and low DPD tumor expression against a very low median survival (9.7 months) in those with low hENT1 and high DPD tumor expression. In patients with high hENT1 expression treated with 5-fluorouracil with folinic acid, no such difference was evident. These results, while consistent with predictive value of DPD tumor expression, should be interpreted with caution because of the limited sample sizes in some subgroups, and will require prospective formal validation. As adjuvant chemotherapy is now firmly established within international guidelines30, a key objective for future clinical studies will be to further elucidate the potential of prospectively guiding the patient to a tailored therapy based on the use of markers such as hENT1 and DPD. From a hypothesis generating point of view, the results presented here suggest that the following.

1. Patients with tumors with high hENT1 expression should be offered gemcitabine.
2. Patients with tumors low with DPD expression should be offered 5-fluorouracil with folinic acid.
3. Patients with tumors with high hENT1 expression and low DPD expression may be offered either gemcitabine or 5-fluorouracil with folinic acid depending on the clinical scenario.
4. Patients with tumors with low hENT1 expression and high DPD expression may derive little benefit from either gemcitabine or 5-fluorouracil with folinic acid, and novel approaches are required for this subgroup.

Whether any of these subgroups in particular would benefit from combination therapy versus standard monotherapy remains to be elucidated although the combination of gemcitabine and 5-fluorouracil with folinic acid might be appropriate in patients with tumour showing hENT1 expression with low DPD expression

The recent results from the ESPAC-4 trial showed that the combination gemcitabine with capecitabine (an orally active 5-fluorouracil prodrug) significantly increased survival compared to gemcitabine monotherapy in the post-operative setting31. To date we do not know whether any subgroup of patients do equally well on standard gemcitabine or 5-fluorouracil/folinic acid as with the gemcitabine/capecitabine combination. Ongoing biomarker analyses of the ESPAC-4 population will assess whether hENT-1, DPD, and/or other targets are suitable for guiding the individual patient to conventional gemcitabine monotherapy. This is important because there is a different toxicity profile the gemcitabine and capecitabine combination as well as additional costs the. Other combination regimens being assessed in the adjuvant setting are FOLFIRINOX32 and the combination of gemcitabine and nab-paclitaxel33.

The validation of clinically reliable protocols for biomarker analyses is essential, a fact that is underlined by the current discussions on conflicting data yielded by different types of hENT1 antibodies14, 34, 35. The importance of strict and reproducible protocols like the DPD protocol presented here should be stressed in future biomarker research.

In conclusion, the present study demonstrates that high intra-tumoral expression of DPD is associated with reduced survival following postoperative 5-fluorouracil with folinic acid adjuvant therapy in pancreatic cancer, while hENT1 expression remains the most robust predictive biomarker for gemcitabine monotherapy. A combined analysis of hENT1 and DPD may assist the clinician in guiding the patient to 5-fluorouracil with folinic acid or gemcitabine. Future prospective trials should evaluate the utility of a predictive biomarker panel to inform the selection of post-operative chemotherapy in pancreatic cancer.

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**REFERENCES**

**1**. Siegel RL, Miller KD, Jemal A: Cancer statistics , 2015 . CA Cancer J Clin 65:5–29, 2015

**2**. Kleeff J, Korc M, Apte M, et al: Pancreatic cancer. Nat Rev Dis Prim 2, 2016

**3**. Rahib L, Smith BD, Aizenberg R, et al: Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer Res 74:2913–21, 2014

**4**. Neoptolemos JP, Stocken DD, Tudur Smith C, et al: Adjuvant 5-fluorouracil and folinic acid vs observation for pancreatic cancer: composite data from the ESPAC-1 and -3(v1) trials. Br J Cancer 100:246–50, 2009

**5**. Neoptolemos JP, Stocken DD, Friess H, et al: A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. N Engl J Med 350:1200–1210, 2004

**6**. Neoptolemos JP, Stocken DD, Bassi C, et al: Adjuvant Chemotherapy With Fluorouracil Plus Folinic Acid vs Gemcitabine Following Pancreatic Cancer Resection. JAMA 304:1073–1081, 2010

**7**. Oettle H, Neuhaus P, Hochhaus A, et al.: Adjuvant chemotherapy with gemcitabine and long-term outcomes among patients with resected pancreatic cancer: the CONKO-001 randomized trial. JAMA 310:1473–81, 2013

**8**. Oettle, H, Post, S, Neuhaus P, et al: Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial. JAMA 297:267–277, 2007

**9**. Valle JW, Palmer D, Jackson R, et al.: Optimal duration and timing of adjuvant chemotherapy after definitive surgery for ductal adenocarcinoma of the pancreas: ongoing lessons from the ESPAC-3 study. J Clin Oncol 32:504–512, 2014

**10**. Neoptolemos JP, Dunn JA, Stocken DD, et al: Adjuvant chemoradiotherapy and chemotherapy in resectable pancreatic cancer: A randomised controlled trial. Lancet 358:1576–85., 2001

**11**. Uesaka K, Boku N, Fukutomi A et al.: Adjuvant chemotherapy of S-1 versus gemcitabine for resected pancreatic cancer: a phase 3, open-label, randomised, non-inferiority trial (JASPAC 01). Lancet 388:248–257, 2016

**12**. Costello E, Greenhalf W, Neoptolemos JP: New biomarkers and targets in pancreatic cancer and their application to treatment. Nat Rev Gastroenterol Hepatol 9:435–444, 2012

**13**. Young JD, Yao SY, Sun L, et al: Human equilibrative nucleoside transporter (ENT) family of nucleoside and nucleobase transporter proteins. Xenobiotica 38:995–1021, 2008

**14**. Greenhalf W, Ghaneh P, Neoptolemos JP, et al: Pancreatic cancer hENT1 expression and survival from gemcitabine in patients from the ESPAC-3 trial. J Natl Cancer Inst 106:20–25, 2014

**15**. Yokota H, Fernandez-Salguero P, Furuya H, et al: cDNA cloning and chromosome mapping of human dihydropyrimidine dehydrogenase, an enzyme associated with 5-fluorouracil toxicity and congenital thymine uraciluria. J Biol Chem 269 (37):23192–6, 1994

**16**. Diasio RB: The role of dihydropyrimidine dehydrogenase (DPD) modulation in 5-FU pharmacology. Oncol 12:23–27, 1998

**17**. Kondo N, Murakami Y, Uemura K, et al: Prognostic impact of dihydropyrimidine dehydrogenase expression on pancreatic adenocarcinoma patients treated with S-1-based adjuvant chemotherapy after surgical resection. J Surg Oncol 104:146–154, 2011

**18**. Kondo N, Murakami Y, Uemura K, et al: Combined Analysis of Dihydropyrimidine Dehydrogenase and Human Equilibrative Nucleoside Transporter 1 Expression Predicts Survival of Pancreatic Carcinoma Patients Treated with Adjuvant Gemcitabine Plus S-1 Chemotherapy after Surgical Resection. Ann Surg Oncol19:646–655, 2011

**19**. Saif MW, Hashmi S, Bell D, et al: Prognostication of pancreatic adenocarcinoma by expression of thymidine phosphorylase/dihydropyrimidine dehydrogenase ratio and its correlation with survival. Expert Opin Drug Saf 8:507–14, 2009

**20**. Miyake K, Imura S, Yoshizumi T, et al: Role of thymidine phosphorylase and orotate phosphoribosyltransferase mRNA expression and its ratio to dihydropyrimidine dehydrogenase in the prognosis and clinicopathological features of patients with pancreatic cancer. Int J Clin Oncol 12:111–119, 2007

**21**. Nakahara O, Takamori H, Tanaka H, et al: Clinical significance of dihydropyrimidine dehydrogenase and thymidylate synthase expression in patients with pancreatic cancer. Int J Clin Oncol 15:39–45, 2010

**22**. Nakamura A, Hayashi K, Nakajima G, et al: Impact of dihydropyrimidine dehydrogenase and gamma-glutamyl hydrolase on the outcomes of patients treated with gemcitabine or S-1 as adjuvant chemotherapy for advanced pancreatic cancer. Exp Ther Med 2:1097–1103, 2011

**23**. Shimoda M, Kubota K, Shimizu T, et al: Randomized clinical trial of adjuvant chemotherapy with S-1 versus gemcitabine after pancreatic cancer resection. Br J Surg 102:746–754, 2015

**24**. McShane LM, Altman DG, Sauerbrei W, et al: Reporting recommendations for tumor marker prognostic studies (REMARK). Nat Clin Pract Oncol 2:416–22, 2005

**25**. Simon RM, Paik S, Hayes DF: Use of archived specimens in evaluation of prognostic and predictive biomarkers. J Natl Cancer Inst 101:1446–52, 2009

**26**. Kaplan E, Meier P: Non-parametric estimation from incomplete observations. J Amer Stat Assoc 53:457–81, 1958

**27.** Peto R, Peto J: Asymptotically efficient rank invariant test procedures. J R Stat Soc Ser A Stat Soc 135:185-207, 1972

**28.** Cox DR: Regression models and life-tables. J R Stat Soc B 34:187-220, 1972

**29**. Chuah B, Goh BC, Lee SC, et al: Comparison of the pharmacokinetics and pharmacodynamics of S-1 between Caucasian and East Asian patients. Cancer Sci 102:478–83, 2011

**30**. Ducreux M, Cuhna AS, Caramella C, et al: Cancer of the pancreas: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 26:v56–v68, 2015

**31**. Neoptolemos JP, Palmer D, Ghaneh P, et al.: ESPAC-4: A multicenter, international, open-label randomized controlled phase III trial of adjuvant combination chemotherapy of gemcitabine (GEM) and capecitabine (CAP) versus monotherapy gemcitabine in patients with resected pancreatic ductal adenocarcin. J Clin Oncol 34:suppl; abstr LBA4006, 2016

**32**. Conroy T, Desseigne F, Ychou M, et al: FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med 364:1817–1825, 2011

**33**. Von Hoff DD, Ervin T, Arena FP, et al: Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med 369:1691–703, 2013

**34**. Poplin E, Wasan H, Rolfe L, et al: Randomized, multicenter, phase ii study of co-101 versus gemcitabine in patients with metastatic pancreatic ductal adenocarcinoma: Including a prospective evaluation of the role of hENT1 in gemcitabine or CO-101 sensitivity. J Clin Oncol 31:4453–4461, 2013

**35**. Sinn M, Riess H, Sinn BV, et al: Human equilibrative nucleoside transporter 1 expression analysed by the clone SP 120 rabbit antibody is not predictive in patients with pancreatic cancer treated with adjuvant gemcitabine – Results from the CONKO-001 trial. Eur J Cancer 51:1546–1554, 2015

**TABLES**

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

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Univariate Analysis** | | | | | | |
|  |  | **Hazard Ratio (95% Confidence Interval)** | | | | |
|  | **Chemotherapy** | | | **Total** |
| **Characteristic** |  | **5-fluorouracil /folinic acid** | | **Gemcitabine** |  |
|  |
| Resection Margin |  | *n*=128 | | *n*=135 | *n*=263 |
| Negative | 1 | | 1 | 1 |
| Positive | 2.03 (1.35-3.03) | | 1.22 (0.84-1.78) | 1.56 (1.19-2.05) |
|  | Wald χ2= 11.74, ***p*=0.001** | | Wald χ2= 1.11 *p*=0.293 | Wald χ2= 10.29, ***p*=0.001** |
| WHO |  | *n*=128 | | *n*=135 | *n*=263 |
| 0 | 1 | | 1 | 1 |
| 1 | 1.48 (0.97-2.25) | | 1.51 (1.00-2.29) | 1.50 (1.12-2.02) |
| 2 | 0.71 (0.34-1.47) | | 1.44 (0.77-2.69) | 1.01 (0.63-1.62) |
|  | Wald χ2= 5.98, ***p*=0.050** | | Wald χ2= 3.86, *p*=0.145 | Wald χ2= 8.47, ***p*=0.015** |
| Lymph Node Status |  | *n*=128 | | *n*=135 | *n*=263 |
| Negative | 1 | | 1 | 1 |
| Positive | 2.51 (1.39-4.52) | | 1.53 (0.91-2.57) | 1.99 (1.35-2.93) |
|  | Wald χ2= 9.39, ***p*=0.002** | | Wald χ2= 2.60, *p*=0.107 | Wald χ2= 11.95, ***p*<0.001** |
| Tumor Stage |  | *n*=127 | | *n*=134 | *n*=261 |
| 1/2 | 1 | | 1 | 1 |
| 3/4 | 1.69 (1.05-2.72) | | 1.42 (0.93-2.16) | 1.54 (1.12-2.10) |
|  | Wald χ2= 4.65**, *p*=0.031** | | Wald χ2= 2.64, *p*=0.105 | Wald χ2= 7.14, ***p*=0.008** |
| Tumor Grade |  | *n*=125 | | *n*=132 | *n*=257 |
| Well | 1 | | 1 | 1 |
| Moderately | 0.60 (0.28-1.32) | | 1.12 (0.54-2.33) | 0.87 (0.51-1.48) |
| Poorly | 0.70 (0.30-1.62) | | 1.37 (0.63-2.99) | 1.04 (0.59-1.84) |
|  | Wald χ2= 1.78, *p*=0.410 | | Wald χ2= 1.09, *p*=0.578 | Wald χ2= 1.43 *p*=0.490 |
| Local Invasion |  | | *n*=128 | *n*=134 | *n*=262 |  | |
| No | | 1 | 1 | 1 |  | |
| Yes | | 1.39 (0.94-2.05) | 1.35 (0.93-1.97) | 1.36 (1.04-1.79) |  | |
|  | | Wald χ2= 2.64, *p*=0.105 | Wald χ2= 2.51, *p*=0.113 | Wald χ2= 5.06 ***p*=0.025** |  | |
| Maximum Tumor diameter |  | | *n*=123 | *n*=130 | *n*=253 |  | |
| <30mm | | 1 | 1 | 1 |  | |
| ≥30mm | | 1.40 (0.94-2.09) | 1.32 (0.89-1.94) | 1.37 (1.04-1.81) |  | |
|  | | Wald χ2= 2.68, *p*=0.102 | Wald χ2= 1.91, *p*=0.167 | Wald χ2= 4.90 ***p*=0.027** |  | |
| Diabetes mellitus |  | | *n*=125 | *n*=132 | *n*=257 |  | |
| No | | 1 | 1 | 1 |  | |
| Yes | | 0.82 (0.49-1.39) | 1.02 (0.66-1.59) | 0.93 (0.66-1.30) |  | |
|  | | Wald χ2= 0.55, *p*=0.460 | Wald χ2= 0.01, *p*=0.925 | Wald χ2= 0.18, *p*=0.672 |  | |
| Gender |  | | *n*=128 | *n*=135 | *n*=263 |  | |
| Male | | 1 | 1 | 1 |  | |
| Female | | 1.08 (0.72-1.60) | 1.10 (0.75-1.60) | 1.09 (0.83-1.43) |  | |
|  | | Wald χ2= 0.13, *p*=0.722 | Wald χ2= 0.24, *p*=0.626 | Wald χ2= 0.38, *p*=0.540 |  | |
| Age, years |  | | *n*=128 | *n*=135 | *n*=263 |  | |
| ≥64 | | 1 | 1 | 1 |  | |
| <64 | | 1.27 (0.85-1.89) | 0.88 (0.60-1.29) | 1.05 (0.80-1.37) |  | |
|  | | Wald χ2= 1.35**,** *p*=0.245 | Wald χ2= 0.44, *p*=0.509 | Wald χ2= 0.12, *p*=0.725 |  | |
| Smoking |  | | *n*=121 | *n*=122 | *n*=243 |  | |
| Never | | 1 | 1 | 1 |  | |
| Ex | | 0.91 (0.58-1.41) | 1.28 (0.84-1.96) | 1.08 (0.79-1.47) |  | |
| Current | | 0.96 (0.55-1.67) | 1.48 (0.82-2.67) | 1.15 (0.77-1.73) |  | |
|  | | Wald χ2= 0.19, *p*=0.907 | Wald χ2= 2.20, *p*=0.332 | Wald χ2= 0.54, *p*=0.762 |  | |
| DPD expression |  | | *n*=115 | *n*=123 | *n*=238 |  | |
| DPYD-0/1 | | 1 | 1 | 1 |  | |
| DPD-2/3 | | 2.07 (1.29-3.33) | 1.47 (0.89-2.41) | 1.74 (1.24-2.44) |  | |
|  | | Wald χ2= 9.15, ***p*=0.003** | Wald χ2= 2.30**,** *P*=0.129 | Wald χ2= 10.11, ***p*=0.002** |  | |
|  |  | |  |  |  |  | |

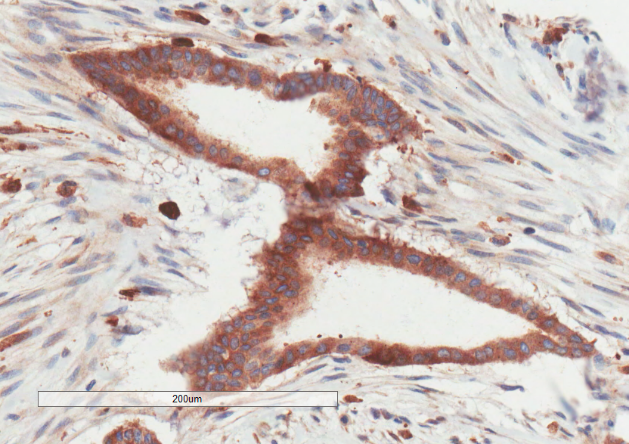
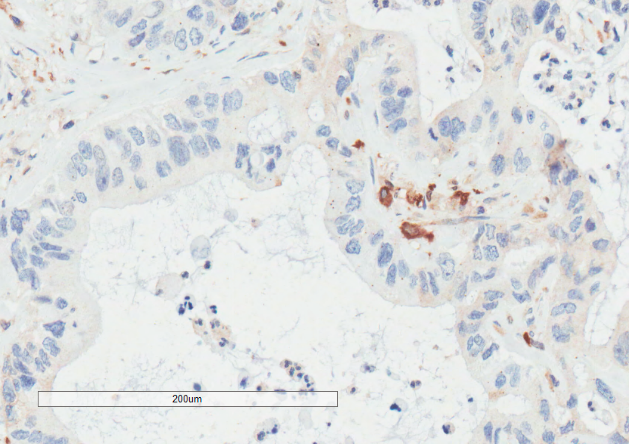
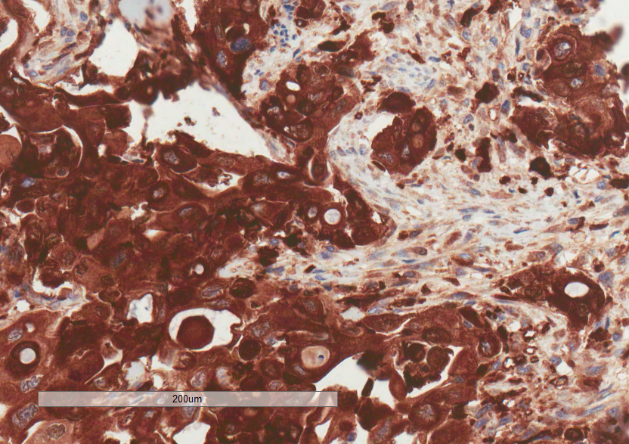
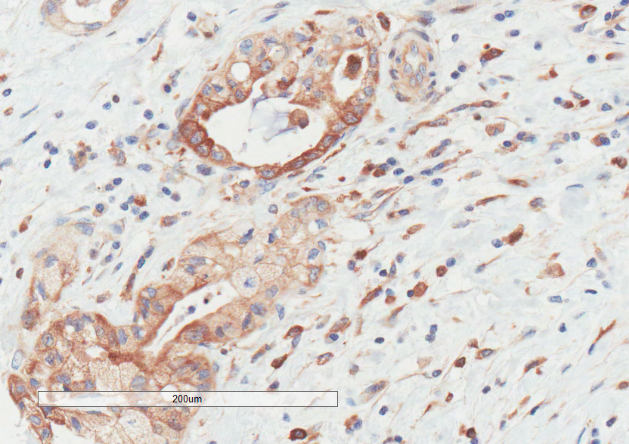
**Table 2.** Multivariate analyses for survival of clinical and pathological risk factors and DPD tumor expression in 5-fluorouracil with folinic acid and gemcitabine treated arms

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Multivariate Analysis** | | | | | | | | | |
|  |  | **5-fluorouracil /folinic acid**  **(*n*=115)** | | | | **Gemcitabine**  **(*n*=123)** | | | |
| **Variable** |  | **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 in combined hENT1 and DPD expression subgroups. HH=hENT1-high, HL=hENT1-low; DH = DPD-high, DL=DPD-low. Comparisons between HH/DH versus HH/DL and HL/DH versus HL/DL in the respective treatment subgroup were performed with log rank testing and subsequent Bonferroni correction

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

**FIGURES**



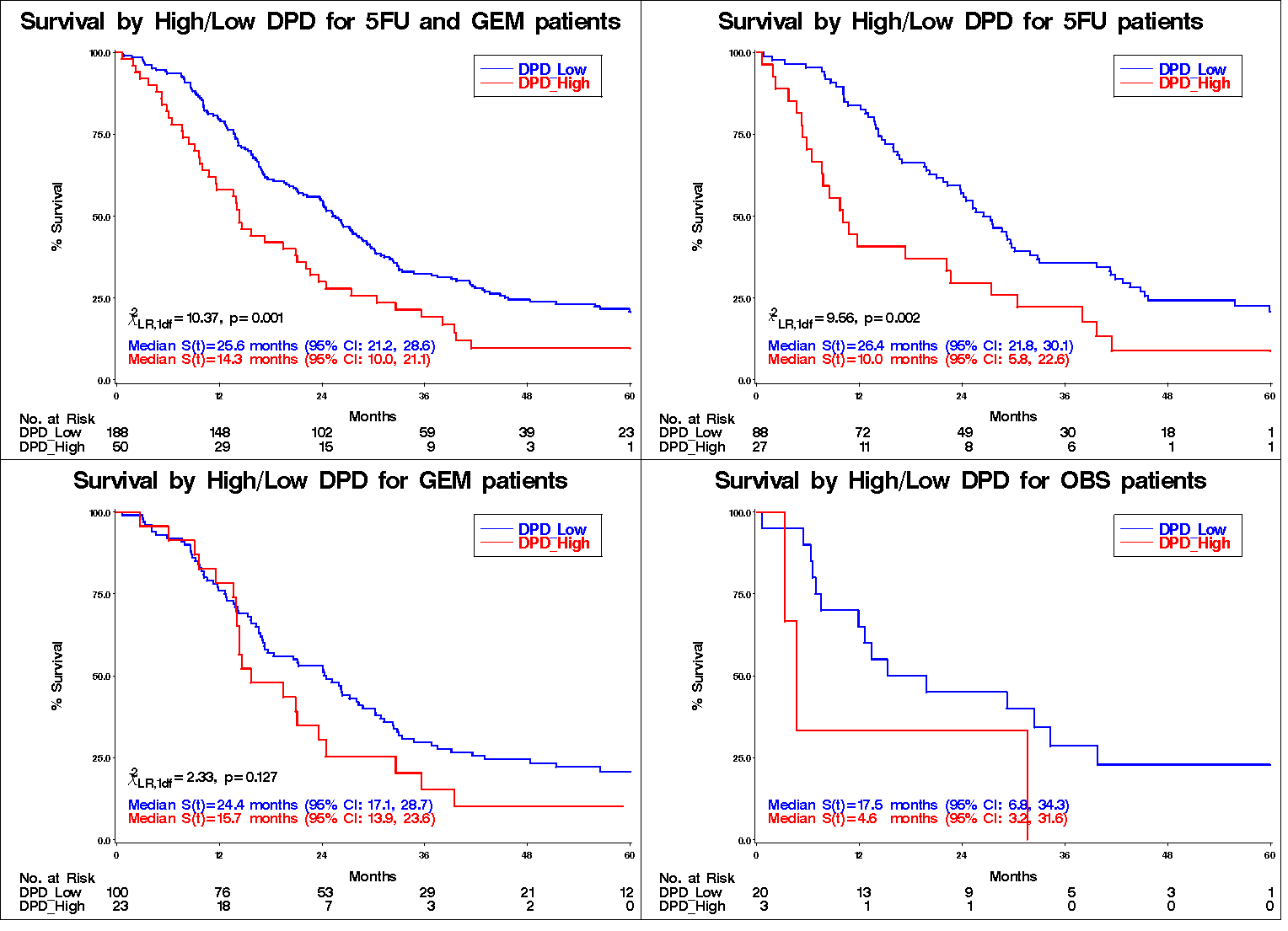
**A**

**B**

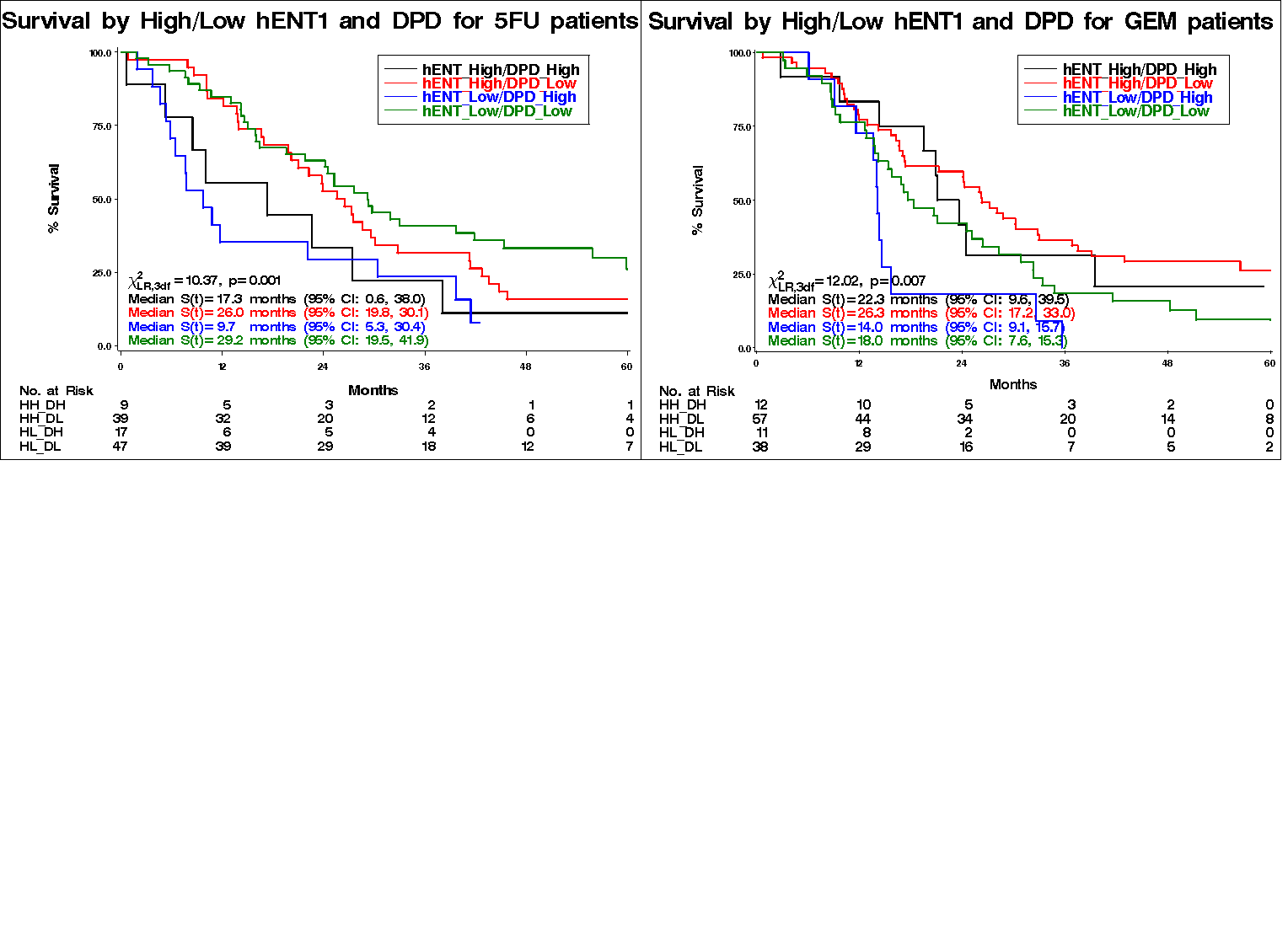
**C**

**D**

**Figure 1.** Representative images of DPD immunostaining in pancreatic adenocarcinomas. A = negative (score ‘0’, *n*=94). B = weak (score ‘1’, *n*=114). C = moderate (score ‘2’, *n*=50). D = strong (score ‘3’, *n*=3). Note that macrophages (arrow) generally stain positive thus serving as internal positive control.

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**Figure 2.** Kaplan-Meier curves depicting time from randomisation until death of any cause in patients in the 5-fluorouracil with folinic acid arm (5-FU), gemcitabine arm (GEM), or untreated (OBS) arms. Patients are stratified into those with low DPD tumor expression (scores 0-1), and high DPD tumor expression (scores 2-3).

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**Figure 3.** Kaplan-Meier survival curves for combined hENT1 and DPD tumor expression. HH=hENT1-high expression, HL=hENT1-low expression; DH=DPD-high expression, DL=DPD-low expression.

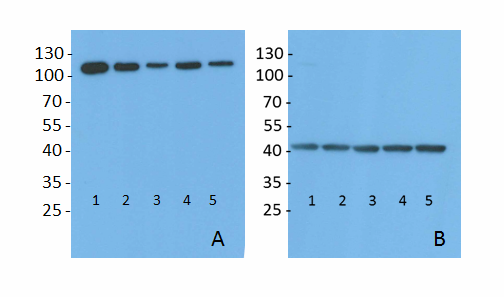
**SUPPLEMENTARY MATERIALS AND METHODS**

**Validation of rabbit-anti-DPD antibody (Abcam ab134922)**

## *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.

**

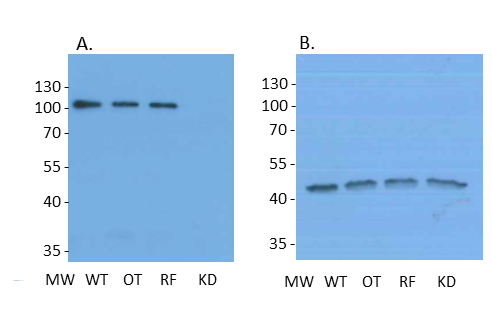
***Supplementary Figure 1.*** *A) Western blot showing the supposed 110 kDa DPD band in five different cell lines. B) The membrane was stripped and reprobed with anti-β-actin-antibody and HRP-conjugated secondary antibody to ensure equal loading. 1) BxPC-3 2) CFPAC 3) MIA PaCa 4) PANC-1 5) SUIT-2.*

*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).

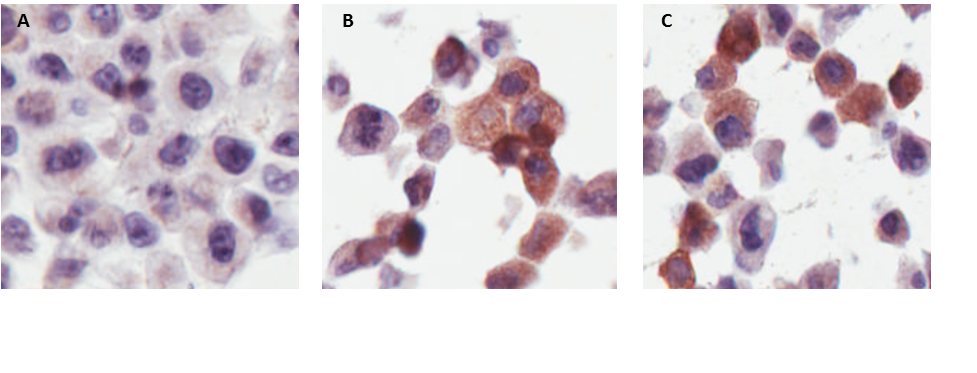
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*Supplementary Figure 2. A) Western blot depicting the disappearance of the 110 kD DPD band following transfection of an anti-DPD siRNApool into SUIT-2 cells. B) The membrane following stripping and reprobing with anti-β-actin antibody. MW: Molecular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

|  |
| --- |
|  |

## *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 stainings. 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 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 TABLES**

***Supplementary Table 1.***Distribution of DPD scoring grades in the patient population (*n* = 261). The mean score for each patient was based on all available core biopsies, and was rounded to the nearest integer

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Arm** | **DPD mean staining** | | | |
| **0** | **1** | **2** | **3** |
| **5-fluorouracil with folinic acid** | 33 | 55 | 26 | 1 |
| **Gemcitabine** | 51 | 49 | 21 | 2 |
| **Observation** | 10 | 10 | 3 | 0 |
| **TOTAL** | 94 | 114 | 50 | 3 |

***Supplementary Table 2.*** Relation between DPD expression tumor scores status and other clinical and pathological factors in all 238 chemotherapy treated patients

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Characteristic** |  | **Number** | **DPD meanr** | | | | ***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%) |
| 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%) |
| Tumor 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 | 43 (34%) | 53 (42%) | 29 (23%) | 2 (2%) | 0.629 |
|  | R1 | 109 | 41 (38%) | 49 (45%) | 18 (17%) | 1 (1%) |
| Maximum Tumor Dia. | <30mm | 103 | 33 (32%) | 49 (48%) | 19 (18%) | 2 (2%) | 0.591 |
|  | >=30mm | 126 | 49 (38%) | 51 (40%) | 25 (20%) | 1 (1%) |
| Diabetes | No | 182 | 69 (38%) | 76 (42%) | 35 (19%) | 2 (1%) | 0.424 |
|  | Yes | 51 | 14 (27%) | 26 (51%) | 10 (20%) | 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) | <64 | 117 | 39 (33%) | 53 (45%) | 23 (20%) | 2 (2%) | 0.871 |
|  | >=64 | 121 | 45 (37%) | 51 (42%) | 24 (20%) | 1 (1%) |