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Hyaluronan heterogeneity in pancreatic ductal adenocarcinoma: Primary tumors compared to sites of metastasis

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Abstract

Background: Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive cancers with poor survival. The dense desmoplastic stroma in PDAC contributes to treatment resistance. Among the components comprising the tumor stroma, hyaluronan (HA) has been demonstrated to play a critical role in tumor progression and survival. Previous preliminary studies have suggested differences in HA expression in primary and metastatic foci of PDAC. However, the effects of treatment and location of HA expression as a biomarker signature remain unknown; this study sought to compare HA expression in primary and metastatic sites of PDAC.

Methods: Tissue from primary and metastatic PDACs were obtained from Cedars-Sinai Medical Center along with associated clinical data. Tissue slides were stained for H&E, HA, and CD44. Associations between HA levels and the evaluated variables were examined including progression free survival and overall survival.

Results: HA score was significantly higher in primary PDACs compared to sites of metastases ($p = 0.0148$). Within the metastases, HA score was significantly higher in liver metastases compared to metastases at other sites ($p = 0.0478$). In the treatment-naive liver metastasis cohort, patients with HA high status had decreased progression free survival and overall survival compared to patients with HA low status ($p = 0.0032$ and $p = 0.0478$, respectively).

Conclusions: HA score is variable between primary PDAC, PDAC metastatic to the liver, and PDAC metastatic to other sites. Within liver metastases, patients with HA high status had

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pan.2021.09.015.

decreased progression free survival and overall survival compared to patients with HA low status. HA levels can serve as a potential biomarker to guide pancreatic cancer treatments and trial design for agents targeting the stroma.

Keywords

Hyaluronan; Hyaluronic acid; Biomarker; Pancreatic cancer; Pancreatic ductal adenocarcinoma; Stromal prognostic

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive cancers with an estimated five-year survival rate of 10% [1]. Despite numerous studies to better understand the biology of PDAC and research into novel treatments, the rate of new cases and deaths has not decreased in over 25 years [1]. This substantiates the need to utilize all resources and new investigations to collectively interrogate the tumor characteristics that make PDAC unique and a significant challenge to treat among all malignancies.

PDAC tumors are comprised of the malignant glandular component typically surrounded by dense stroma constituting a rich fibro-inflammatory microenvironment. Hyaluronan (HA) is one of the components that comprises this stromal compartment within the tumor microenvironment. Among cancers, HA accumulation is greatest in PDAC [2]. HA plays important roles within the extracellular matrix and has been demonstrated to interact with CD44 and CD168 cell surface receptors that activate subsequent downstream signaling cascades involved in tumor progression and survival [3]. Normally, matrix deposition of HA is degraded by a number of hyaluronidase enzymes including HYAL1–3, KIAA1199, and PH20 to prevent excessive accumulation [4,5]. In PDAC, however, signaling is skewed to favor an excess of HA within the extracellular matrix contributing to increased interstitial fluid pressure and decreased vascular permeability, which promote cancer progression [2,6].

Recent studies have evaluated the effects of targeting HA in the tumor microenvironment. Preliminary studies have indicated similarities and differences in HA expression between primary and metastatic foci of pancreatic cancer [7,8]. KPC animal models that spontaneously developed pancreatic intraepithelial neoplasia and progress to metastatic PDAC were treated with a novel targeting agent against HA, polyethylene glycol-conjugated (pegylated) recombinant human hyaluronidase PH20 (PEGPH20), and demonstrated efficacy of PEGPH20 [2,6]. PEGPH20 degraded intratumoral HA, increased intratumoral blood vessel permeability, and demonstrated increased survival when used in combination with a commonly used PDAC chemotherapeutic, gemcitabine [2,6]. These studies provided the foundation to carry out a phase IB/II randomized clinical trial using FOLFIRINOX with or without PEGPH20 followed by a phase 3 randomized controlled clinical trial using gemcitabine and nab-paclitaxel with or without PEGPH20 in patients with metastatic PDAC [9,10]. The results of the phase 3 trial did not show a benefit for patient survival as had been suggested by earlier studies, and PEGPH20 was halted for further therapeutic development. To completely understand why PEGPH20 was not effective and to allow success for future stromal targeting agents, further research into PDAC tumor biology, appropriate patient

selection, and the rich stromal microenvironment is necessary. In this context, the effect of prior treatment and location of metastasis on HA expression, as well as the role of HA receptors like CD44, on HA as a biomarker signature remains unknown. The goal of the current study was to expand the understanding of HA expression and interactions in the context of PDAC, to better understand differences in primary and metastatic foci of pancreatic cancer. This study hypothesized that the amount of HA may be variable in the primary tumor compared to sites of metastasis. HA heterogeneity may impact patient treatment, selection for stromal targeting studies and ultimately PDAC patient clinical outcomes.

2. Materials and methods

2.1. Patient tissue and clinical data

Primary (n = 43) and metastatic (n = 66) PDAC tissues were obtained from 100 patients at Cedars-Sinai Medical Center (Flow diagram FigureAppendix A); 9 patients had matched tissues from the primary tumor and site of metastasis. Clinical data was obtained from patient medical records. Clinical and pathological variables abstracted from the medical record included age, sex, race, CA 19–9 level, tumor location, and stage at diagnosis. All procedures were carried out in accordance with institutional review board protocol Pro 00039754.

2.2. Immunohistochemical staining and pathological grading

Tissue slides were stained with hematoxylin and eosin (H&E) for histological analysis, HA using a biotin-TSG-6-deltaHep-Fc histochemical assay [11], and CD44 by immunohistochemistry (clone SP37). HA staining was considered positive for tumor stroma staining at an intensity greater than background stroma. HA was scored as the percentage of tumor stroma staining, and HA status was defined as ≥50% staining being HA high and <50% being HA low [12]. CD44 was assessed by light microscopy, quantifying the amount of tumor cells staining (to the nearest 10%), evaluating the intensity of staining in tumor cells compared to positive controls (graded from 0 as weakest to 3 as strongest), and multiplied together to generate an H-score (which ranged from 0 to 300).

2.3. Statistical analysis

Data are presented as frequency (percentage, %) for categorical variables and mean (±standard deviation) or median (range) for continuous variables. Associations between HA status and the evaluated variables were examined with *t*-test, Wilcoxon rank-sum test, Chi-squared test, or Fisher's exact test where appropriate. Kaplan-Meier curves were created to assess progression free survival and overall survival and the log-rank test was used to compare Kaplan-Meier curves. Time to event was defined as the time from PDAC diagnosis to last follow-up, progression, or death. Pearson correlation was performed to explore the association between HA score and CD44 score. Furthermore, a Cox regression model with HA score and CD44 score as covariates was used to assess their relationship with overall survival and progression free survival. Analyses were performed using SAS 9.4 (SAS Institute, Inc., Cary, North Carolina) with two-sided tests and a significance level of 0.05.

3. Results

It has been previously demonstrated that HA accumulation is associated with low tumor grade and nodal metastases in PDAC [13]. The current study built upon this previous data with an additional cohort of treatment-naïve PDACs that metastasized to the liver. Clinical and pathological features of these 45 liver metastasis tissues are summarized in Table 1. The patients were grouped by HA high status vs HA low status and compared for each characteristic. The mean age was 67 and 72 years for HA high and low statuses, respectively. The number of males (n = 12) was equal for the respective groups; and the number of females was distributed as evenly as possible with 11 and 10 with HA high versus low status, respectively. The majority of tissues were from patients of Caucasian race with a minority of African American, Asian, and Hispanic race. For those patients with CA 19–9 levels measured, the median HA score trended lower for those patients with HA high scores (424 U/mL) versus those with HA low scores (5008 U/mL). CA19–9 values with values above their limit of detection (i.e. >18000) were expressed as their limits (changed to 18000) to make this a continuous variable. The primary PDAC tumor location of the metastatic liver tissues was more commonly localized in the body and tail region, and fewer were localized to the uncinate, head, and neck or a combination of these respective sites. These liver metastatic tissues were predominantly scored based on stage at diagnosis as stage 4 and a few were stage 2B.

HA scores were compared among PDAC tissue samples. Using the newly analyzed naïve liver-metastatic PDACs in combination with the previous cohort of primary pancreas tumor tissue and tissue from a myriad of metastatic sites (n = 109), median HA score was shown to be significantly higher in the primary tumors; 50 (5.0–90.0) compared to 40 (0.0–80.0) in metastatic tumors (p = 0.0148). All metastatic sites include previously treated and naive liver (n = 59), lung (n = 3), stomach (n = 1), peritoneum (n = 1), retroperitoneum (n = 1), and porta hepatis lymph nodes (n = 1). Representative H&E images of primary pancreas tumor tissues and sites of metastasis demonstrate histological differences of the stromal quality confirmed by differential HA distribution as visualized using the HA histochemical assay (Fig. 1). To further the understanding of HA accumulation among the various metastatic sites, HA score was compared for liver metastases (n = 59) and other sites of metastasis (n = 7). Liver metastases were found to have a significantly higher median HA score compared to other sites of metastasis as specified; 40 (0.0–80.0) versus 20 (5.0–50.0), respectively (p = 0.0478). Furthermore, combined pre-treated and naïve liver metastasis tissue (n = 59) compared to primary pancreas tumor tissue (n = 43) were shown to have a lower median HA score of 40 (0.0–80.0) versus 50 (5.0–90.0), respectively (p = 0.0412). A visual comparison of the number of tissues within each HA score grouping for liver metastasis, other sites of metastasis, and primary pancreas tissue was plotted (Supplemental Fig. 1). Within the liver metastases tissue, naïve liver metastasis tissues demonstrated a trend toward higher median HA scores compared to previously treated tissues, 50 compared to 30, respectively; however, this difference was not statistically significant (Table 2, p = 0.0622).

A classification of HA status was used to divide our naïve metastatic liver tissues to evaluate survival, as was used in the clinical trials [10,12]. HA high status was defined as ≥50% staining and HA low status was defined as <50% staining. Progression free survival was

significantly shorter for patients with HA high tissue status (<100 days) compared to those with HA low tissue status (>800 days) (Fig. 2, p = 0.0032). Similarly, overall survival was demonstrated to be significantly shorter for patients with HA high tissue status in naïve liver metastasis (<750 days) compared to those with HA low tissue status (>1250 days) (Fig. 3, p = 0.0478).

The entire cohort of liver metastasis tissues that included naïve and previously treated to evaluate survival was also examined. As most of the tissues were from patients diagnosed with stage 4 PDAC, progression free survival for HA high and HA low was evaluated. There was a trend toward patients with stage 4 tumor tissues having a HA high status, correlating with shorter progression free survival compared to those with HA low status (Supplemental Fig. 2, p = 0.4581). Among patients with stage 4 PDAC, those patients with HA high status appeared to have shorter overall survival compared to those with HA low status (Supplemental Fig. 3, p = 0.3894). Survival was also evaluated for PDAC stages 1–3, and similar trends were seen in patients with tissues having a HA high status, correlating with shorter progression free survival and overall survival compared to those with HA low status (Supplemental Figs. 4–5, p = 0.6854 and p = 0.9446, respectively).

The association of HA in the extracellular matrix with the CD44 cell surface receptor, known to activate subsequent downstream signaling, was assessed in a subset of these primary and liver metastasis PDAC tissues. HA score and CD44 H-score were plotted and did not show a positive correlation (Supplemental Fig. 6, p = 0.4941). Further analysis examined only the primary pancreas tissues with CD44 or alternatively only the liver metastasis tissue with CD44 and did not show a positive correlation (p = 0.9364 and p = 0.2682, respectively). Using the HA score and CD44 H-score as covariates, Cox regression models were used to predict overall survival and progression free survival. Prediction of overall survival was not significant for HA score or CD44 H-score (Supplemental Table 1, p = 0.1248 and p = 0.3047, respectively). Prediction of progression free survival was significant for HA score, but not CD44 score (Supplemental Table 1, p = 0.0049 and p = 0.8879, respectively). Thus, for each unit increase in HA score, the risk of recurrence or progression increases by 4.4% at any fixed point in time adjusting for CD44 H-score. The proportional hazards assumption was met for both of the Cox models. CD44 H-score was further compared between liver metastasis tissue (H-score of 100; 0.0–300.0) and primary tumor tissue (H-Score of 100; 20.0–300.0), but there was no difference in median score (p = 0.8599). In addition, mean CD44 H-score in metastatic pancreatic tissue was compared for tissue with HA high (n = 8) versus HA low (n = 7) status and was not found to show significant differences with HA status (tumor CD44 H-score of 93.8 (95.52) versus 174.3 (130.0), respectively; p = 0.1907). Median CD44 H-score in primary pancreatic tissue was also compared for tissue with HA high (n = 9) versus HA low (n = 5) status and was not found to show significant differences with HA status (tumor CD44 H-score of 100.0 (20.0–300.0) versus 100.0 (20.0–140.0); p = 0.4988).

4. Discussion

The current study shows that HA levels may serve as a prognostic biomarker in pancreatic cancer. Specifically, for patients with treatment-naive liver metastasis, it demonstrated that

HA high status is associated with decreased progression free and overall survival compared to HA low status (Figs. 2 and 3). The majority of PDAC patients are diagnosed with advanced stage, metastatic cancer. In addition, the most common site of metastasis in PDAC patients is the liver. Importantly, the current results show significance for the most abundant group of PDAC patients at diagnosis. These results also corroborate those previously reported showing that patients with HA high status have poorer survival compared to patients with HA low status [8]. The use of HA status as a biomarker can potentially be used to preemptively suggest the use of more aggressive therapy for those patients with HA high status as our data highlights their significantly shorter survival. Conversely, for those patients with HA low status, their treatment course may be planned to take into account therapy that will be sustainable for a longer treatment course to allow them to receive full benefits and potentially attain longer survival.

We also demonstrated that HA levels found in pancreatic cancer primary tumor tissue are significantly different compared to liver and other sites of metastasis. These results align with a previously reported study that showed similarities in HA levels when primary PDAC tissue was compared to metastatic PDAC [8]. Whatcott et al. demonstrated that the level of desmoplasia, which was in part characterized by HA levels, in matched primary versus metastatic PDAC tumors was statistically different, which aligns with our study showing significant differences using mostly unmatched and previously untreated patient tissues [8]. Of note, the status of neoadjuvant treatment or treatment naïve was unknown in the Whatcott et. al. study, which may have lessened the significant differences of HA levels in their study. HA staining methodology varied between our studies. However, they also showed that patients with high levels of HA compared to those with low levels of HA had significantly shorter survival, which is in agreement with our results looking at naïve liver metastases tissue [8]. The differences between our studies may be due to the limited sample sizes, matched versus unmatched tissues, unknown neoadjuvantly treated vs mostly naïve tissues, staining methodology, and differences in sites of PDAC metastasis. High HA levels have been shown to correlate with more aggressive cancer characteristics in hepatocellular carcinoma, head and neck cancer, ovarian cancer, breast cancer, prostate cancer and in cell and mouse models of pancreatic cancer [3,14–18]. Higher levels of HA in liver metastasis tissue compared to other sites of metastasis in the current study correlates with decreased survival of PDAC patients with liver metastasis compared to other sites of metastasis [19]. The significance of varying levels of HA among the primary tumor site, liver metastasis, and other sites of metastasis suggests that therapy may need to be tailored to these different tumor sites. A patient with higher HA levels in the liver may respond differently to the same therapy as a patient with low HA levels in the lung or other sites of metastasis. While treatment options are currently limited in PDAC, this HA heterogeneity among the various tumor sites may be more important when novel targeted treatments are developed. Additionally, patient selection for studies based on HA level taking into account disease stage being localized versus metastatic will be important for future studies aimed at targeting the stroma or tumor microenvironment (TME). It is known that as a pancreatic tumor develops from a pancreatic intraepithelial neoplasia to metastatic PDAC, inflammation and HA deposition in the TME contribute to this disease process [20]. As progression of PDAC ensues, the inflammatory TME recruits and activates stromal cells

including cancer associated fibroblasts, stellate, and immune cells at varying levels in the primary tumor and metastatic organ sites, which enhance HA formation and deposition in the TME. Treating the evolving PDAC tumor that dynamically adjusts to the TME requires a multi-pronged approach to target the tumor cells, inflammation, HA deposition and stromal cell infiltration that enhance the desmoplastic matrix deposition and promotion of feedback signaling that perpetuates the progressive evolution of PDAC.

Previous studies have shown that when HA is bound to CD44 this contributes to cancer progression and inflammation [21,22]. In this study, a predictive model of progression free survival was generated using HA score and adjusted for CD44 H-score (Supplemental Table 1). This highlights the importance of HA and CD44 to play a role in tumor progression. Although predictive models using CD44-H score in our data set were not significantly correlated to outcomes, CD44 is one of many well-known variables in HA signaling and processing. These players include variant CD44 proteins, multiple other HA degrading enzymes, and biological activity of HA degradation products [4,5,23,24]. To better predict patient survival using HA as a biomarker, its relationship with CD44 needs to be further studied and understood in PDAC.

Although our study demonstrates HA heterogeneity among various sites of PDAC there are some limitations that must be taken into consideration for future studies to expand upon these results. While the number of primary and liver metastatic PDAC tissues assessed is comparable to previous studies, the number of tissues from other sites of metastasis (n = 7) is small. We included this small number of tissues as the information gathered from these tissues is crucial for a well-rounded analysis of the varied PDAC TME. Implicit within this analysis is the reflection of the sites of PDAC metastasis with liver being the main site of metastasis. Although the PDAC TME involves far more components than HA and CD44, the scope of this paper focused on these as HA is thought to play a critical role in PDAC progression through its CD44 receptor. Further work to analyze additional players within the TME through additional studies is needed to broaden our knowledge on additional matrix components, stromal, and immune cell types.

Over the years, several studies have tried to target HA in the tumor microenvironment as a therapeutic option. Animal models and early preclinical studies demonstrated PEGPH20 was effective at degrading intratumoral HA, promoting increased intratumoral blood vessel permeability, and was shown to increase survival when used in combination with PDAC chemotherapeutics [2,25]. This prompted a phase 3 randomized controlled clinical trial using PEGPH20 with or without gemcitabine and nab-paclitaxel [10]. The results of the phase 3 trial did not show benefit for patient survival, and PEGPH20 was halted for further therapeutic development. The results from the current study suggest that the failure of PEGPH20 may in part be due to a lack of understanding of HA heterogeneity in the tumor microenvironment of the primary tumors compared to other sites of metastasis. The findings of this study coupled with the clinical results of the HALO 301 trial also suggests that aggressiveness and treatment resistance of PDAC are likely due to other immunosuppressive elements of the TME and stromal heterogeneity among patients. Targeting of HA is not a one-size-fits-all-tumors treatment option. Thus, the intricacies of the varying tumor microenvironment landscapes, such as HA levels within each organ need

to be better understood within the context of other tumor microenvironment elements, such as immunosuppressive (Treg, TAM, MDSCs) cell composition and clinical burden of disease (localized vs metastatic), in order to better guide trial design and patient selection. This study demonstrates the importance of HA heterogeneity among PDAC primary tumors and sites of metastasis that may serve as a prognostic biomarker.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational relevance

Treatment resistance in pancreatic ductal adenocarcinoma (PDAC) results in part from the dense desmoplastic stroma. Studies to understand these stromal components critical for tumor progression including hyaluronan (HA) are underway. This study investigated the potential of HA as a biomarker in primary PDAC and metastases. HA score was used to demonstrate, for the first time, significant heterogeneity across the various tissue sites; expression was greatest in primary PDAC, followed by PDAC metastatic to the liver, and then PDAC metastatic to other sites. Within the naive liver metastases tissue, patients with HA high status had decreased progression free survival and overall survival compared to patients with HA low status. Future agents targeting the stroma will need to consider HA heterogeneity across tissue sites for optimal outcomes. HA levels can serve as a potential biomarker to guide treatments and trial design.

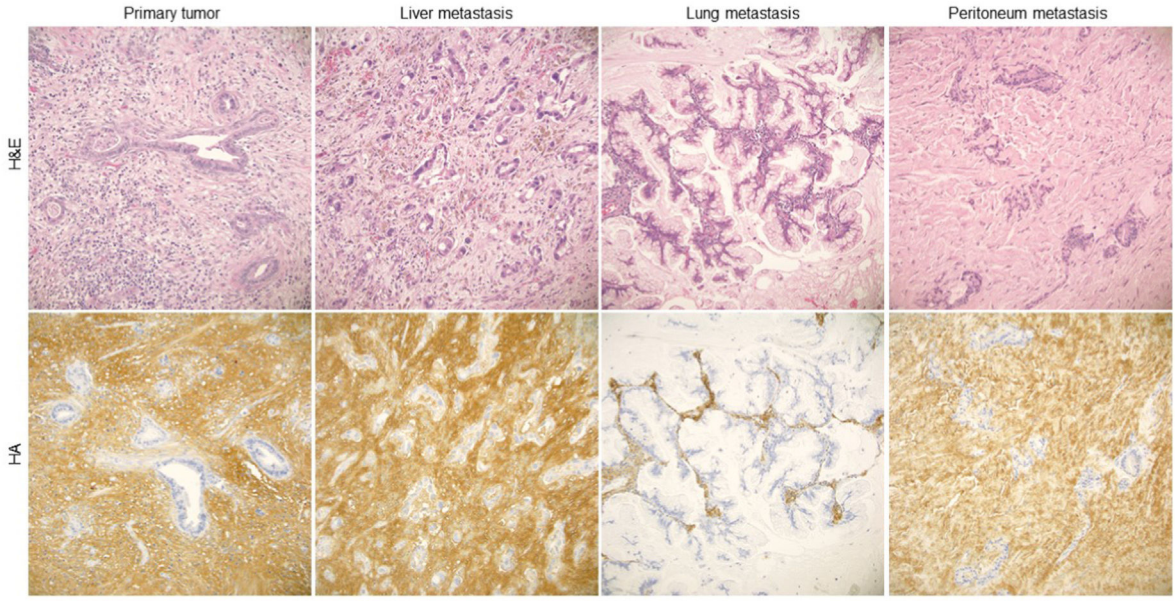

Fig. 1.

Representative tissue histology demonstrating primary and metastatic pancreatic cancer. Hematoxylin and eosin (H&E)-stained tissues are shown in the top row for the tumors at the indicated sites. HA staining (brown) in the lower row is counterstained with hematoxylin (blue). 200× magnification.

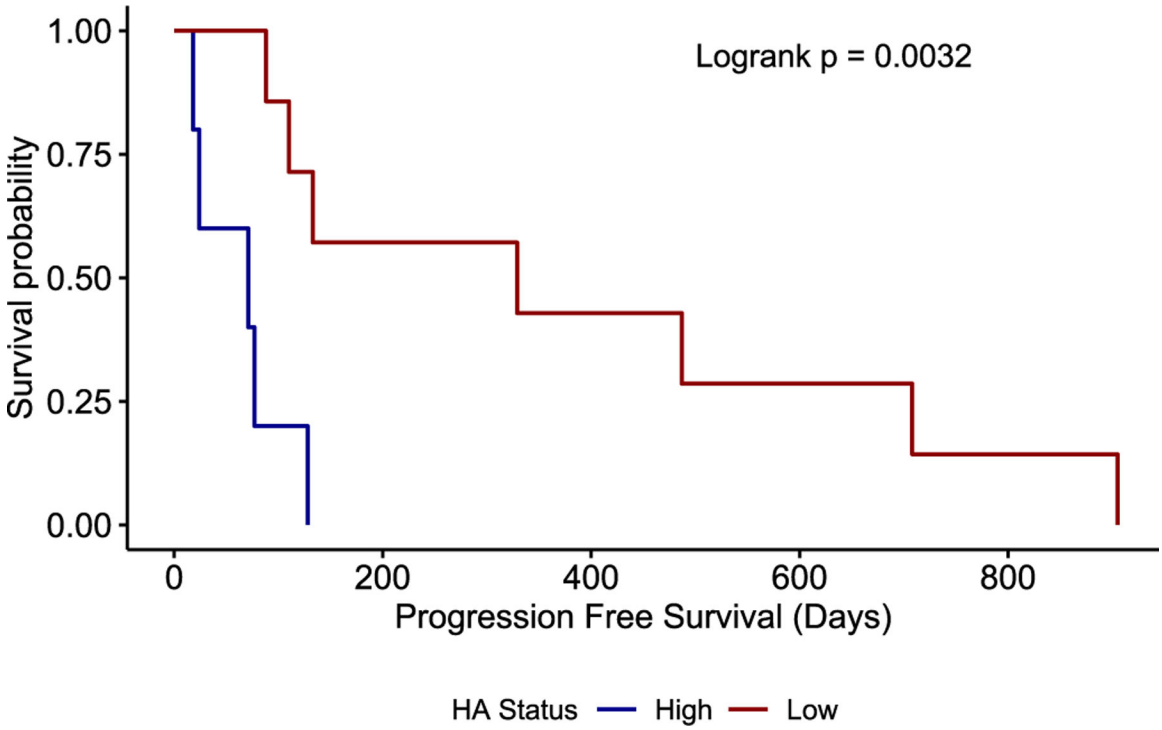

Fig. 2.

Naïve liver metastasis progression free survival, product-limit survival estimates. Progression free survival is plotted (days) along the x-axis for HA high (blue) and HA low (red) status. Survival probability is plotted along the y-axis.

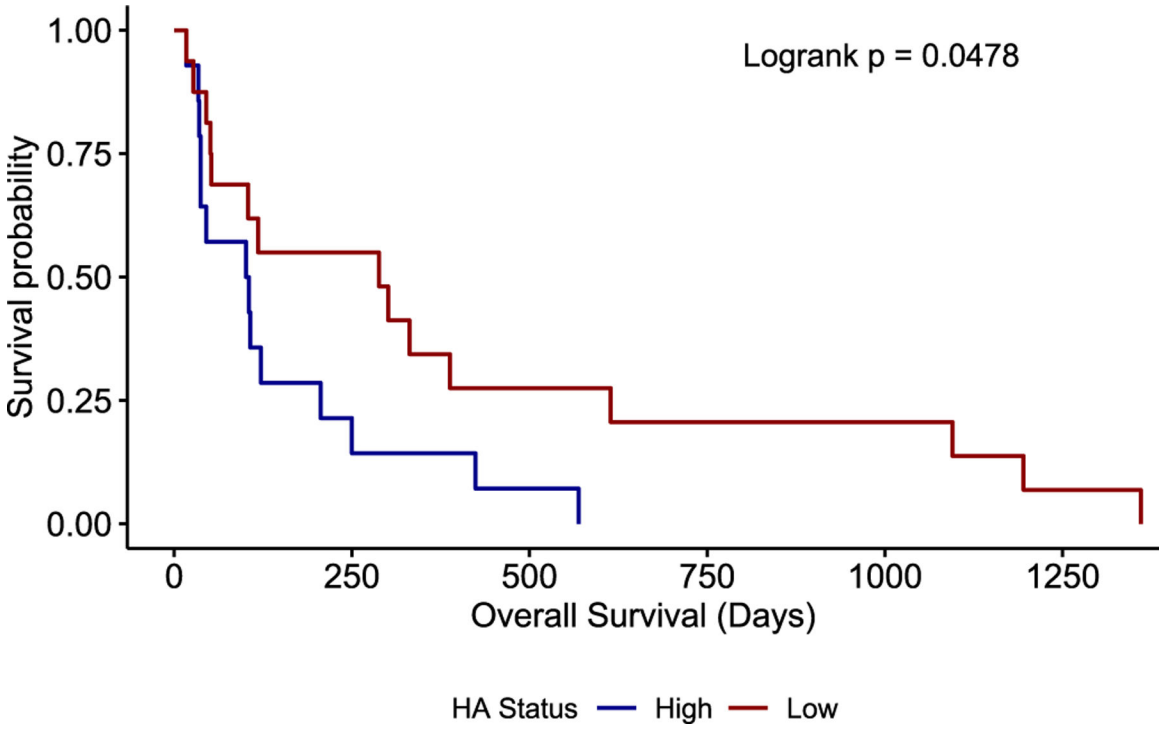

Fig. 3.

Naïve liver metastasis overall survival, product-limit survival estimates. Overall survival is plotted (days) along the x-axis for HA high (blue) and HA low (red) status. Survival probability is plotted along the y-axis.

Table 1

Clinical and pathologic characteristics comparing HA low vs. HA high status in treatment-naïve pancreatic liver metastasis tissue.

	High (N = 23)	Low (N = 22)	Total (N = 45)	p value
Age	66.9 (15.2)	72.0(11.0)	69.4 (13.4)	0.2024
Sex (n, %)				0.8734
Male	12 (52.2%)	12 (54.5%)	24 (53.3%)	
Female	11 (47.8%)	10 (45.5%)	21 (46.7%)	
Race (n, %)				0.2484
Caucasian	12 (52.2%)	17 (77.3%)	29 (64.4%)	
African American	8 (34.8%)	3 (13.6%)	11 (24.4%)	
Asian	1 (4.3%)	0 (0.0%)	1 (2.2%)	
Hispanic	2 (8.7%)	2 (9.1%)	4 (8.9%)	
Initial CA19–9	424 (1.0–24000.0)	5008 (1.0–100000.0)	1322 (1.0–100000.0)	0.2012
Tumor Location				0.7945
Uncinate, head, neck	6 (31.6%)	7 (36.8%)	13 (34.2%)	
Body, tail	12 (63.2%)	10 (52.6%)	22 (57.9%)	
Combined	1 (5.3%)	2 (10.5%)	3 (7.9%)	
Stage at diagnosis				0.2220
2B	0 (0.0%)	2 (9.5%)	2 (4.5%)	
4	23 (100.0%)	19 (90.5%)	42 (95.5%)	

Table 2

HA score comparison between naïve and previously treated pancreatic cancer liver metastases tissue.

	Naïve (N = 45)	Previously treated (N = 14)	Total (N = 59)	p value
HA Score				0.0622
Median	50.0	30.0	40.0	
Q1,Q3	25.0, 70.0	25.0, 50.0	25.0, 60.0	
Range	(10.0–80.0)	(0.0–60.0)	(0.0–80.0)	
HA status				0.1393
High	23 (51.1%)	4 (28.6%)	27 (45.8%)	
Low	22 (48.9%)	10(71.4%)	32 (54.2%)	