**Title:** Dihydropyrimidine dehydrogenase and hENT1 tumor expression predict survival from adjuvant chemotherapy in pancreatic cancer.

**Running Title:** DPD and hENT1 as biomarkers in pancreatic cancer.

**Authors:** Nils O. Elander, M.D. Ph. D., Karen Aughton, Ph.D., Paula Ghaneh, M.D., John P. Neoptolemos, M.D., Daniel H. Palmer, M.D. Ph. D., Trevor F. Cox, Ph.D., Fiona Campbell, M.D., FRCPath., Eithne Costello, Ph.D., Christopher M. Halloran, M.D., John R. Mackey, M.D., Andrew G Scarfe, M.D., Juan W. Valle, M.D., Alexander C. McDonald, M.D., Ross Carter, M.D., Niall C. Tebbutt, Ph.D., David Goldstein, M.B., Jennifer Shannon, Ph.D. FRACP., Christos Dervenis, M.D., Bengt Glimelius, M.D., Mark Deakin, M.D., Richard M Charnley, Alan Anthoney, M.D., Markus M. Lerch, M.D., Julia Mayerle, M.D., Attila Oláh, M.D., Markus W. Büchler, M.D., William Greenhalf, Ph.D., for the European Study Group for Pancreatic Cancer.

From the Cancer Research U.K. Liverpool Cancer Trials Unit, University of Liverpool, Liverpool, United Kingdom (N.O.E., K.A., P.G., J.P.N., D.H.P., T.F.C., F.C., E.C., C.M.H.,W.G.); Cross Cancer Institute and University of Alberta, Canada (J.R.M. and A.G.S.); University of Manchester / The Christie NHS Foundation Trust, Manchester, United Kingdom (J.W.V.); The Beatson West of Scotland Cancer Centre, Glasgow, Scotland, United Kingdom (A.C.M.); Glasgow Royal Infirmary, Glasgow, Scotland, United Kingdom (R.C.); Austin Health, Melbourne, Australia (N.C.T.); Prince of Wales hospital and Clinical School University of New South Wales, Australia (D.G); Nepean Cancer Centre and University of Sydney, Australia (J.S.); the Agia Olga Hospital, Athens, Greece (C.D.); Department of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden (B.G.); University Hospital, North Staffordshire, United Kingdom (M.D.); Freeman Hospital, Newcastle upon Tyne, United Kingdom (R.M.C.); St James's University Hospital, Leeds, United Kingdom (A.A.); Department of Medicine A, University Medicine Greifswald, Greifswald, Germany (M.M.L., J.M.); the Petz Aladar Hospital, Gyor, Hungary (A.O.); the Department of Surgery, University of Heidelberg, Heidelberg, Germany (M.W.B.).

**Corresponding Author:**

Dr William Greenhalf, Ph.D.,

Liverpool Cancer Research UK and Clinical Trials Unit:

University of Liverpool,

1st floor Block C, Waterhouse Building,

3 Brownlow Street, Liverpool,

L69 3GL, UK

Tel: +44 (0)151 794 8383

Fax: +44 (0)151 794 8931

Email: greenhaf@liv.ac.uk

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**Conflict of Interest**

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**ABSTRACT**

**Purpose:** Adjuvant treatment with gemcitabine or 5-fluorouracil plus folinic acid (5FU/FA) prolongs patient survival following resection of pancreatic cancer. Dihydropyrimidine dehydrogenase (DPD) tumor expression may provide added value to human equilibrative nucleotide transporter-1 (hENT1) tumor expression in predicting improved survival to the type of chemotherapy treatment.

**Methods:** DPD and hENT1 immunohistochemistry and scoring was performed on tumor cores from 272 patients with pancreatic cancer in the ESPAC-3(v2) trial, randomized to either postoperative gemcitabine or 5FU/FA.

**Results:** Cores from 238 (88%) patients were suitable for scoring. DPD tumor expression was associated with reduced overall survival in the entire chemotherapy population (HR=1.73 [95% CI = 1.21-2.49], *p*=0.003). This difference remained statistically significant in the 5FU/FA arm, but not in the gemcitabine arm (HR=2.07 [1.22-3.57], *p*=0.007 and HR=1.47 [0.91-3.37], *p*=0.119, respectively). High hENT1 tumor 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). In patients with hENT1-high tumor expression, DPD tumor expression status added little value. In patients with hENT1-low tumor expression, DPD-low tumor expression was associated with increased median survival with 5FU/FA treatment compared to DPD-high tumor expression (29.2 [19.5-41.9] vs. 9.7 [5.3-30.4] months, *p* = 0.002). The latter subgroup also had poor median survival with gemcitabine (14.0 [9.1-15.7] months).

**Conclusion:**DPD tumor expression was shown to be a negative prognostic biomarker. Together with tumor expression of hENT1, DPD tumor expression defined specific patient subgroups that benefitted from either 5FU/FA or gemcitabine following resection of pancreatic adenocarcinoma, requiring further prospective evaluation.

**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 treatment1–3. Following multicenter 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 survival4–10. Adjuvant S-1, an orally active drug containing tegafur (another 5FU prodrug) has also improved survival in patients from Japan11.

Although both 5FU/FA and gemcitabine are efficient at the cohort level, specific individuals may benefit more from gemcitabine than 5FU/FA 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 5FU/FA arm14. 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*15, that catabolizes 5FU into dihydrofluorouracil16. Metabolites of 5FU interfere with cell function by inhibition of DNA synthesis and repair, RNA transcription and DNA methylation16. 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-beta-ureidopropionate and subsequently to fluoro-beta-alanine16. Thus we could hypothesize that low intra-tumoral DPD expression would favor the production of cytotoxic 5FU metabolites and prolong survival. This hypothesis has received some support in small retrospective studies predominantly involving the composite drug S-117–23. Gimeracil, a component of S-1, is an inhibitor of DPD that maintains a high concentration of 5FU 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 being randomized 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 aimed to test whether DPD expression could add to the predictive value of hENT1 expression in selecting patients for either gemcitabine or 5-FU adjuvant therapy14.

**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. The ESPAC-3 trial randomized 551 patients to 5FU/FA and 537 to gemcitabine7,8. This was 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 SOP’s as previously reported14. The arrays contained cores from patients entirely from ESPAC-3(v2) randomized to 5FU/FA or gemcitabine, or patients from the ESPAC-1/ESPAC-3(v1) trials randomized to observation only. 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 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). 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 Materials and Methods). 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. The previously collected hENT1 scores for the tumors were added to the data set in order to investigate a possible relationship with the DPD scores. DPD score and hENT1 Hscore were uncorrelated (Pearson correlation = - 0.01).

**Statistical considerations**

Cox proportional hazards modeling (Cox PH) was used to find prognostic/predictive models for survival calculated from date of randomization to death. Models were compared using Akaike’s Information Criterion (AIC). Subsequently, Kaplan-Meier curves26 and log-rank tests27 were calculated for sub-groups of patients based on dichotomization of variables. The predictive potential of a biomarker was assessed by the treatment/biomarker interaction within a Cox PH model28. A 2-sided significance level of *P* < 0.05 was used throughout. Analyses were carried out using STATA v14 (StataCorp).

**RESULTS**

***Immunohistochemical staining and scoring***

We stained tissue cores from 303 patients: 272 patients randomized and treated in the chemotherapy arms of the ESPAC-3(2) trial7, and 31 patients randomized to observation in the combined ESPAC-1/ESPAC-3(v1) trials4-7. 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 (86.14%) were scored including 238 chemotherapy-treated patients, 115 (20.9% originally randomized) given 5FU/FA and 123 (20.9% originally randomized) given gemcitabine plus 23 patients randomized to observation. Demographics,shown in Table 1, are similar to those previously reported for the whole trial population6,14. DPD expression tumor scores in relation to clinical and pathological variables are shown in Table 2. Representative images of the different scores and their frequencies are presented in Figure 1.

***Cox regression univariate analyses***

Cox proportional hazards univariate analyses of survival by clinico-pathologic risk factors, DPD tumor 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 3. Significant prognostic factors for the entire chemotherapy treated population (both gemcitabine and 5FU/FA) were resection margin status, WHO performance status, lymph node status, tumor stage, tumor invasion into nearby organs, and DPD expression. High DPD expression was associated with reduced survival (HR 1.73, 95% CI 1.21-2.49, *p*=0.003). This difference was also 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). Tumor expression of DPD was not significantly associated with any of the other clinical or pathological factors analyzed (Table 2). Tumor 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 and interaction between biomarkers and treatment arms***

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 4). 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 the gemcitabine treated group (HR 1.62; 95% CI 0.97-2.69; p=0.065

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

In the combined chemotherapy treated populations (5-FU/FA and gemcitabine), the median 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, Figure 2). This difference remained statistically significant in the 5-FU/FA 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). Overall survival in gemcitabine treated patients was not significantly different according to DPD status (median survival 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).

Patients with high and low hENT1 tumor expression were subdivided according to high and low DPD tumor expression (Table 5 and Figure 3). As we have previously reported, high hENT1 expression was associated with favorable survival in gemcitabine-treated patients (14). DPD added no additional predictive value to hENT-1 status in gemcitabine-treated patients. Thus, in hENT1-high patients treated with gemcitabine, both DPD-low and DPD-high showed a favorable median overall survival of 26.3 (17.2-33.0) and 22.3 (9.6-39.5) months, respectively (*p*=0.360). Low hENT1 was associated with shorter survival when treated with gemcitabine, irrespective of DPD status (median overall survival for hENT1-low/DPD-low vs.hENT1-low/DPD high, 18.0 (7.6-15.30) months and 14.0 (9.1-15.7) months respectively (p=1.000)).

In hENT1-high patients treated with 5FU/FA, similarly no significant difference between DPD-high and DPD-low was observed (median overall survival 17.3 (0.6-38) and 26.0 (19.8-30.1) months respectively, p=1.000). However, in patients with low hENT1 treated with 5-FU/FA, additional analysis of DPD added significant predictive value. Thus, patients with low hENT1 and low DPD tumor expression treated with 5FU/FA had a median 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 tumor expression (χ2LR = 9.28, *p* [raw] = 0.002, *p* [post Bonferroni correction] = 0.014).

**DISCUSSION**

In the present study, intratumoral DPD expression status was analysed in the ESPAC-3(v2) population of patients with pancreatic adenocarcinoma randomized to postoperative chemotherapy with 5FU/FA or gemcitabine. Given the key role of DPD in the catabolism of 5FU, we hypothesized that low intratumoral expression of DPD would result specifically in increased overall survival in patients treated with 5FU/FU. Overall, DPD had prognostic value. However, this remained statistically significant only in patients treated with 5-FU/FA and not those treated with gemcitabine, suggesting a potential predictive role for 5-FU/FA treatment.

Given the previously reported predictive value of hENT1 tumor expression for adjuvant gemcitabine, we explored the additional predictive value of DPD tumor expression in hENT1-high and hENT1-low tumor expression subgroups. In patients with hENT1-high tumor expression treated with gemcitabine, both DPD-low and DPD-high tumor expression showed a favorable median overall survival. Similarly, in 5-FU/FA treated patients with high hENT1 tumor expression no significant difference between DPD-high and DPD-low tumor expression was observed. This suggests that if hENT1 tumor expression is high, evaluation of DPD tumor expression will not add any useful information, and these patients should generally be recommended for gemcitabine based therapy given the more tolerable toxicity profile.

In patients with hENT1-low tumor expression treated with gemcitabine, survival was poor irrespective of DPD tumor expression. These data confirm that hENT1 tumor expression is a potentially useful predictive biomarker for improved survival with adjuvant gemcitabine. However, for patients with hENT1-low tumor expression treated with 5-FU/FA, evaluation of DPD tumor expression provided additional predictive value. Patients with DPD-low tumor expression treated with 5-FU/FA survived significantly longer than patients with DPD-high tumor expression. This suggests that there is a subgroup of patients with hENT1-low tumor expression and with low DPD tumor expression that derive significant survival benefit from adjuvant 5FU/FA. Conversely, the subgroup of patients with hENT1-low tumor expression and with high DPD tumor expression has a poor survival outcome whether treated with 5FU/FA or gemcitabine. We hypothesize that the additional prognostic information from tumoral 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 hENT1-high tumor expression are likely to derive a survival benefit from gemcitabine therapy irrespective of DPD tumor expression status, and based on the toxicity profiles then gemcitabine should be the drug of choice.
2. In patients with hENT1-low tumor expression status, gemcitabine is less efficacious for improved survival. For these patients DPD tumor expression may be analysed for additional prognostic information.
   1. Patients with hENT1-low and with DPD-low tumor expression have a favorable prognosis with 5FU/FA treatment (median overall survival = 29.2 months).
   2. Patients with hENT1-low and DPD-high tumor expression have a poor prognosis whether given 5FU/FA or gemcitabine (9.7 and 14 months median overall survival respectively. In this case novel agents or combination regimens may be needed to improve survival in this subgroup,

Earlier studies investigating DPD tumor 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 11,17-23. Asian individuals handle the metabolism of fluoropyrimidines quite differently from Europeans in part due to genotypic differences such as in CYP2A6 (which converts tegafur in S-1 to 5FU)29. The present study provides novel evidence as it was performed in a randomized controlled setting in patients who were primarily of European origin, and notably receiving clearly defined single agent regimens.

Planned biomarker analyses of the ESPAC-4 population9 will assess whether hENT1, DPD and/or other tumor 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 tumor expression may be resistant to gemcitabine and 5-FU individually and also 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, intratumoral DPD expression was a negative prognostic biomarker for patients with pancreatic adenocarcinoma undergoing postoperative chemotherapy. Intratumoral hENT1 expression was confirmed to be a predictive marker for gemcitabine treatment, and the additional prognostic value of DPD tumor expression may be used to estimate the survival in patients with hENT1-low tumor expression, where DPD-low tumor expression indicates better prognosis at least for patients treated with 5FU/FA. Patients with hENT1-low and DPD-high tumor expression present a particular challenge, and novel agents and/or combination regimens will be needed to improve survival for this subgroup. Further prospective evaluation is warranted in order to define the role for hENT1 and/or DPD tumor expression in personalized approaches to adjuvant therapy in pancreatic cancer.

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**LEGENDS**

**Tables**

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

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

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

**Table 4.** Multivariate analyses for survival of clinical and pathological risk factors and DPD tumor expression in 5-fluorouracil plus folinic acid and gemcitabine treated arms.

**Table 5.** Median overall survival durations in subgroups based on combined hENT1 and DPD tumor expression status.

**Figures**

**Figure 1.** Representative images of DPD immunohistochemical staining. A = DPD-0 (negative). B = DPD-1 (weak). C = DPD-2 (moderate). D = DPD-3 (strong).

**Figure 2.** Kaplan-Meier survival curves and median overall survival for DPD-low vs. DPD-high tumor expression in the entire chemotherapy treated population (5FU/FA plus gemcitabine), 5FU/FA treated patients, gemcitabine treated patients, and the small observational (OBS) population.

**Figure 3.** 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 tumor expression status.

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**TABLES**

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

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| 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) |

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| 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%) |
| Tumor 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 | 44 (34%) | 55 (42%) | 29 (22%) | 2 (2%) | 0.696 |
|  | **R1** | 109 | 41 (38%) | 49 (45%) | 18 (17%) | 1 (1%) |
| Maximum Tumor 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%) |

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

**Table 3.** Cox proportional hazards univariate analyses of survival by clinical and pathological risk factors, DPD tumor expression (low, score=0-1; high, score=2-3), and hENT1 tumor expression (high vs. 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** |
| Tumor 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** |
| Tumor 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 Tumor 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 4.** Multivariate analyses for survival of clinical and pathological risk factors and DPD tumor expression in 5-fluorouracil with folinic acid and gemcitabine treated arms.

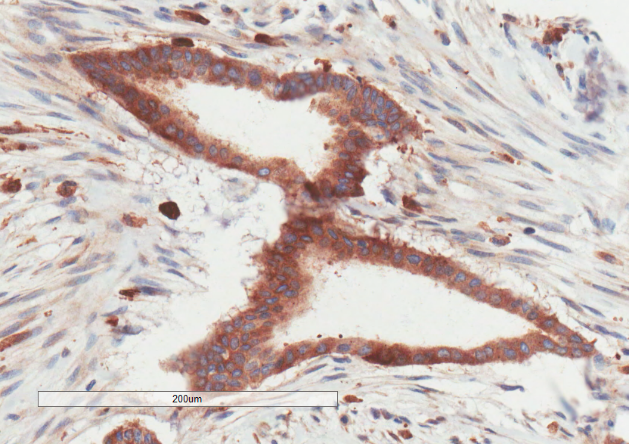
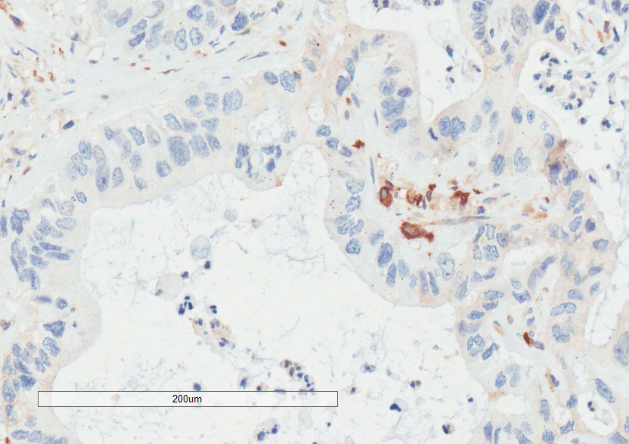
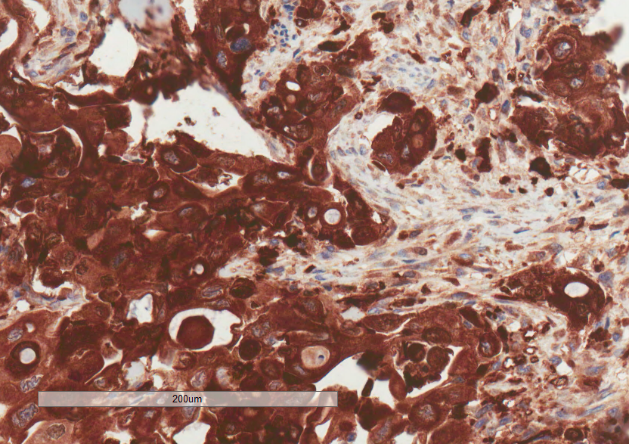
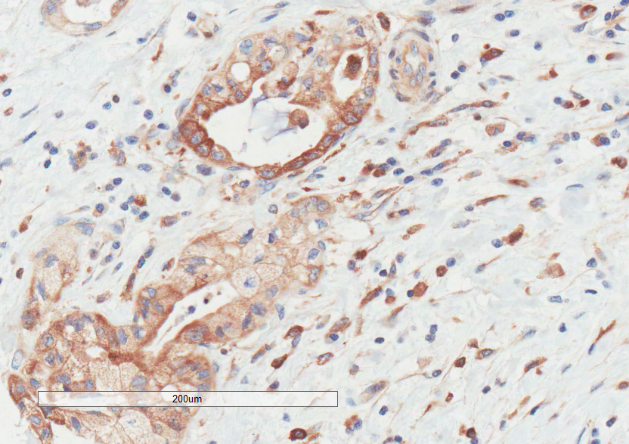
|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Multivariate Analysis | | | | | | | | |
| Variable |  | **5-fluorouracil /folinic acid**  **(*n*=115)** | | | **Gemcitabine**  **(*n*=123)** | | | |
|  | **HR (95% CI)** | **Wald χ2** | ***p*** | **HR (95% CI)** | **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 5.** Median overall survival durations in subgroups based on combined hENT1 and DPD tumor 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.** Representative images of DPD immunohistochemical staining. A = DPD-0 (negative). B = DPD-1 (weak). C = DPD-2 (moderate). D = DPD-3 (strong).



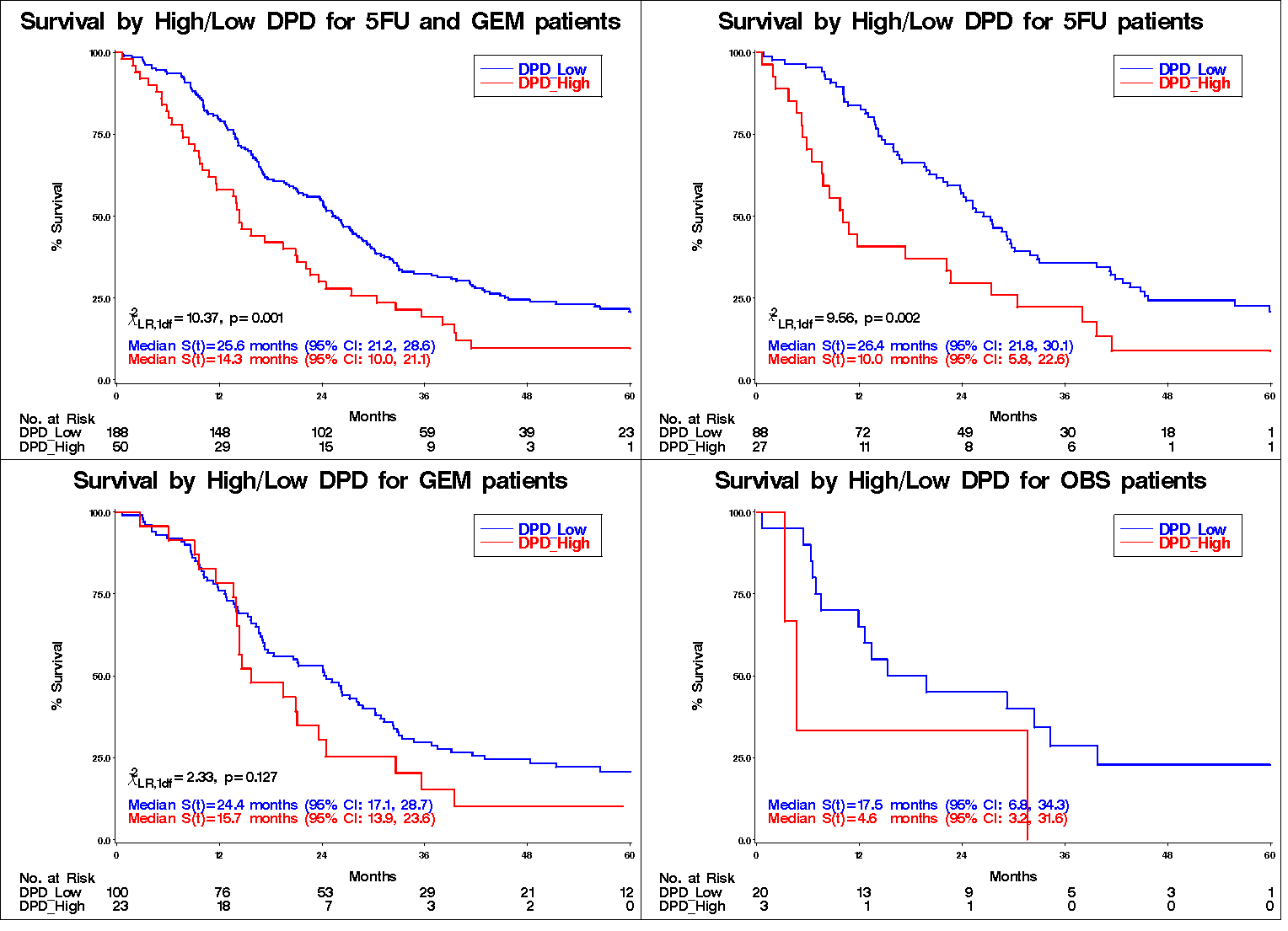
**A**

**B**

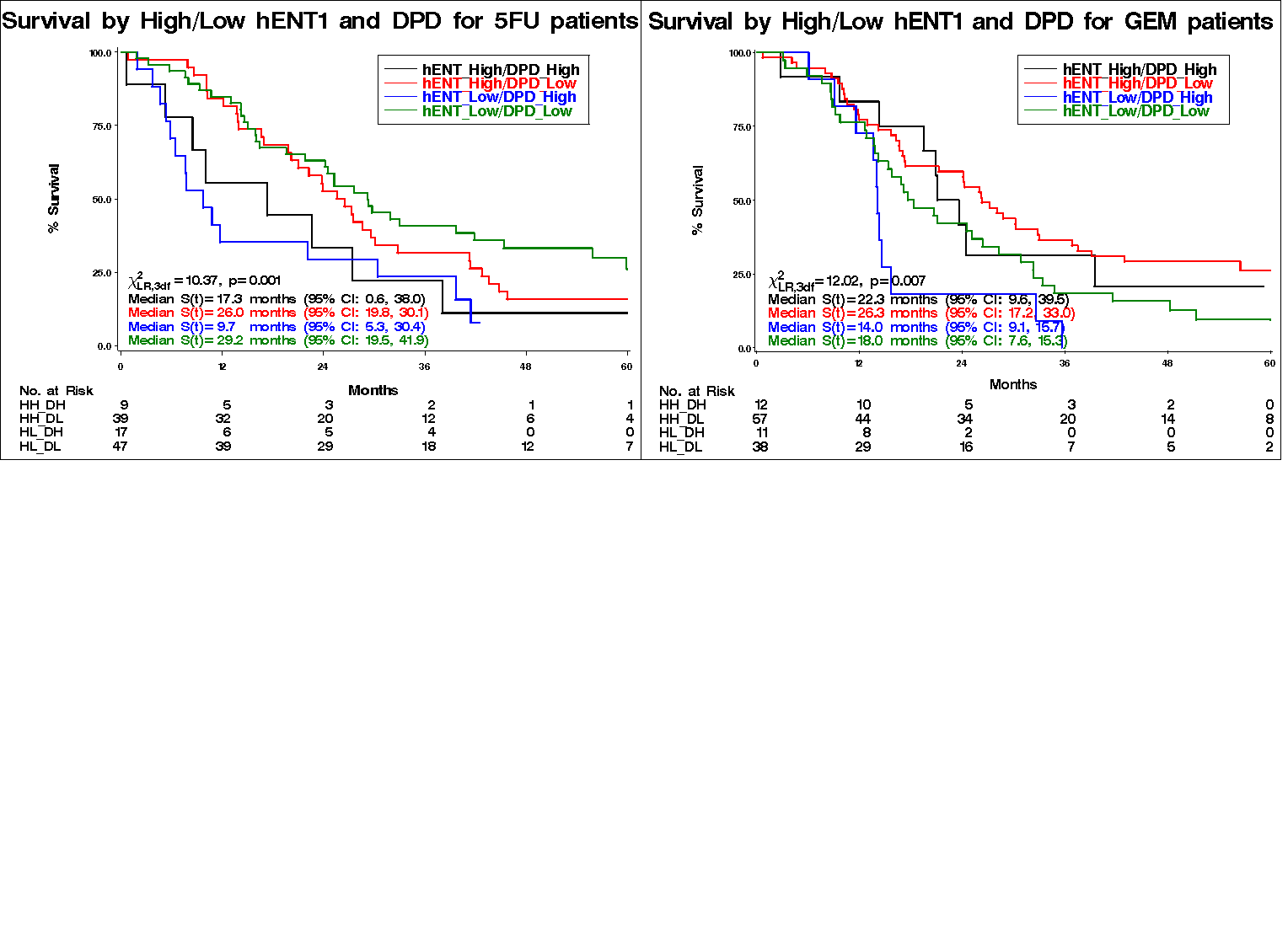
**C**

**D**

**Figure 2.** Kaplan-Meier survival curves and median overall survival for DPD-low vs. DPD-high tumor 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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**Figure 3.** 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 tumor expression status.

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