A novel biomarker panel examining response to adjuvant pancreatic cancer therapy in NRG Oncology RTOG 9704

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Running head: Pancreatic Cancer Biomarkers from NRG Oncology RTOG 9704

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Abstract

Background: NRG Oncology RTOG 9704 was an adjuvant pancreatic cancer trial randomizing resected patients to fluorouracil (5-FU) and chemoradiation versus gemcitabine and chemoradiation.

Methods: Of 451 participants successfully enrolled in the trial, 213 have archived baseline serum samples available for study. Fifty-six potential protein biomarkers in pancreatic cancer were identified, and by using the proximity ligation assay (PLA), these proteins were quantified from each of the serum samples. A univariate Cox proportional hazards model was used to examine the impact of each PLA biomarker on overall survival.

Results: Fifty-six proteins were quantified from 213 NRG Oncology RTOG 9704 patient serum samples. Elevated CEA, CA 125, and CA 19-9 levels above the median were prognostic and associated with worse outcome. MMP-7 was predictive of response to adjuvant gemcitabine and chemoradiation, but not adjuvant 5-FU and chemoradiation. The median survival for patients in the gemcitabine and chemoradiation arm with MMP-7 levels below the median was 24 months, but only 18 months with MMP-7 levels above the median ($p=0.0001$).

Conclusions: PLA is a powerful technique for identifying potential biomarkers from small volumes of archived serum.

Impact: MMP-7 may be a predictive biomarker in determining benefit from adjuvant gemcitabine chemotherapy.

Introduction

Pancreatic cancer is the fourth most lethal malignancy in the United States, with over 48,000 new diagnoses expected in 2015 [1]. Though there has been recent improvement in the treatment of metastatic disease, the best chance for cure remains with surgical resection of early-stage disease. Even with surgical resection, patients are at significant risk for both local and systemic recurrence. Multiple strategies have been employed to reduce this risk though with limited efficacy. Systemic chemotherapy after surgery has provided the most benefit for patients, but even these improvements are modest - six months of adjuvant gemcitabine only improved 5-year survival from 10% to 21% [2]. Radiation with concurrent chemotherapy has also been explored, but the marginal benefit when given in addition to a course of systemic therapy has not been fully defined [3-5]. The ongoing RTOG 0848 trial is attempting to clarify the role of chemoradiation.

Improved adjuvant strategies are needed – either via novel drugs, or by using the chemotherapy and targeted agents available today more effectively. Given the heterogeneity in tumor and patient phenotype, tailoring therapy based on specific molecular pathways may be the best approach for maximizing the therapeutic ratio. These tailored treatment strategies can be developed in many ways, but the most straightforward is to analyze completed clinical trials, obtain archived specimens from those trials, and identify biomarkers that can predict response to therapy. A challenge in this approach is conserving but maximizing the limited amount of archived specimens available.

The proximity ligation assay (PLA) is a powerful tool that can be used to identify new biomarkers, as it is capable of quantifying dozens of proteins from microliter volumes of archived serum. To detect a protein of interest, two protein-specific antibodies that have been

conjugated with engineered oligonucleotides bind the protein in close proximity to each other (Figure 1). The conjugated oligonucleotides on the adjacent antibodies can then approximate end-to-end and bind a third "linker" oligonucleotide added to the media, forming dsDNA. A positive signal is achieved once this strand of dsDNA is formed. A unique "barcode" sequence engineered within the dsDNA can be quantified using qPCR techniques. PLA is sensitive and specific because it yields minimal background and requires two antibodies to generate a signal [6-8].

Figure 1. *Whereas ELISA requires one primary antibody to generate a signal (A), PLA requires two antibodies (B). If only one antibody binds the protein of interest in PLA, no signal is generated (C).*

NRG Oncology RTOG 9704 was an adjuvant randomized phase III clinical trial using a combination of chemoradiation and systemic chemotherapy to treat patients with resected pancreatic cancer [9]. Of 451 patients successfully enrolled in the study, 230 were randomized to 5-FU, followed by 5-FU with radiation and more 5-FU, while 221 received gemcitabine, followed by 5-FU with radiation and more gemcitabine (Figure 2). There was no significant difference in efficacy between the two treatments. For patients with pancreatic head lesions, there was a trend toward improved survival in the gemcitabine arm. Serum samples drawn at baseline were archived and made available for further analysis.

Figure 2. *CONSORT diagram for NRG Oncology RTOG 9704*

To identify biomarkers that predict response to adjuvant therapy, a panel of PLA probes capable of quantifying serum proteins of interest in pancreatic cancer was developed. This panel was then applied to baseline serum samples from the adjuvant pancreatic cancer trial NRG Oncology RTOG 9704, and the results were correlated with the clinical outcome data.

Methods

Target Identification

Biomarkers of interest were identified through query of the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) for encoding genes upregulated in pancreatic adenocarcinoma and by literature review. Due to the lack of commercially available antibodies for many of the biomarkers identified, PLA probes could only be built for a subset of these targets.

PLA Probe Construction

Two sets of PLA probes were used in this project.

The first set was used in prior publications [7,8]. These probes were constructed by Solulink (San Diego, CA) using proprietary technology with a mixture of monoclonal and polyclonal antibodies and have been stored long term at -20°C.

The second set of probes was constructed specifically for this project using Antibody-Oligonucleotide All-in-One Conjugation Kits (Solulink). Antibodies were affinity purified, polyclonal (R&D Systems). Oligonucleotides were prepared by the Stanford University Protein and Nucleic Acid Facility and by Integrated DNA Technologies (Coralville, IA).

PLA Probe Validation

To validate the newly constructed probes, serum PLA was performed as described previously (6-8). In place of the target antigen, species-specific secondary antibodies were used to hold the PLA probe antibodies together in close proximity to generate a signal. For example, to validate PLA probes built with goat antibodies, bovine anti-goat antibodies were used as a

substitute for the costly purified target protein. Probes were considered validated if they were able to generate a standard curve under this condition.

Probe Panel Construction

A 56-probe PLA panel was designed based upon the previously identified targets of interest in pancreatic cancer (Table 1). The panel contained seven eight-probe subpanels, which were assayed individually. Each probe within a subpanel had a unique DNA barcode, and only validated probes were included. Probes in each subpanel were carefully selected to ensure that there was no cross-reactivity with other probes in the subpanel.

Blood samples from NRG Oncology RTOG 9704 were drawn up to 21 days prior to randomization, or if after randomization, prior to protocol treatment. Of 213 baseline serum samples provided, 121 samples had been previously thawed once, and 92 samples had been previously thawed twice. The former samples were thawed on ice for eight minutes, then at room temperature (RT) for 14 minutes. Once thawed, samples were placed back into storage at -80°C so that all samples had undergone the same number of freeze-thaw cycles.

To prepare for the assay, the serum samples were randomly organized over three 96-well plates. The serum was thawed at RT, mixed 1:1 with a proprietary blocking solution for ten minutes at RT, divided among eight sets of three 96-well plates, and stored at -80°C. Seven of the sets were intended for the assay, with one reserved for back-up.

Proximity Ligation Assay

For each of the seven PLA probe subpanels, one set of three 96-well serum plates was thawed at RT. PLA probe mixture was added to the blocked serum and incubated at 37°C for two hours. ssDNA probe splints with ligase were then added by robotic pipette (Velocity11,

Agilent Technologies), followed by incubation at 30°C for 15 minutes. Uracil-DNA Excision Mix (Epicentre) was added to stop ligation, again using robotic pipetting to ensure consistent timing. The resulting bar-coded DNA was amplified with 13 cycles of PCR. All serum samples for each probe panel were amplified simultaneously under identical conditions using a Tetrad thermal cycler (Bio-Rad). The PCR product was diluted in Tris EDTA buffer (TE), divided among eight sets of second-generation plates (one set for each PLA probe), and stored at -20°C for future use.

To determine the signal of one PLA probe, a set of second-generation plates was brought to room temperature. The PCR product in TE was transferred to a 384-well plate by robotic pipette, combined with iTaq SYBR Green Supermix (Bio-Rad) and primers appropriate for the PLA probe of interest, and amplified using quantitative PCR (Model 7500, Applied Biosystems). A DNA standard curve was used to transform the qPCR output into a relative measure of PLA units.

Statistical Analysis

The raw PLA unit data was initially screened in the laboratory. The assay included 56 PLA probes, though two became desiccated in storage, and 12 yielded inadequate signal leaving 42 available for subsequent analysis.

The central screen to identify potential prognostic or predictive factors used a univariate Cox proportional hazards model examining the impact of each PLA biomarker on overall survival (analyzing the PLA biomarker as a continuous variable). Survival was measured from the date of randomization through the date of last follow-up or death. As the scales of different PLA biomarkers varied substantially, each biomarker was normalized to its interquartile range (difference between 1st and 3rd quartile) prior to analysis. This normalization does not change the

analysis, though it makes the individual PLA hazard ratios more comparable to each other. To reduce the false positive rate of this initial screen, a Bonferroni corrected p-value was used to account for multiple statistical testing. Forty-two probes were analyzed, and a significant p-value in the initial screening analysis was $p<0.00119$. Prognostic biomarkers were defined as those that impacted survival in both treatment arms, whereas predictive biomarkers were defined as those with a significant interaction between the biomarker and overall survival. Analyses were conducted with SAS (version 9.2).

Results

Among the 451 patients in NRG Oncology RTOG 9704, 213 (47%) had serum samples drawn prior to treatment. In total, 101 were in the gemcitabine arm, and 112 were in the 5-FU arm. There were no statistically significant differences between patients with and without available serum in terms of age, gender, race, tumor location, performance status, stage, tumor size, surgical margins, or treatment arm (data not shown).

The screening of the PLA markers to identify prognostic or predictive factors is summarized in Figure 3. Three of the 42 probes analyzed demonstrated prognostic value for the entire patient population: increased CA 19-9, CEA and CA 125 were all associated with decreased survival. MMP-7 was the sole predictive biomarker ($p=0.00117$). Low levels of MMP-7 predicted improved survival in the adjuvant gemcitabine group (HR 1.39, 99.88% CI 1.05-1.83, $p=0.0001$) but not in the 5-FU group (HR 0.96, 99.88% CI 0.73-1.25). Figure 4 demonstrates disease-free survival and overall survival for MMP-7 divided by its median value and stratified by treatment arm. Among patients in the gemcitabine arm, those with low MMP-7 ($<$ median value) had a median survival of 24 months compared to 18 months among those with high MMP-7 ($\geq$ median value). MMP-7 remained an independent significant predictor of

survival in gemcitabine treated patients on multivariate analysis controlling for age, gender, race, tumor location, T-stage, N-stage, margin status, and pre-treatment CA 19-9 (HR 1.45, 95% CI 1.23-1.70, p<.0001).

Figure 3. *Univariate Survival Analysis of PLA Biomarkers (Hazard ratios with 99.88% CI). Filled in diamonds indicate statistically significant data.*

Figure 4. *Survival Curves for Matrix Metalloproteinase-7 (MMP-7)*
MMP-7 < median: MST = 1.99 years
MMP-7 ≥ median: MST = 1.52 years

Discussion

Pancreatic cancer continues to be a highly lethal malignancy. Though recent advances have made significant improvements in the treatment of metastatic disease, these advances have yet to translate into benefit in the adjuvant setting. Adjuvant gemcitabine with or without chemoradiation remains the standard of care with median overall survival of 20-23 months [2,10].

This study sought to identify biomarkers from the completed NRG Oncology RTOG 9704 clinical trial to predict benefit from adjuvant therapy. Of 56 intended probes, only 42 yielded adequate signal for analysis. Of these, only four probes yielded statistically significant data – CA 19-9, CEA, CA 125, and MMP-7.

CA 19-9 is a sialylated form of Lewis A antigen that is highly expressed in the tumors of many pancreatic cancer patients [11]. In this study, high levels of CA 19-9 were prognostic for decreased survival. CA 19-9 levels had been assayed previously from baseline NRG Oncology RTOG 9704 serum samples using ELISA, and high levels were also prognostic for decreased survival [12]. Multiple benign conditions can elevate CA 19-9, including hepatitis, gallstones, and pancreatitis [13,14]. Five percent of the population is unable to produce Lewis A antigen

and is therefore unable to produce CA 19-9 [11]. A meta-analysis including 2,283 patients demonstrated that elevated CA 19-9 levels have a median sensitivity of 70% and specificity of 82% for the diagnosis of pancreatic cancer [15].

Carcinoembryonic antigen (CEA) and CA 125 have also been described previously as biomarkers in pancreatic cancer, and high levels were prognostic for decreased survival. CEA and CA 125 are cell surface glycoproteins, and both are associated with multiple tumor types. Duraker et al reported 181 patients with malignant and non-malignant pancreatic disease, and the sensitivity of elevated CEA for the diagnosis of pancreatic cancer was 39.0% with a specificity of 91.4%, while the sensitivity of elevated CA 125 was 56.9% with a specificity of 77.6% [16]. Like CA 19-9, serum CEA and CA 125 levels can be influenced by multiple benign conditions. In particular, CA 125 can be affected by ascites due to liver disease and pleural fluid due to heart failure [17,18], while CEA levels can be affected by smoking, cirrhosis, and hepatitis [19,20].

Matrix metalloproteinase-7 (MMP-7) is a protease expressed on 98% of pancreatic cancers and is thought to influence ductal metaplasia in pre-malignant pancreatic lesions [21]. MMP-7 promotes invasiveness and metastasis, and high serum MMP-7 levels correlate with decreased survival [22,23]. In this study, high MMP-7 serum levels predicted worse disease-free and overall survival in patients treated with gemcitabine, but not 5-FU. Clinically, these data predict that the subset of patients with low MMP-7 levels benefit the most from gemcitabine-based chemotherapy. The mechanism to explain these results is not clear but these data imply that gemcitabine may be active in a cytotoxic pathway that is modulated by regulating MMP-7.

It is important to note that serum PLA is currently intended for research purposes. The hallmark of this particular experiment was that all of the samples were assayed simultaneously under nearly identical conditions so that the results could be compared to each other in a relative

manner. Rigorous controls and standards would be needed to process samples individually for comparison. PLA is an ideal assay for use in biomarker discovery because multiple proteins may be assayed for simultaneously from small amounts of starting material.

It is also important to note that this particular PLA panel was built as a screening assay. Though the probes were built with a variety of control measures, they were not tested against commercially-available target peptides due to prohibitive cost. The assumption was that the affinity-purified polyclonal antibodies used to build the probes would bind their target antigens as per the stated claims of their commercial sources. Biomarkers that yielded negative results need to be evaluated cautiously, as it is possible that the cognate PLA probe did not function properly due to technical reasons.

Conclusion

PLA is a powerful tool for identifying potential biomarkers from small volumes of archived serum. Low MMP-7 levels may predict benefit from adjuvant gemcitabine in patients with resected pancreatic cancer. Prospective validation of the results is warranted.

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Figure 1. Whereas ELISA requires one primary antibody to generate a signal (A), PLA requires two antibodies (B). If only one antibody binds the protein of interest in PLA, no signal is generated (C).

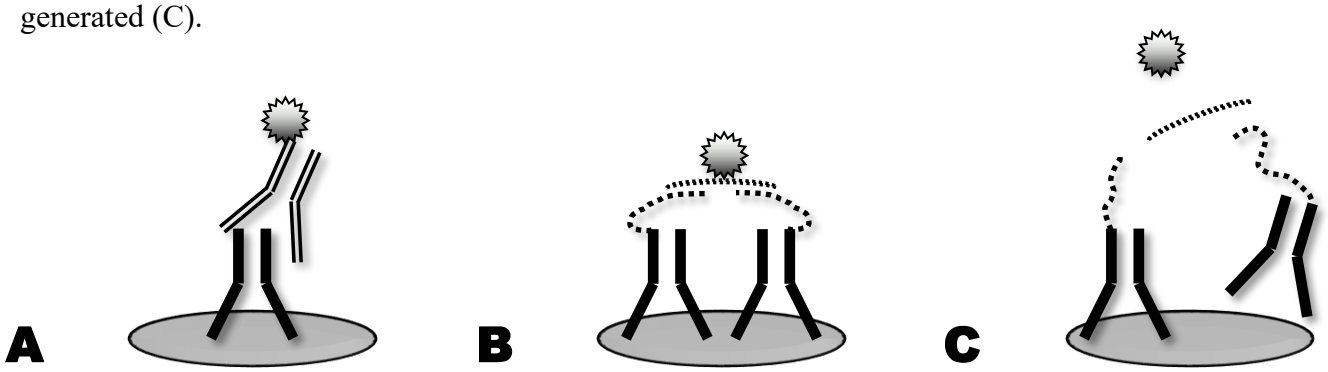
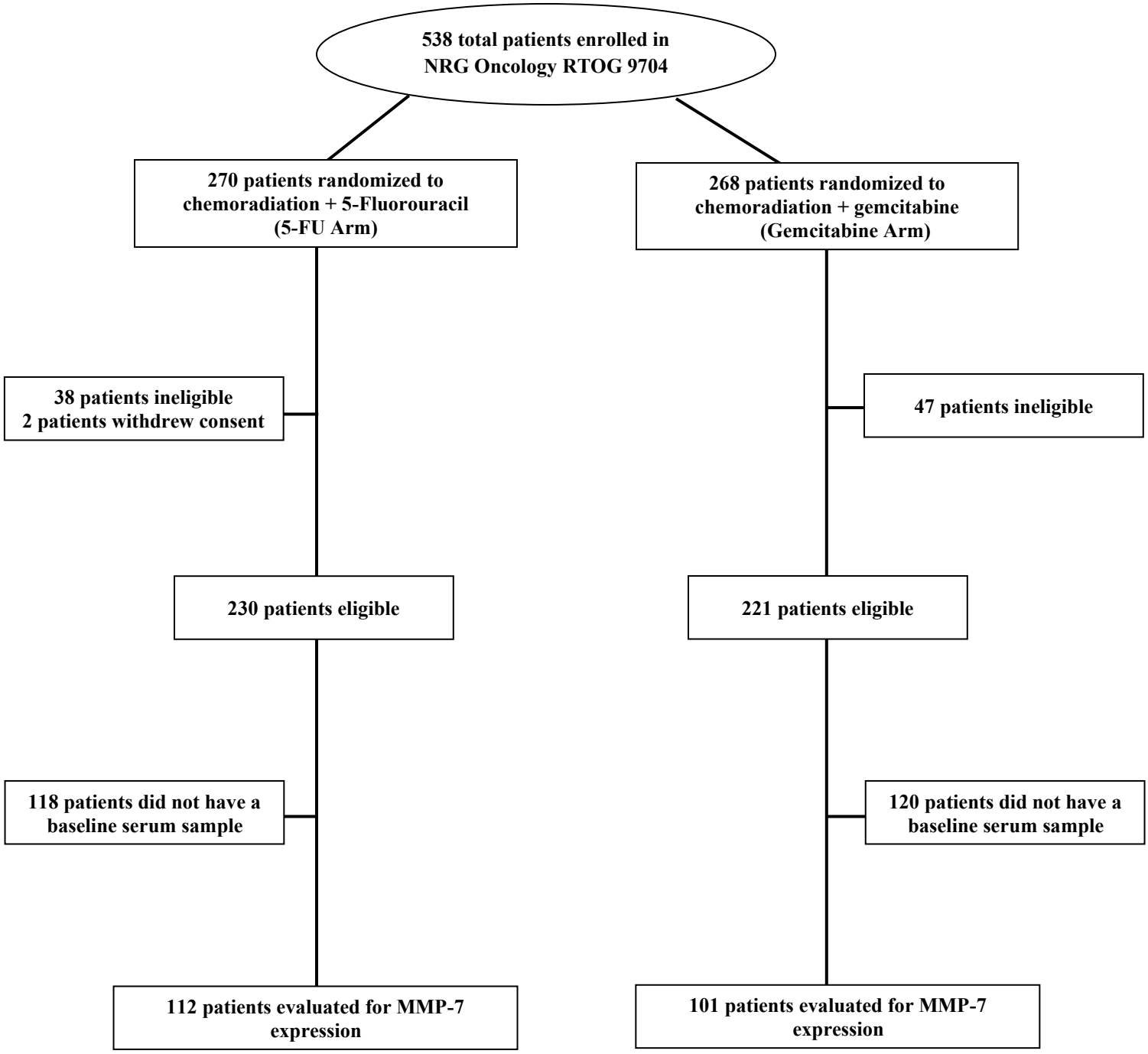


Figure 2. CONSORT diagram for NRG Oncology RTOG 9704



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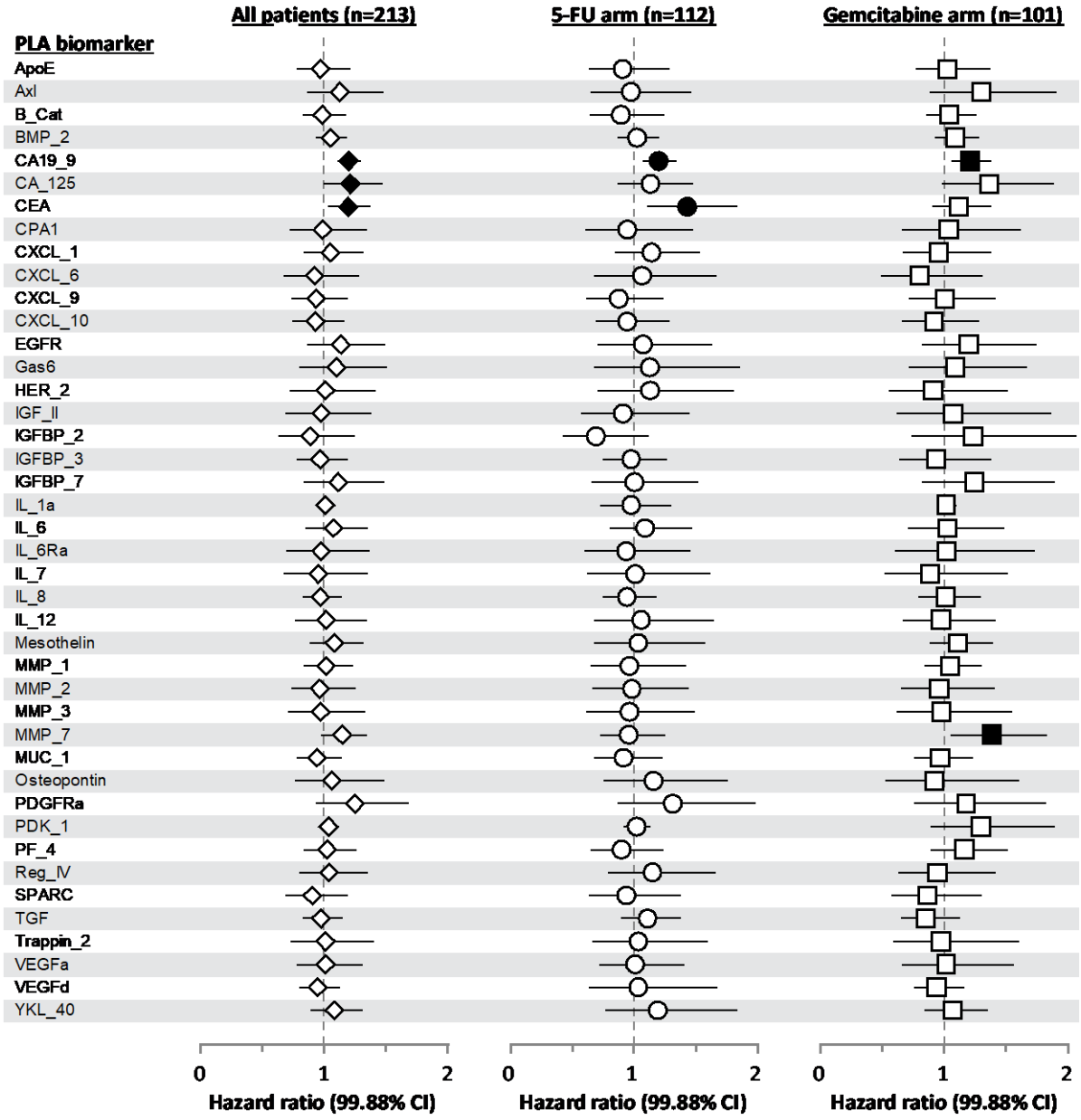

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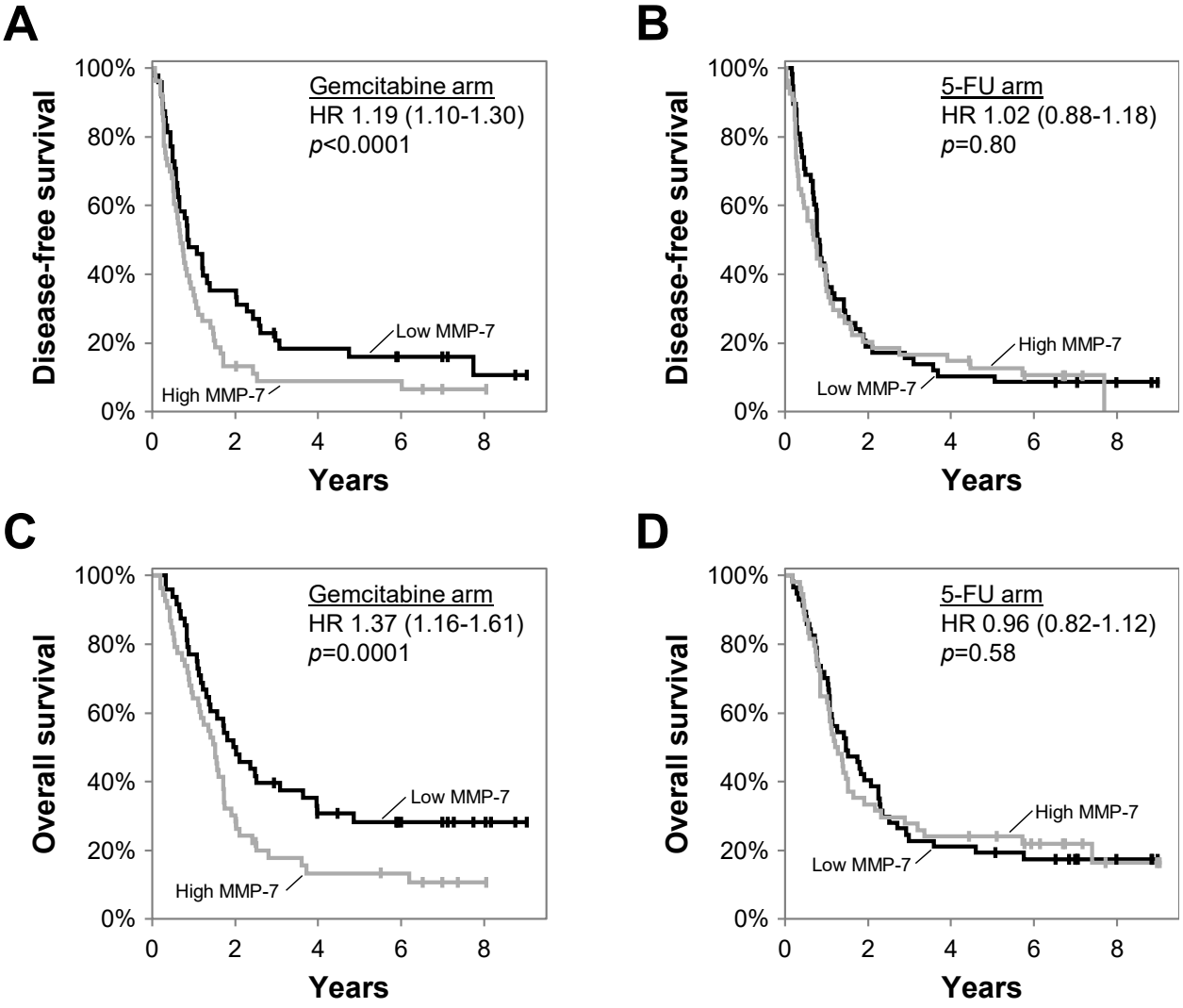

Table 1. Pancreatic cancer PLA probe panel

Subpanel	PLA Probes							
1	VEGFa	YKL-40	β-Cat	PLN	IL-8	ApoE	MMP-1	CXCL1
2	VEGFb	HIF-1α	CA 125	FGF	CA 19-9	EpCAM	MMP-2	CXCL6
3	VEGFc	MIF	BMP-2/4	HGF	IL-1α	IL-7	MMP-3	CXCL9
4	VEGFd	OPN	Trappin-2	TGF	Mesothelin	PDGFRα	MMP-7	CXCL10
5	CTGF	MMP-9	SPARC	HDGF	CPA1	IL-1β	CEA	IGFBP-2
6	IGF-2	MMP-13	Reg4	IL-6Rα	Gas6	IL-6	Her2	IGFBP-3
7	EGFR	PDK-1	PF4	Axl	MUC-1	IL-12	TNFα	IGFBP-7

Serum Sample Preparation