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**Cytoplasmic HuR Status Predicts Disease-Free Survival in Resected Pancreatic Cancer:**

**A Post-Hoc Analysis from the International Multi-Institutional Phase III**

**ESPAC-3 Clinical Trial**

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Running Head: HuR as a Predictive Marker in PDA

**MINI-ABSTRACT**

The mRNA binding protein HuR (*ELAVL1*) was evaluated as a predictive marker for response to chemotherapy in patients with resected pancreatic cancer. Immunohistochemical evaluation of tissue samples from the multi-institutional ESPAC-3 trial determined that high cytoplasmic HuR expression correlated with reduced disease-free survival in patients receiving adjuvant gemcitabine, but improved disease-free survival in patients receiving 5-fluorouracil.

**ABSTRACT**

**Objective:** We tested cytoplasmic HuR (cHuR) as a predictive marker for response to chemotherapy by examining tumor samples from the international, multi-institutional ESPAC-3 trial in which patients with resected pancreatic ductal adenocarcinoma (PDA) received either gemcitabine (GEM) or 5-fluorouracil (5-FU) adjuvant monotherapy.

**Background:** Previous studies have implicated the mRNA binding protein, HuR (*ELAVL1*), as a predictive marker for PDA in the adjuvant setting. These studies, however, were based on small cohorts of patients outside of a clinical trial, or a clinical trial where patients received multi-modality therapy with radiation.

**Methods:** Tissue samples from 379 patients with PDA enrolled in the ESPAC-3 trial were immunolabeled with an anti-HuR antibody and scored for cytoplasmic HuR (cHuR) expression. Patients were dichotomized into groups of high versus low cHuR expression.

**Results:** There was no association between cHuR expression and prognosis in the overall cohort (Disease-Free Survival [DFS], p=0.44; Overall Survival [OS], p=0.41). Median DFS for patients with high cHuR was significantly greater for patients treated with 5-FU compared to GEM (20.1 months, 95% CI: 8.3-36.4 vs. 10.9 months, 95% CI: 7.5-14.2; p=0.04). Median DFS was similar between the treatment arms in patients with low cHuR (5-FU, 12.8 months, 95% CI: 10.6-14.6 vs. Gem, 12.9 months, 95% CI: 11.2-15.4).

**Conclusion:** Patients with high cHuR expressing tumors may benefit from 5-FU based adjuvant therapy as compared to GEM, while those patients with low cHuR appear to have no survival advantage with GEM vs. 5-FU. Further studies are needed to validate HuR as a biomarker in both future monotherapy and multi-agent regimens.

**INTRODUCTION**

By the year 2030, pancreatic ductal adenocarcinoma (PDA) will become the second leading cause of cancer-related death in the United States.1 PDA is often asymptomatic at a curable stage, while the majority of patients present with local invasion or metastatic disease.2 Moreover, adjuvant chemotherapy after resection for PDA offers a proven, albeit limited, overall survival benefit.3-5 To date, 5-fluorouracil (5-FU), gemcitabine (GEM), and their derivatives (e.g., capecitabine) are the best available drugs for PDA treatment in the adjuvant setting and represent the current standard of care.6, 7 Previous studies have explored gene expression transcripts (i.e., *hENT1, CDA, dCK, RRM1, RRM2*) as markers to guide adjuvant therapy.8-11 However, to date there is no standardized predictive biomarker for adjuvant therapy approved for clinical use. While better, targeted treatment options are in the pipeline, strategies to select resected patients for the optimal adjuvant therapy (i.e., GEM vs. 5-FU) may provide short-term strategies to favorably improve outcomes.

We have previously evaluated the mRNA binding protein, HuR (*ELAVL1*), as a predictive marker for PDA response to adjuvant 5-FU and GEM in three separate cohorts (Table 1).12-14 Under conditions of stress in the tumor microenvironment (e.g., hypoxia, glucose deprivation, chemotherapy), HuR translocates from the nucleus to the cytoplasm and binds U- or AU- rich sequences in the 3’ untranslated region (UTR) of select target mRNAs (e.g., WEE1, PIM1, TRAIL) important for PDA cell survival and chemotherapy resistance.15-17 Our first two earlier studies suggested that increased cytoplasmic HuR expression was a poor prognostic marker in a retrospective cohort of patients undergoing resection for PDA overall.12, 13 However, in an ad hoc review of data from a phase III clinical trial by McAllister et al where patients received multi-modality therapy, HuR was not predictive of survival after either adjuvant GEM or 5-FU.14 (Table 1)

Based on this previous work, we sought to study HuR in a larger cohort of patients from a two armed, multi-institutional randomized controlled phase III trial. The European Study Group of Pancreatic Cancer trial (ESPAC3) was a National Cancer Institute (NCI)-funded, phase III trial, which randomized patients with resected pancreatic cancer to one of two approved first-line adjuvant chemotherapies.18 The results of this trial demonstrated a very limited and non-significant difference in median progression free survival (PFS) and OS between 5-FU and GEM (PFS, 14.1 vs. 14.3 months, p=0.53; OS, 23 vs. 23.6 months, p=0.39). Short of a prospective biomarker-driven randomized trial, this study served as the best cohort of patients available to study HuR as a prognostic and predictive biomarker. The absence of adjuvant radiation in this study was particularly compelling for this biomarker study since external beam radiation treatment engages HuR biologically through mechanisms that are independent from those impacted by chemotherapy.19

**METHODS**

After study approval from the Institutional Review Board at Thomas Jefferson University 1,233 tissue microarray (TMA) samples were obtained from a total of 379 patients with resected PDA enrolled in the ESPAC-3 trial. Patients included in the study received either adjuvant gemcitabine or 5-fluorouracil.

*Tissue Microarray Preparation*

An experienced pathologist evaluated tumor samples from patients enrolled in the ESPAC-3 trial after hematoxylin and eosin staining of sections. TMA samples were generated from a representative area of each tumor with four to eight cores arrayed for each patient. In total, 1,233 TMA samples were generated from the 379 patients, 589 from 186 patients who received 5-FU and 644 from 193 patients who received GEM.

*Immunohistochemical Analyses*

Antigen retrieval was performed using Discovery CCI (Ventana cat #950-500) for a total application time of 64 minutes. Primary immunolabeling was performed using HuR antibody (3A2) (Santa Cruz Biotechnology #sc-5261) at a 1:300 dilution with Ventana Antibody Dilution Buffer (cat #ADB250) for a 44-minute incubation at room temperature. Immune complexes were visualized using the ultraView Universal DAB (diaminobenzidine tetrahydrochloride) Detection Kit (Ventana cat #760-500), which uses a rabbit Horseradish Peroxidase (HRP) multimer cocktail for secondary immunolabeling. Slides were then washed with a Tris based reaction buffer (Ventana cat #950-300) and stained with Hematoxylin II (Ventana cat #790-2208) for 8 minutes.

*Scoring of the TMA*

A pathologist (WJ) with special interest in pancreatic pathology scored HuR immunolabeling in a blinded manner using a previously published scoring system.13 The pathologist assessed labeling intensity (strong vs. weak), percentage of cell labeling, and cellular localization of labeling (nuclear vs. cytoplasmic). Each sample was then graded based on the following scale: 0, no labeling; 1, weak diffuse labeling; 2, strong labeling in less than 50% of tumor cells; 3, strong labeling in greater than 50% of tumor cells. Between one and eight samples were graded per individual in the study. The graded cytoplasmic scores for each patient were averaged to obtain a cHuR score. These were analyzed in dichotomized groups identified as low cHuR (score <1.5) and high cHuR (score ≥1.5), similar to previously published reports.13

*Statistical Analyses*

All analyses were carried out using SAS version 14.0 (SAS Institute Inc., North Carolina, USA). Univariate Cox regression was carried out for all control and treatment variables for the response variables of overall survival (OS) time and disease-free survival (DFS) time. Control variables included HuR labeling, age, gender, smoking status, lymph node status, margin status, and clinical stage while treatment variables included 5-FU and GEM. Kaplan-Meier survival curves were used to explore the relationship between HuR labeling, treatment, and survival arms. Multivariable Cox regression models were built to investigate associations that appeared in exploratory analysis. These models included HuR labeling levels, treatment, and relevant control variables as covariates. The predictive utility of HuR was evaluated by testing the significance of the interaction between treatment and dichotomized HuR labeling in a Cox proportional hazards regression model. Univariate tests for association of potential confounders with HuR labeling levels were performed using Student’s T-test for normally distributed continuous variables, Wilcoxon Rank-Sum test for non-normal continuous variables, and Pearson’s chi-squared test for categorical variables. Variables associated with HuR labeling at a threshold value of p ≤ 0.2 were considered for inclusion in multivariable models.

**RESULTS**

*Evaluation of Cytoplasmic HuR Expression and Correlation with Clinicopathologic Features*

We received TMAs that included specimens from 379 patients enrolled in the ESPAC-3 trial, of which 186 were treated with 5-FU and 193 with GEM. Of these 379 patients, 75 (19.8%) had tumors with high cHuR expression and 304 (80.2%) had tumors with low cHuR expression (Figure 1). Patient demographics, treatment group allocation, and clinicopathologic features were similar between patients with high and low cHuR expression (Table 2).

*Correlation of Cytoplasmic HuR Expression and Response Outcomes*

In evaluating the entire cohort, there was no association between cHuR expression and DFS (HR=1.11, CI=0.85-1.46, p=0.44) or OS (HR=0.89, CI=0.68-1.18, p=0.41), limiting HuR’s utility as a prognostic marker in a mixed population of patients treated with 5-FU and GEM. As expected based on results from the original ESPAC-3 trial, positive lymph nodes and positive resection margins were prognostic for worse DFS and OS by univariate analysis (Table 3).

Kaplan-Meier survival curves demonstrated a differential response in DFS (Figure 2A) when stratified by cHuR expression and treatment arm (p=0.04). Patients with high cHuR who were treated with 5-FU had a near doubling of median DFS as compared to those treated with GEM (5-FU, 20.1 months, CI=8.3-36.4 vs. GEM 10.9 months, CI=7.5-14.2, p=0.012). However, median DFS was similar between the treatment arms in patients with low cHuR (5-FU, 12.8 months, CI=10.6-14.6 vs. GEM, 12.9 months, CI=11.2-15.4, p=0.44). Overall, the worst DFS was observed in patients with high cHuR who were treated with GEM. A significant differential response was not seen in OS (p=0.24), although there was a persistent trend toward improved survival in patient with high cHuR who were treated with 5-FU as compared to GEM (5-FU 27.6 months, CI=14.2-39.7 vs. GEM, 20.7 months, CI=13.9-26, p=0.08) (Figure 2B).

Unadjusted Cox regression was used to evaluate cHuR as a predictive marker for DFS and OS. This analysis found cHuR to be predictive of DFS when stratified by the treatment arm (p=0.012) (Table 4). Specifically, high cHuR was associated with improved DFS in patients treated with 5-FU as compared to GEM (HR=0.51, CI=0.31-0.85, p=0.01). In patients treated with GEM, high cHuR was associated with reduced DFS when compared to low cHuR (HR=1.54, CI, 1.08-2.2, p=0.02). Overall, cHuR was not a significant predictive marker for OS (p=0.29). There was a trend toward improved OS in patients with high cHuR treated with 5-FU as opposed to GEM (HR=0.63, CI=0.38-1.06, p=0.08). As seen with DFS, there was again a trend toward reduced DFS with GEM in patients with high cHuR (HR=1.4, CI=0.97-1.99, p=0.07).

Multivariable Cox regression was performed, adjusting cHuR expression for lymph node status and resection margins (Table 5). After adjustment, cHuR expression was no longer a significant predictive marker for DFS (p=0.11) or OS (p=0.27). There was however a trend in the multi-variate model towards improved DFS in patients with high cHuR treated with 5-FU (HR=0.64, CI=0.39-1.08, p=0.09), as well as reduced DFS with GEM (DFS, HR=1.51, CI=1.06-2.16, p=0.02; OS, HR=1.39, CI=0.95-2.05, p=0.09).

**DISCUSSION**

The search for a robust reproducible predictive biomarker for pancreatic cancer has eluded investigators for decades. In a previous study of 40 resected PDA specimens from our group, low cHuR correlated with a 7-fold increased risk in mortality in patients receiving adjuvant GEM therapy (HR=7.34, CI=2.05-26.22, p=0.0022).13 These findings were later supported in a smaller cohort from the same institution.12 In a group of 24 patients who received adjuvant GEM monotherapy, low cHuR was predictive of worse OS as compared to high cHuR (HR=2.84, CI=1.04-7.74, p=0.04). These results were attributed to the role of HuR in upregulating and activating dCK, an enzyme responsible for metabolizing GEM to its active metabolites. These data were validated by *in vitro* experiments, and supported a model where HuR sensitized tumor cells to the action of GEM by enhancing the prodrug’s conversion to its active form. Recently, McAllister et. al. assessed the predictive value of cHuR in 165 patients from the RTOG 9704 trial.14 While this study had the advantage of including a large sample of patients from a randomized trial, radiation therapy administration was interpreted as a confounder.24 Ionizing radiation complicates HuR biology by inducing phosphorylation of HuR by checkpoint kinase 2 (CHK2), which causes dissociation of HuR-mRNA complexes, altered downstream gene expression, and improved cell survival in vitro.19 In the RTOG 9704 cohort, there was no difference between low and high HuR expressing tumors with respect to DFS or OS in either arm. It was presumed that the radiation effect might have contributed to the lack of any interaction between cHuR and outcome.

However, since the publication of these studies, we and others have demonstrated through *in vivo* and *in vitro* models that HuR actually supports a therapeutic resistant phenotype in PDA through HuR-mediated overexpression of key pro-survival proteins. 20-23 Most recently, Elebro et. al. reported an association between high cHuR expression and reduced OS in gemcitabine-treated patients with pancreaticobiliary-type periampullary tumors compared to untreated controls (HR 2.07, 95% CI 1.03-4.17, p=0.028).23 These later studies conflict with the earlier work, and indicate that HuR status is more likely to promote resistance to treatment than it is to enhance drug sensitivity. For this reason, we specifically sought to analyze a cohort of samples from a rigorously accrued phase III trial with robust patient-follow up and no radiation therapy arm.

Herein, we evaluated TMA samples generated from tumors of patients enrolled in the ESPAC-3 phase III trial, where patients received either GEM or 5-FU monotherapy, without any radiation. cHuR was evaluated objectively as a prognostic marker and as predictive marker for each specific treatment. cHuR expression alone was not an informative prognostic marker for DFS or OS in the total cohort. However, in the 20% of our cohort with high cHuR expression, a significant survival advantage was seen in patients receiving 5-FU, while these patients’ tumors were relatively resistant to GEM. This translated to a near doubling in median DFS (20.1 vs. 10.9 months, p=0.04). Notably, these values were above and below the median DFS for the entire ESPAC-3 cohort, (14.3 months).7 Patients with low cHuR had no difference in median DFS with respect to treatment arm, and the values approximated the abovementioned median value for the ESPAC-3 trial. This observation provides a measure of validation for the current study, as the subgroup with low cHuR comprised the majority of the current cohort (80.2%, n=304).18 Once adjusted for lymph nodes status and positive resection margins cHuR was no longer a significant predictive marker. However there remained a trend toward improved DFS with 5-FU treatment in patients with high cHuR. The loss of statistical significance is most likely due to the small sample size of patients with high cHuR (19.8%, n=75) relative to the overall large cohort studied.

GEM and 5-FU (including capecitabine, a prodrug of 5-FU) therapy have remained the standard of care for PDA treatment in the adjuvant setting based on the results of ESPAC-3, and other phase III clinical trials.3-5, 18, 24, 25 Results from the CONKO-001 trial demonstrated a significant improvement in both DFS and OS in patients with PDA who received adjuvant GEM versus observation (DFS, 13.4 vs. 6.7 months, p<0.001; OS, 22.8 vs. 20.2 months, p=0.01).4, 5 Soon after, the Radiation Therapy Oncology Group (RTOG) 9704 trial randomized patients to receive 5-FU based chemoradiation sandwiched between GEM or 5-FU monotherapy and ultimately showed no survival benefit between the two treatment groups, but OS was on par with patients receiving adjuvant therapy in CONKO-001. ESPAC-3 evaluated 5-FU and GEM without radiation therapy in a large, phase III, randomized controlled trial and found there to be no significant difference in median progression free survival (PFS, 14.1 vs. 14.3 months, p=0.53) or OS (23 vs. 23.6 months, p=0.39) between the two treatment groups.18 Recently reported, ESPAC-4 found a small benefit in median survival with combination GEM/capecitabine (a 5-FU derivative) compared to GEM alone (28 vs. 25.5 months, p=0.032).7 Additional GEM and 5-FU based multi-agent regimens are currently being tested in phase II and III trials, providing alternative treatment strategies (NCT02243007, NCT01964430). Thus, there remains an opportunity to better select patients for specific regimens, in order to optimize treatment efficacy and minimize treatment toxicity.

Compared to our previous studies, we strongly believe this current cohort represents the best population available to study in the adjuvant setting, as all patients were enrolled in a randomized trial with long-term follow-up and no radiation confounder. We acknowledge limitations of our analysis. One limitation is that our analysis remains underpowered to detect a true difference in survival with respect to HuR status. In this cohort, only 19.8% of patients had tumors with high cHuR expression. Underscoring this point, detecting high cHuR in less than 20% of patients from ESPAC-3 does not correlate with previous scoring patterns where high cHuR was detected in closer to half the tumors evaluated in the study.12-14 Another limitation of using HuR as a biomarker is the reliance on immunohistochemistry and a subjective scoring system. Scoring is dependent on a trained pathologist and may be subject to variable interpretation by different pathologists. This current study only evaluated patients who received adjuvant 5-FU and GEM monotherapy and cannot be applied to patients receiving combination therapies (FOLFIRINOX, GEM/nab-paclitaxel), or to those with locally advanced or metastatic tumors. Still, this study provides evidence that cHuR may be predictive for 5-FU efficacy in patients with resectable PDA and high cHuR. If validated, these findings challenge prior work highlighting HuR as promoting chemotherapy resistance.20, 21 Perhaps HuR regulates specific transcripts that contribute to 5-FU sensitivity. Alternatively, high cHuR may be a marker of rapid cell division, and these cells may be particularly susceptible to 5-FU cytotoxic therapy.14

Regardless of the exact mechanism, there is an opportunity to optimize outcomes using the drugs that we currently have available, and offer an opportunity to personalize treatments for patients with PDA. As a next step, patients enrolled in the Adjuvant Pancreatic Adenocarcinoma Clinical Trial (APACT) and ESPAC4 trial would be preferred cohorts to test the predictive value of cHuR in the context of relevant combination therapies, and provide a basis for a prospective, personalized trial where treatments are based on cHuR expression.

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**FIGURE LEGENDS**

Figure 1. Pancreatic Ductal Adenocarcinoma Tissue Microarray from the ESPAC3 trial. Nuclear HuR labeling marked by solid arrow, cytoplasmic HuR labeling marked by dashed arrow A. Sample TMA, low magnification B. No cytoplasmic HuR (only nuclear HuR detected), high magnification C. Low cytoplasmic HuR (weak labeling in <50% of cells), high magnification C. High cytoplasmic HuR (strong labeling in almost 100% of cells), high magnification.

Figure 2. Kaplan Meier survival curves for A. Disease Free Survival (DFS) and B. Overall Survival (OS), stratified by average cytoplasmic HuR score group and treatment arm.