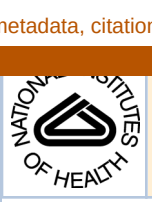

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Identification of Potential Prognostic Biomarkers in Patients with Untreated, Advanced Pancreatic Cancer from a Phase III Trial (CALGB 80303)

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Abstract

BACKGROUND—Patients with advanced stage adenocarcinoma of the pancreas have a poor prognosis. The identification of prognostic and/or predictive biomarkers may help stratify patients so that therapy can be individualized.

METHODS—Serum samples from patients enrolled in the CALGB 80303 Phase III trial, "Randomized Study of Gemcitabine with Versus without Bevacizumab in Patients with Locally Advanced or Metastatic Adenocarcinoma of the Pancreas" were used to discover novel biomarkers. For the discovery phase, 40 sera were selected based on length of survival and type of therapy, and subjected to LC-MS/MS analysis. The top features (proteins) were then further selected for validation by ELISA.

RESULTS—Quantitation by nano-LC-MS/MS resulted in 1452 peptides mapping to 156 proteins across all 40 samples, 92 of which had 2 or more peptides. After curation of the data we selected one putative prognostic protein, alpha-1 antichymotrypsin (AACT), and two putative predictive proteins, histidine-rich glycoprotein (HRG) and complement factor H (CFH) for validation by ELISA. AACT was found to be negatively correlated with overall survival ($\tau = -0.30$ (-0.38, -0.22); $p<0.00001$). There was no evidence for interaction with bevacizumab and HRG, but there was some evidence for a weak positive correlation of HRG with overall survival ($\tau = 0.11$ (0.03, 0.19); $p<0.01$). CFH was found to be neither a predictive nor a prognostic factor for overall survival.

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CONCLUSION—AACT may be a useful prognostic marker in patients with advanced stage pancreatic carcinoma, although additional validation studies are needed.

Keywords

pancreatic neoplasms; prognosis; biological markers; bevacizumab; alpha 1-antichymotrypsin

INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer related death in the United States[1]. In 2010, it is estimated that there will be 46,000 new cases of pancreatic cancer, resulting in 37,000 deaths. Less than 20% of patients have early-stage disease and the overall 5-year survival rate is less than 5%[2].

Gemcitabine has been the standard systemic therapy for advanced pancreatic cancer for the past fifteen years[3]. Many studies have explored the value of the addition of other agents to gemcitabine in an effort to improve outcomes. To date, only the oral tyrosine kinase inhibitor erlotinib (that targets the epidermal growth factor receptor pathway) has been shown to improve overall survival when added to gemcitabine, although the benefit is measured only in weeks[4].

Bevacizumab, an anti-vascular endothelial growth factor (VEGF) monoclonal antibody that has been shown to augment the effects of chemotherapy in patients with advanced cancers of the colon, breast, and lung[5–7], has also been evaluated in pancreatic cancer patients in combination with gemcitabine[8, 9]. A phase II trial of the combination of gemcitabine plus bevacizumab in patients with stage IV pancreatic cancer demonstrated an overall response rate of 21% and a median overall survival of 8.8 months[8]. These data led the Cancer and Leukemia Group B (CALGB) to evaluate this combination in CALGB 80303, a Phase III double-blind, placebo-controlled randomized trial of gemcitabine with or without bevacizumab in locally advanced or metastatic pancreatic adenocarcinoma[9].

Despite the encouraging pilot data, CALGB 80303 failed to demonstrate a survival benefit from the addition of bevacizumab to gemcitabine[9]. However, as happens in clinical practice as well as in clinical trials, some patients in either arm had appreciably longer survival than the median while others did much worse than the median. We hypothesized that the evaluation of the serum proteome of these outlier patients from the CALGB 80303 trial might lead to the discovery of novel prognostic and predictive biomarkers that could be used to stratify patients and individualize treatment.

PATIENTS AND METHODS

Patient Samples

Baseline serum samples from select patients enrolled in CALGB 80303 Phase III[9] were collected at the time of enrollment into the trial, and stored at −80 °C in a central repository. Of the 602 patients enrolled, all 253 usable serum samples from patients who signed informed consent documents were available for analysis; sera from 129 males and 124 females with a mean age of 64.2 years (range 35.8–84.2 years) were included. Clinical data including overall survival and treatment arm are shown in Table 1.

For the discovery phase, we selected sera from patients in four groups of 10 patients each, based on length of survival (long or short) and treatment arm (gemcitabine plus bevacizumab or gemcitabine plus placebo). For each arm, the 10 patients were selected at random from the tails of the survival distribution estimated by Kaplan-Meier. The “long”

and “short” survival groups had overall survival greater than 10 months and less than 2 months, respectively. This “feature selection” approach allowed us to compare different combinations of groups and search for both prognostic and predictive markers. The clinical features of each of the four discovery groups, including overall survival, treatment arm, age and gender, are shown in Table 2.

Sample Preparation for Mass Spectrometry

Sera were first depleted of 14 abundant serum proteins using Multiple Affinity Removal System (MARS)-14 spin cartridges (Agilent, Santa Clara, CA). An equal amount of protein from each of the 40 depleted samples was submitted to the Duke Proteomics Facility for trypsin digestion and LC-MS/MS analysis. Rapigest SF (Waters, Milford, MA) was added to a concentration of 0.1% (w/v), samples were reduced with 10 mM dithiothreitol at 80 °C for 15 min and then alkylated with 20 mM iodoacetamide at room temperature in the dark for 30 minutes. Proteins were then digested with modified porcine trypsin (Promega Gold, Promega, Madison, WI) at a protein to trypsin ratio of 50:1 overnight at 37 °C. Proteolysis was quenched and Rapigest was cleaved with the addition of trifluoroacetic acid and acetonitrile to 1% and 2% (v/v), respectively. The samples were each spiked with 50 fmol yeast alcohol dehydrogenase (MassPrep, Waters) per μg sample protein as an internal standard.

Mass Spectrometry Analysis and Data Processing

Liquid chromatography-Tandem Mass Spectrometry (LC-MS/MS) analysis was performed independently on each of the 40 patient samples, in random order, using a nanoAcquity liquid chromatograph (Waters) coupled via nanoelectrospray ionization to a QToF Premier mass spectrometer (Waters). Thirty-seven samples were analyzed once using a method that alternated between an MS scan and MS/MS interrogation of the single most intense multiply-charged ion, in order to obtain at least 10 points across each chromatographic peak for accurate label-free peptide quantification. Three randomly selected samples were analyzed in this fashion, but in triplicate in order to assess analytical variability of the LC-MS/MS platform. Finally, 15 samples were chosen for an additional analysis with another MS/MS method that interrogated the top 3 precursors by tandem MS in real-time, to deepen proteome coverage for the overall experiment. These particular runs were used only to supplement identifications and were not included in the quantitative analysis.

All data from the 61 LC-MS/MS runs were loaded into Rosetta Elucidator v3.3 (Rosetta Biosoftware, Seattle, WA) for alignment, feature extraction and quantification. The Elucidator package also generated 93,874 MS/MS spectra, which were searched against the Swissprot database with human taxonomy (v57.1, www.expasy.org) using the Mascot v2.2 search engine (Matrix Science, Inc., Boston, MA). Search tolerances were 20 ppm precursor and 0.04 Da product ion tolerance, semitryptic enzyme specificity, and a maximum of two missed cleavages. Cysteine was searched as a fixed carbamidomethyl derivative, and dynamic modification was allowed on methionine (oxidation), asparagine (deamidation) and glutamine (deamidation). Validation of MS/MS spectra was performed using PeptideProphet and ProteinProphet algorithms, as implemented in Elucidator v3.3; peptides with a PeptideProphet score greater than 0.67 were annotated. This corresponded to a 0.5% peptide FDR, as determined with reverse decoy database validation. This analysis resulted in the quantification of 1452 peptides in 156 proteins across all 40 samples, 92 of which had 2 or more peptides.

Analysis of Peptide Data

The objective of this phase was to select features from among these peptides for further validation in the entire patient cohort. To identify features with the potential to discriminate

patients with respect to survival (long versus short), the Wilcoxon rank sum test was used. To identify features potentially predictive for long survival the interaction term from a two-way multiplicative analysis of variance quasi-logistic model was used. The features were ranked with respect to absolute values of the corresponding test statistics. The top features were then further selected for validation based on the quality of the peptide data, as well as biological relevance to cancer.

ELISA

Following analysis, three proteins were selected for validation by ELISA. Serum levels of complement factor H (CFH), histidine-rich glycoprotein (HRG), and alpha 1-antichymotrypsin (AACT) were determined. Quantitative competitive ELISAs were used to measure the levels of CFH and HRG, and a sandwich ELISA was used for AACT. For the competitive ELISAs, purified CFH (Complement Technology, Inc., Tyler, TX) or HRG were biotinylated (EZ-Link Sulfo-Biotin Kit, Thermo Scientific, Waltham, MA), mixed with diluted serum, and allowed to interact with immobilized capture antibody specific for each protein. HRG was purified from a healthy volunteer's serum by affinity capture on Ni-NTA His-Bind Resin (EMD Chemicals, Gibbstown, NJ) in the presence of 20 mM imidazole, removing unbound or loosely bound proteins by sequential washes with 20 and 100 mM imidazole, and elution with 500 mM imidazole. Captured biotinylated proteins were detected with a streptavidin-HRP conjugate and ABTS/H_2O_2. The amount of CFH or HRG in the serum was calculated from standard curves consisting of serial dilutions of purified protein interacted with capture antibody in the presence of biotinylated protein. Standard curves were run on each ELISA plate at the same time as the experimental samples using the same conditions.

Serum AACT was quantified using a matched pair of capture and detection antibodies (H00000012-AP11, Novus, Littleton, CO) as recommended by the manufacturer. Standard curves were constructed using purified AACT (GenWay, San Diego, CA).

ELISA Data Analysis

For each marker, the association between the serum level measured by ELISA and overall survival was estimated using the Kendall tau correlation coefficient. As overall survival is subject to right-censoring, the extension proposed by Akritas and Siebert[10] was used. The null sampling distribution of the test statistic was approximated using B=100,000 permutation replicates. For each association parameter, a 95% confidence interval was constructed using B=100,000 bootstrap replicates. For each marker, the interaction between serum level and bevacizumab was tested within the framework of a log-linear multiplicative Cox model. The p-values and confidence intervals provided have not been adjusted for multiple testing.

Statistical analyses were conducted by CALGB statisticians. A flow diagram describing the sequence of events is shown in Fig. 1.

RESULTS

Discovery Phase: Protein Identification and ELISA

Quantitation by nano-LC-MS/MS resulted in 1452 peptides mapping to 156 proteins across all 40 samples, 92 of which had 2 or more peptides. Feature selection to identify putative prognostic or predictive markers was performed initially on peptide data. This analysis identified 184 peptides, mapping to 26 proteins, whose abundances were significantly different between long and short survivors, indicative of prognostic markers. The analysis also identified 60 peptides, mapping to 17 proteins, whose abundances were seemingly

correlated with response to bevacizumab, indicative of predictive markers. After these assignments were made, we eliminated from further consideration all proteins that had any of the following characteristics: 1) Proteins that were targeted for depletion by the MARS-14 spin cartridges, or 2) whose peptide intensities were essentially indistinguishable from baseline, or 3) whose statistically significant prognostic or predictive peptides represented 15% or fewer of the total number of peptides identified for that protein, or 4) that were identified by 2 or fewer total peptides, or 5) that had some prognostic and some predictive peptides, or 6) that had sufficient homology to depleted proteins as to make their concentration after depletion suspect, or 7) whose level is known to be affected by coagulation in the blood collection tube.

Validation Study

The serum concentrations of AACT, CFH and HRG, as determined by ELISA, were correlated with overall survival for 253 patients (Fig. 2). AACT was found to be negatively correlated with overall survival ($\tau = -0.30$ (-0.38, -0.22); $p < 0.00001$) (Table 3). Although HRG was initially identified as a potential predictive marker in the discovery phase, there was no evidence for interaction with bevacizumab when the levels of the intact protein were used in place of individual peptide levels. There is some evidence for a weak positive correlation of HRG with overall survival ($\tau = 0.11$ (0.03, 0.19); $p < 0.01$. There was no evidence that CFH, identified as a potentially predictive marker, was either a predictive or prognostic marker for overall survival.

DISCUSSION

The identification of prognostic and/or predictive biomarkers has the potential to refine patient selection so that therapy can be individualized. For example, biomarkers could help determine which patients might avoid unnecessary therapy either because they already have a favorable prognosis without treatment, or because their tumor is not predicted to respond to treatment. In other patients, biomarkers may predict that therapy could prolong survival and that knowledge could outweigh the risk of treatment-related toxicity.

Because there was no therapeutic difference amongst the arms of CALGB 80303, serum from outliers on either treatment could be analyzed. For the current study we used a quantitative proteomics platform to search for prognostic and predictive biomarkers in patients with advanced pancreatic carcinoma. This was accomplished by subjecting the sera to depletion of the 14 most abundant proteins, followed by nano-LC-MS/MS. The validation phase, however, employed unaltered serum, with the goal of developing an assay that would be useable in clinical practice.

Analysis of the MS data produced several candidate markers, and we focused our validation studies on the three proteins that survived robust manual curation of the data. Our results suggested that increasing levels of AACT are associated with a poorer outcome, and that patients with a low level of the protein had longer survival. These results need to be confirmed in independent validation trials, but are similar to those of a previous study that found an association between AACT and pancreatic cancer[11]. Additional studies have shown correlations between the concentration of AACT and both advanced stage and poor prognosis in gastric cancer[12] and lung adenocarcinoma[13].

AACT is a member of the serpin (*ser*ine *p*rotease *in*hibitor) superfamily. Serine proteases expressed in the tumor microenvironment are essential to the tissue remodeling seen in malignant progression. While some protease inhibitors inhibit tumor progression (*e.g.*, maspin, which is down-regulated in invasive breast carcinoma[14]), others, including AACT, appear to promote tumor progression[15]. It was shown that tumors from a metastatic breast

cancer cell line had higher levels of expression of AACT than those produced from a non-metastatic cell line[16]. Additionally, malignant breast tumor cells induced local AACT expression by host cells at primary and secondary tumor sites[16]. Although these experiments indicate a role for AACT in tumor progression, the exact mechanism of action has not been elucidated.

Of the two putative predictive markers found from the MS-based discovery phase, neither retained a significant relationship between response to combined therapy and outcome when tested with whole serum by ELISA. The serum concentration of HRG did, however, display a marginal association with overall survival. Since HRG has been found to possess antiangiogenic properties in tumors, a positive association with survival is understandable[17]. A more thorough elucidation of the role of HRG in pancreatic cancer will require additional investigation.

While the literature is replete with protein expression profiling studies in search of biomarkers, it is becoming apparent that there are fundamental limitations to this type of discovery approach. First, most discovery studies measure altered protein abundance, but do not evaluate post-translational modifications of proteins or alterations in protein amino acid sequence as a result of mutation. Thus, important distinguishing features of tumor protein expression may be missed. Second, there can be methodological limitations to biomarker discovery. We observed an inconsistent correlation between the peptide abundance by quantitative nano-LC-MS/MS and the protein level by ELISA. While it has been shown that label-free direct quantitation, as was used here, can be an effective method to compare relative peptide abundances among comparable samples[18], it is not guaranteed that these peptide abundances will necessarily correlate with those of the intact proteins to which the peptides map. One possible reason for this is ion suppression, which occurs when co-eluting peptides compete for ionization and, hence, detection by the mass spectrometer[19, 20]. This can result in the underestimation of the concentration of one or more peptides relative to the amount of intact protein present in the original complex mixture.

In addition, we depleted the most abundant proteins from serum for the discovery phase but used whole serum for validation by ELISA. While antibody-based depletion methods, like the MARS columns used in the current study, have been shown to be robust and reproducible with respect to the targeted proteins, problems with depletion of off-target proteins remain[21]. In our studies, peptides mapping to HRG exhibited a statistically significant correlation with response to bevacizumab yet the intact protein, as quantified by ELISA did not. HRG is known to bind immunoglobulins[21] and could have been co-depleted to varying degrees along with the intended proteins. Likewise, the serum level of CFH could have been altered during the targeted depletion of complement C3, which is a known binding partner.

Finally, and just as importantly, it is uncertain whether differentially expressed tumor proteins will have sufficient abundance to produce a significant change in systemic levels. Thus despite significant advances in proteomic technology, discovery programs may be limited to a common set of relatively abundant host response proteins, which may be important for tumor formation or inhibition, but may lack specificity to define a tumor cell type.

While the theoretical considerations for employing biomarkers appear logical, the reality of introducing relevant diagnostics has been problematic. Since tumors are heterogeneous, it is unlikely that a single biomarker will have sufficient power to govern clinical practice. The solution has been to develop a panel of differentially expressed markers from platforms that interrogate a large number of proteins. In this discovery scenario, it is not difficult to find

potential markers as there are a large number of features (*i.e.*, peptide signals in this study) relative to the number of subjects.

Additionally, mining algorithms can be unstable and may overfit the data in this setting. This generates a number of potential leads, thus requiring significant resources for assay development and validation in independent trials. When not accounting for multiple testing, this approach is certain to yield a significant number of false positive results, which do not come to fruition on further analysis. Although proteomic techniques are becoming increasingly advanced, it still appears that novel strategies will be needed if this field is to make significant advances in clinical diagnostics.

By incorporating a detailed correlative sciences plan into the design of CALGB 80303, biological specimens could be probed and information could be gained despite the lack of a treatment effect of the experimental arm. This enabled the identification of a possible prognostic marker (AACT) for pancreatic cancer, which can now be tested in a prospective trial. If it is eventually validated, future trials could possibly use AACT in conjunction with other markers to stratify patients in the effort to enrich for patient populations more or less likely to benefit from given treatments.

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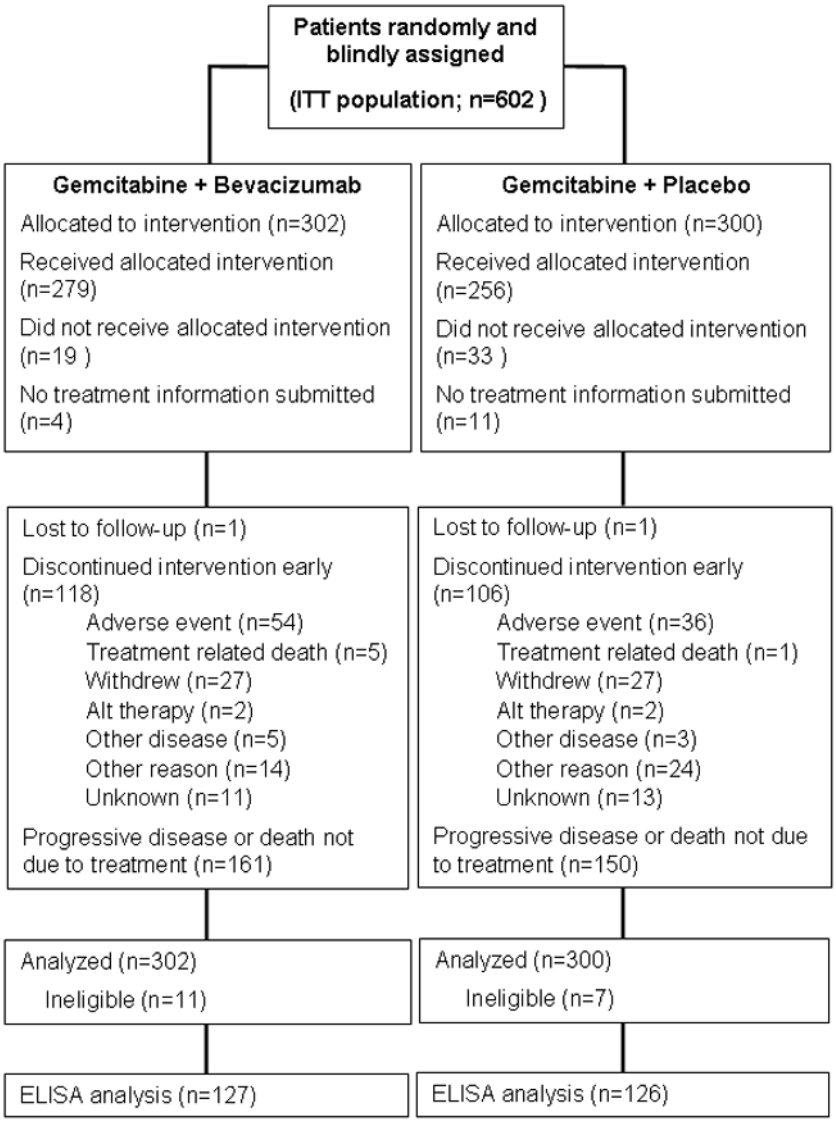

Figure 1.
Patient cohort and analysis flowchart.

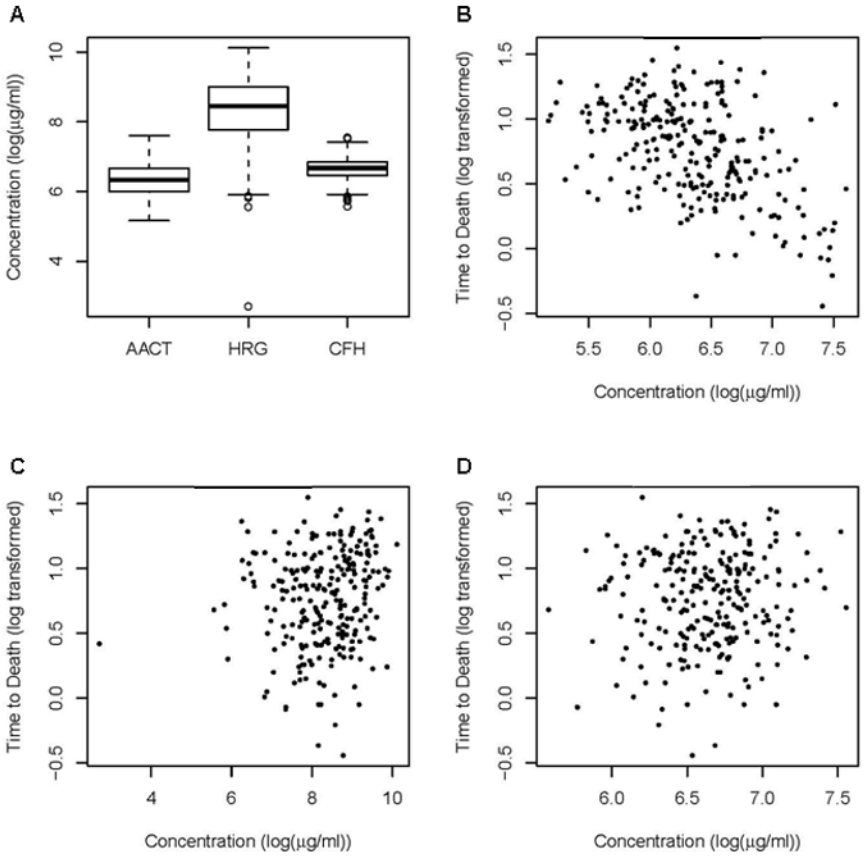

Figure 2.

Empirical distribution of ELISA concentrations (log transformed) for AACT, HRG and CFH in the study population (panel A); Scatter plot of time to death in years (log base 10 transformed) and concentrations of AACT, HRG and CFH (log transformed (panels B, C and D).