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Lauren E. Colbert 4/7/14

APPROVAL SHEET

Nuclear Hypoxia-Inducible Factor 1 Alpha as a Predictor of Recurrence Pattern and Overall Survival in Resected Pancreatic Adenocarcinoma

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Master of Science

Clinical Research

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ABSTRACT COVER PAGE

Nuclear Hypoxia-Inducible Factor 1 Alpha as a Predictor of Recurrence Pattern and Overall Survival in Resected Pancreatic Adenocarcinoma

By

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B.A., Hope College, 2009

Advisor: Walter J. Curran, Jr., MD

An abstract of a thesis submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Master of Science

in Clinical Research

2014

ABSTRACT

Purpose:

Nuclear Hypoxia-Inducible Factor 1 Alpha (HIF1a) has been implicated in development of cancer metastases. The goal of this analysis was to evaluate HIF1a expression as a prognostic factor for overall survival (OS), distant recurrence (DR) and local recurrence (LR) following resection for pancreatic adenocarcinoma (PAC).

Methods:

Specimens were collected from 98 patients with PAC who underwent resection without neoadjuvant therapy between January 2000 and December 2011 at one institution. Immunohistochemical (IHC) staining was performed using anti-HIF1a antibody and scored using a previously defined scoring system by an independent pathologist blinded to patient outcomes. Univariate analysis was used to compare HIF1a scores for LR and DR groups and for OS. Multivariate logistic regression was used to determine predictors of LR and DR. A receiver operating characteristic (ROC) curve was conducted for predictive power of HIF1a expression on DR.

Results:

8 (8%) patients demonstrated isolated LR, 26 (26.5%) patients had isolated DR, and 13 patients had both LR and DR. 53 (54%) patients had high HIF1a expression and 45 (46%) patients had low HIF1a expression. High HIF1a expression was significantly associated with DR (p=0.03) and low HIF1a expression was significantly associated with isolated LR (p=.02). In multivariate logistic regression analysis adjusting for nodal status,

margin status, tumor grade, perineural invasion, lymphovascular invasion and use of adjuvant therapy, high HIF1a was the only significant predictor of DR (OR=3.10 [95% CI 1.18-8.10]; p=.02). In patients with a recurrence, the AUC of high HIF1a score for DR was 74.8%. A HIF1a score $\geq$ 2.5 demonstrated a specificity of 100% for DR in these patients.

Conclusions:

High HIF1a expression is a predictor of distant failure versus isolated local failure in patients undergoing resection of pancreatic adenocarcinoma. HIF1a expression may have utility as a clinical decision-making tool to determine candidates for adjuvant local therapy and systemic chemotherapy.

COVER PAGE

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INTRODUCTION & BACKGROUND

Pancreatic adenocarcinoma (PAC) remains the fourth leading cause of cancer death in the United States today and carries an extremely poor prognosis, with 5-year survival rates remaining near 5%. Out of the estimated 44,000 newly diagnosed cases of pancreatic cancer in 2012, 37,400 deaths are expected eventually.(1) Although incremental gains have been made in treatment strategies, complete resection remains the only hope for cure. Methods for stratifying patients and tailoring treatment for pancreatic adenocarcinoma rely mainly on descriptive clinicopathologic features such as Tumor Node Margin (TNM) TNM stage, margin status, perineural invasion and CA19-9, all of which have modest prognostic value. One accepted treatment algorithm after pancreatic resection is to administer adjuvant chemoradiation.(2, 3) Even with curative resection, five-year survival rates remain around 20%. At least 40% of patients have metastatic disease at presentation, and another 30-40% have locally advanced disease not lending itself to curative intent resection. In order to better understand which patients may develop metastatic disease, several potential molecular biomarkers have been studied in pancreatic cancer with varying degrees of clinical utility. Some of these biomarkers include hypoxia markers.

Rapidly growing solid tumors exhibit areas of hypoxia where the rate of growth outpaces blood vessel formation at the growing edge of the tumor. One of the pathways linking hypoxia to angiogenesis is the Hypoxia-Inducible Factor 1 (HIF-1) pathway, which is composed of subunits HIF-1α and HIF-1β. Under physiologic conditions, HIF-1α is recognized by the von Hippel-Lindau tumor suppressor (pVHL) and tagged for

degradation via the ubiquitin pathway. Under hypoxic conditions HIF-1α is *not* recognized by pVHL, leading to accumulation of HIF-1α and dimerization with HIF-1β. The dimer then translocates to the nucleus. Once inside the nucleus, HIF-1α transcribes several genes exhibiting multiple cellular signaling effects. While HIF-1β is a common subunit among many bHLH-PAS proteins and is constitutively expressed, HIF-1α is uniquely regulated in this way by available oxygen (O_2).(4) O_2 deprivation leads to increased signaling of nuclear HIF-1α, which in turn causes tumor cells to experience an adaptive switch to angiogenic signaling, glycolytic metabolism, and subsequent micrometastases. This ability to respond to microenvironmental stress leads in turn to tumor resistance to available therapeutic approaches. High levels of HIF-1α activity have been shown to increase tumor growth, vascularization and glucose metabolism and also reflect more clinically aggressive tumors due to an association with p53 mutations. HIF-1α expression has been well-documented as a clinical marker of aggressive disease, poor prognosis and treatment resistance in other cancers, such as ovarian tumors,(5) cervical cancer(6) and more recently in other gastrointestinal malignancies such as esophageal and colorectal cancer,(7) potentially through regulation of hypoxia-based tumor-stromal interactions(8). The aim of this study was to evaluate the role of HIF-1α expression in prognosticating survival and patterns of failure in patients with resectable PAC.

METHODS

I. Research Goal

a. The goal of this study was to evaluate a) the association of hypoxia biomarkers HIF1a, HSP90, CAIX and iNOS with overall survival, isolated local recurrence (LR) and distant recurrence (DR)

II. Study Design

a. Patient Selection

i. The study population consisted of one hundred patients with early-stage PAC who underwent pancreaticoduodenectomy with curative intent between January of 2000 and December of 2011 at Emory University Hospital.

ii. Inclusion criteria were pathologically diagnosed pancreatic adenocarcinoma and adequate tissue available for evaluation. Two patients who received neoadjuvant chemotherapy or radiation were excluded to avoid bias in biomarker interpretation. Approximately 30 of these patients were included in previous reports by the authors.(9-11)

iii. Overall survival and follow-up time were calculated using date of surgery as the initial contact. Chart review was conducted to obtain patient demographics, treatment characteristics and pathologic

characteristics. Patients were followed for up to five years after surgery or until date of death.

iv. Local recurrence was defined as radiographic evidence or pathologic confirmation of recurrent disease in the pancreas, pancreatic bed or associated nodal regions on standard post-treatment surveillance scans. Distant recurrence was defined as radiologic or pathologically confirmed recurrent disease in other sites at any time post-treatment. Permission was obtained from the Institutional Review Board and patient confidentiality was maintained according to the Health Insurance and Patient Accessibility Act of 1996.

b. Immunohistochemical Analysis

i. Representative sections of tumor were selected using formalin-fixed paraffin embedded slides reviewed by an expert pancreatic pathologist (NA), and tissue microarray (TMA) blocks were created and reviewed for homogeneity. TMA cores were stained for nuclear HIF-1α expression using anti- HIF-1α monoclonal mouse antibody (NB100-105, Novus Biologicals, Littleton, CO). Nuclear expression levels of HIF-1α were determined by an experienced pancreatic pathologist (SB) blinded to patient outcomes and clinical data. Additional hypoxia biomarkers were stained including iNOS using anti-iNOS polyclonal rabbit antibody (NB300-605, Novus Biologicals, Littleton, CO), HSP90 using

monoclonal mouse antibody (NBP1-97529, Novus Biologicals, Littleton, CO), and CAIX using polyclonal rabbit antibody (NB100-417, Novus Biologicals, Littleton, CO). Immunohistochemical (IHC) expression scores were calculated based on a previously defined scoring system incorporating intensity and percent of cells staining.(10-13) In addition to analysis of the calculated scores as a continuous variable, scores were divided into low and high expression levels. Low expression level tumors were defined as tumors with a score less than the median for each biomarker and high expression level tumors were defined as those with a score greater than or equal to the median for each biomarker.

c. Statistical Analysis

i. Descriptive characteristics were generated for all clinicopathologic covariates, patient demographics, clinical outcomes and biomarker intensity, percent and score. Medians and ranges were generated for continuous variables and frequency statistics were generated for categorical variables. Descriptive characteristics were generated for the endpoints of recurrence pattern, death or loss to follow-up for all patients.

ii. Univariate analysis was performed for association of clinicopathologic covariates and biomarkers with isolated local recurrence (LR) and with distant recurrence (with or without local

recurrence) (DR). T-test or Wilcoxon signed rank test were used for continuous variables where appropriate. Chi-square or Fisher's exact test were used for categorical variables where appropriate. Covariates included HIF-1α percentage, intensity and expression score, CAIX percentage, intensity and expression score, HSP90 percentage, intensity and expression score, and iNOS percentage, intensity and expression score, in addition to age, sex, ethnicity, receipt of adjuvant chemotherapy and/or radiation, tumor size, margin status, nodal status, grade, perineural invasion (PNI), and lymphovascular invasion (LVI). Univariate analysis was also performed for association of each covariate with HIF-1α expression level in order to assess for possible interaction between variables.

iii. Univariate Kaplan-Meier analyses were also performed for each covariate with overall mortality. Covariates included HIF-1α percentage, intensity and expression score, CAIX percentage, intensity and expression score, HSP90 percentage, intensity and expression score, and iNOS percentage, intensity and expression score, in addition to age, sex, ethnicity, receipt of adjuvant chemotherapy and/or radiation, tumor size, margin status, nodal status, grade, perineural invasion (PNI), and lymphovascular invasion (LVI). If any of the biomarkers were significant on

univariate analysis, a multivariate survival analysis would be performed using Cox proportional hazard modeling.

iv. Multivariate stepwise logistic regression modeling was performed for LR and DR in all patients adjusting for all clinically relevant covariates.

v. A subgroup analysis was performed including only patients with known recurrence before death or patients alive with disease. This excluded patients with no documented recurrence due to loss to follow-up or whose death was due to non-cancer causes. Multivariate logistic regression modeling was performed for association of HIF-1α score with LR and DR in this cohort, using stepwise elimination with $\alpha < 0.1$ as a threshold. Receiver operating characteristic (ROC) curves and predictive probability curves were generated for biomarker expression as a predictor of LR and DR.

vi. In order to assess the correlation between biomarkers, spearman correlation were estimated between HIF-1α score and HSP90, iNOS and CAIX scores.

RESULTS

Descriptive Statistics

8 (8%) of patients exhibited local recurrence alone, 13 (13%) of patients exhibited distant recurrence alone, 26 (27%) of patients exhibited both local and distant recurrence and 51 (52%) of patients exhibited no known recurrence (Table 1, Figure 2b). 65 patients died during follow-up with a median overall survival of 30.6 months. Median follow-up time was 30.6 months. 53 (54%) patients exhibited high HIF-1α expression, 58 (59%) exhibited high CAIX score, 33 (34%) exhibited high HSP90 score and 46 (47%) exhibited high iNOS score. 79 (81%) underwent adjuvant chemotherapy and 17 (17%) underwent adjuvant radiation therapy. Median tumor size was 3.5 cm (range 1-7cm), 26 (26%) patients had positive surgical resection margins and 70 (70%) patients had positive nodal status at resection. HIF-1α was differentially expressed in patient samples, and this can be seen in figure 1. The remainder of the patient characteristics can be seen in table 1.

On univariate association of covariates with HIF-1α score (table 5), the only predictor that was significantly different between those with high HIF-1α and those with low HIF-1α was age;patients with high HIF-1α were older on average compared to patients with low HIF-1α (p=.02).

Local Recurrence

On univariate analysis for association of covariates with local recurrence alone in all patients (table 2), the only covariates significantly associated were HIF-1α percent (p=0.04), HIF-1α score (p=0.03) with the local recurrence group having lower HIF-1α percent and scores compared to everyone else. On multivariate adjusted logistic regression for local recurrence in all patients (table 6), only HIF-1α score remained significant (p=0.05) with an odds ratio for local recurrence of 0.50 (95% CI 0.22-0.95) for high expression level versus low expression level.

Distant Recurrence

In univariate analysis of covariates associated with distant recurrence in all patients (table 3), HSP90 percent was associated with distant recurrence (p=0.02), but SP90 overall score was not (p=0.18). CAIX score was also associated with distant recurrence (p=0.03), but neither of these effects remained significant when adjusted for adverse tumor characteristics in multivariate logistic regression analysis (data not shown). In multivariate logistic regression for distant recurrence in all patients, adjusted for all clinically relevant covariates (table 7), age was the only significant predictor of distant recurrence, with an odds ratio of 0.96 per year younger (95% CI 0.92-0.998; p=0.04). On subset analysis of all patients with recurrence, Low HIF-1α score was associated with increased risk of distant recurrence on both univariate (p=0.03) and multivariate logistic regression (table 8) analyses. In multivariate logistic regression analysis, high HIF-1α

score yielded an estimated Odds Ratio of 2.35 (95% CI 1.08-6.18) for high HIF-1α score over low HIF-1α score for risk of distant recurrence.

In this subset, ROC curves for distant recurrence predicted b HIF-1α score (figure 2d) exhibited an area under the curve (AUC) of 75.21. Predicted probability curves (figure 2c) exhibit a specificity of 100% for distant recurrence in patients with HIF-1α score > 2.5.

Survival Analyses

On log-rank Kaplan-Meier analysis for all-cause overall mortality (table 4), increased risk of death was associated with positive lymphovascular invasion (median survival 14.01 months vs 18.58 months; p=0.04) and lack of adjuvant therapy (median survival 10.33 months vs 17.92 months; p=0.03). Multivariate survival analysis was not performed because no biomarkers were associated with increased mortality, including HIF-1α (p=0.66), HSP90 (p=0.56), iNOS (p=0.96) and CAIX (p=0.85).

Correlation of Biomarkers

In Figure 3, spearman correlation showed that HIF-1α score was not associated with HSP90 (p=0.61), CAIX (p=0.67) or iNOS score (p=0.97).

DISCUSSION/CONCLUSIONS

In this study, high nuclear HIF-1α expression was highly specific as a predictive biomarker for distant recurrence of disease. Many biomarkers have been studied in pancreatic cancer, including other biomarkers involved in angiogenesis, tumor growth and invasion, and chemotherapy metabolism.(14, 15) Like the majority of these biomarkers, the hypoxia biomarkers in this study failed to show a significant survival advantage or disadvantage in these patients. However, few of these markers have been linked to specific patterns of failure in pancreatic cancer, as HIF-1α has been in this study. The loss of one such biomarker, SMAD4, was associated with increased pattern of distant failure in patients with advanced pancreatic cancer in a rapid autopsy series(16, 17), but this association did not stand in patients with earlier stage resectable pancreatic cancer(18), potentially due to less aggressive tumor biology or earlier disease in these patients. This makes nuclear HIF-1α, to our knowledge, one of the first biomarkers to identify specific patterns of failure in early-stage pancreatic cancer patients.

Despite this novelty, HIF-1α has been associated with both metastatic disease in other tumor types(19-22) and with aggressive tumor histology in cell line and tissue studies of pancreatic cancer (4, 14, 15, 23). Nuclear HIF-1α is expressed in approximately 88% of pancreatic ductal carcinomas versus only 16% of normal pancreas tissues, and is expressed by adjacent stroma to tumor in 43% cases, which suggests a role of HIF-1α in metastatic tumor behavior(24, 25). Recent studies have further elucidated a potential link between tumor hypoxia and HIF-1α activated stromal proliferation that may make tumors with high HIF-1α expression prone to metastatic disease(8). It has

been unclear whether nuclear HIF-1α expression levels are a result of treatment response by tumors or a marker of aggressive tumor behavior, thus this study analyzed only tissue samples from patients that had not been previously exposed to chemotherapy or radiation therapy.

A pancreatic cancer biomarker that is predictive of failure patterns may be very timely as the role of radiation therapy versus isolated adjuvant chemotherapy in resected pancreatic cancer remains under debate. Several large studies have shown an advantage to adjuvant chemotherapy alone over adjuvant chemoradiation(26) yet 30% of PAC patients still die from locally aggressive disease rather than widespread metastatic disease (27). Radiation therapy can provide excellent local control in these patients and should be utilized in selected patients. Identifying those patients with resected PAC who are likely to develop isolated local or distant metastatic disease may have two-fold clinical benefit: prognostic information may help select patients for local radiation therapy and/or more aggressive systemic chemotherapy, and the development of targeted agents may prevent distant metastases and thus increase the effectiveness of local therapies and improve survival. With further investigation, it is also possible that this information could help stratify patients with resectable and borderline resectable tumors who would benefit from neoadjuvant therapy.

This data also justifies the further development of potential therapeutic targets, including HIF-1α inhibitors and HSP90 inhibitors,(24, 25) as both systemic agents and as radiosensitizing agents. When radiation is administered to a hypoxic tumor, such as pancreatic adenocarcinoma, the radiation dose must be nearly tripled in order to achieve comparable cell killing as compared to oxygenated tissue, which also increases normal

tissue toxicity. Pancreatic adenocarcinoma is among the most hypoxic of all solid tumors, and HIF-1α inhibitors have been investigated pre-clinically as one method of increasing radiation response by decreasing resistance to radiation-induced apoptosis.(24, 25) HIF-1α expression has also been associated with gemcitabine resistance in hypoxic environments, indicating HIF-1α may have a role in potentiating systemic therapeutic agents.(28)

This study has demonstrated that nuclear HIF-1α expression level at time of resection for early-stage PAC is a strong predictor of eventual distant failure versus local failure. While this analysis provides important hypothesis-generating data supported by previous studies of HIF-1α expression in PAC, future studies are necessary to prospectively evaluate the role of HIF-1α expression in a larger, more homogeneous patient cohort, such as evaluation as a secondary endpoint in a future cooperative group trial.

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Table 1. Patient Demographics and Clinicopathologic Characteristics (n=98)

Patient Demographics		**n**	**%**
Ethnicity			
	Black	21	21.4
	White	62	63.3
	Other	6	6.1
Age <60		27	28.7
Age >=60		67	71.3
Clinical Characteristics		**n**	**%**
Tumor Size		35 mm (10-70)*	
Surgical Margin Positive		26	26
Surgical Margin Negative		71[+]	72
Tumor Grade:			
	I	4	4
	II	77	79
	III/IV	17	17
Perineural Invasion		83	85
Lymph Node (LN) Positive		70	70
Lymphovascular Invasion		55	55
High HIF-1α Expression[α]		53	54
High CAIX Expression		58	59
High HSP90 Expression		33	34
High iNOS Expression		46	47
Treatment Characteristics		**n**	**%**
Adjuvant Chemotherapy (Yes)[+]		79	81
Adjuvant Chemotherapy (No)		14	14
Radiation Therapy (Yes)		17	17
Patterns of Failure			
Local Recurrence Alone[+]		8	8
Distant Recurrence Alone		13	13
Distant & Local Recurrence		26	27
No Known Recurrence		51	52
Deaths Reported: 65 deaths			
Median Follow-up: 8.27 months			
Median Overall Survival 30.6 months			

* Median (Range) given where appropriate

+ Denotes category with missing data

[α] High expression defined as all values greater than nearest whole integer to median for each individual biomarker

Table 2. Univariate Association of Covariates with Local Recurrence Alone (n=98)

Variable	No Local Recurrence	Local Recurrence	p-value
	Wilcoxon Score	**Wilcoxon Score**	
CAIX Intensity	50.10*	43.00*	0.47
CAIX Percent	50.10	42.94	0.50
CAIX Score	50.43	38.94	0.27
HSP90 Intensity	50.14	42.31	0.40
HSP90 Percent	49.75	46.75	0.77
HSP90 Score	50.13	42.44	0.45
iNOS Intensity	49.56	48.88	0.95
iNOS Percent	50.45	38.88	0.27
iNOS Score	50.21	41.50	0.40
HIF1a Intensity	50.60	37.25	0.16
HIF1a Percent	51.29	29.38	**0.04**[+]
HIF1a Score	51.36	28.63	**0.03**
Tumor Size	48.40	43.69	0.65
	N (%)	**N (%)**	**P-value**
Ethnicity			
Black	20 (24.39)	1 (14.29)	0.62
White	57 (69.51)	1 (14.29)	
Other	5 (6.10)	5 (71.43)	
Grade			0.53
I	4 (4.44)	0 (0.00)	
II	69 (76.67)	8 (100.00)	
III	17 (18.89)	0 (0.00)	
Nodal Status			0.69
Positive	25 (27.78)	3 (37.50)	
Negative	65 (72.22)	5 (62.50)	
PNI			0.60
Positive	77 (85.56)	6 (75.00)	
Negative	13 (14.44)	2 (25.00)	
LVI			0.72
Positive	51 (56.67)	4 (50.00)	
Negative	39 (43.33)	4 (50.00)	
Adjuvant Therapy			1.00
Yes	72 (84.71)	7 (87.50)	
No	13 (15.29)	1 (12.50)	

* Wilcoxon Score (Rank Sums) reported unless otherwise noted

[+] Bold font denotes statistical significance (alpha <.05)

Abbreviations: LN (lymph node), LVI (lymphovascular invasion), PNI (perineural invasion), R0 (negative surgical margins), R1/R2 (microscopic/ macroscopic positive surgical margins)

Table 3. Univariate Association of Covariates with Distant Recurrence (n=98)

Variable	No Distant Recurrence	Distant Recurrence	p-value
	Wilcoxon Score	**Wilcoxon Score**	
CAIX Intensity	45.56	55.46	0.07
CAIX Percent	45.09	56.12	0.06
CAIX Score	44.41	57.21	**0.03**
HSP90 Intensity	50.05	48.67	0.79
HSP90 Percent	55.13	41.00	**0.02**[+]
HSP90 Score	52.55	44.90	0.18
iNOS Intensity	50.92	47.36	0.49
iNOS Percent	51.02	47.21	0.52
iNOS Score	50.75	47.62	0.59
HIF1a Intensity	48.60	50.90	0.67
HIF1a Percent	46.60	53.90	0.22
HIF1a Score	46.85	53.51	0.25
Tumor Size	46.78	49.82	0.60
	N (%)	**N (%)**	**P-value**
Ethnicity			0.28
Black	9 (17.65)	12 (31.58)	
White	38 (74.51)	2 (5.26)	
Other	4 (7.84)	24 (63.16)	
Grade			0.78
I	3 (5.08)	1 (2.56)	
II	45 (76.27)	32 (82.05)	
III	11 (18.64)	6 (15.38)	
Nodal Status			0.95
Positive	42 (71.20)	28 (71.80)	
Negative	17 (28.81)	11 (28.21)	
PNI			0.98
Positive	50 (84.75)	33 (84.62)	
Negative	9 (15.25)	6 (15.38)	
LVI			0.96
Positive	33 (55.93)	22 (56.41)	
Negative	26 (44.07)	17 (43.59)	
Adjuvant Therapy			0.73
Yes	47 (83.93)	5 (13.51)	
No	9 (16.07)	32 (86.49)	

* Wilcoxon Score (Rank Sums) reported unless otherwise noted

[+] Bold font denotes statistical significance (alpha <.05)

Abbreviations: LN (lymph node), LVI (lymphovascular invasion), PNI (perineural invasion), R0 (negative surgical margins), R1/R2 (microscopic/ macroscopic positive surgical margins)

Table 4. Univariate Association of Covariates with Overall Mortality		
Covariate	**Median Survival (in months) (95% CI)**	**Log-rank p-value**
HIF1a Score		0.66
Low Expression[α]	16.04 (11.90-26.01)	
High Expression	16.9 (8.48-22.72)	
HSP90 Score		0.56
Low Expression	16.90 (8.68-20.98)	
High Expression	15.81 (12.00-30.61)	
iNOS Score		0.96
Low Expression	15.22 (8.68-20.38)	
High Expression	17.98 (11.90-29.72)	
CAIX Score		0.85
Low Expression	18.58 (8.58-25.74)	
High Expression	16.90 (10.82-22.72)	
Ethnicity		0.28
Black	30.02 (11.90-56.71)	
Other	13.30 (1.48-.)*	
White	16.90 (9.99-20.98)	
Grade		0.97
I	18.31 (4.08-32.38)	
II	16.90 (11.54-20.38)	
III	13.86 (6.58-37.87)	
Nodal Status		0.06
Positive	14.01 (9.07-19.69)	
Negative	26.01 (11.90-37.87)	
PNI		0.44
Positive	17.98 (11.90-22.72)	
Negative	13.84 (4.10-19.70)	
LVI		**0.04**[+]
Positive	14.01 (9.96-20.38)	
Negative	18.58 (13.84-35.31)	
Adjuvant Therapy		**0.03**
Yes	17.92 (12.00-25.74)	
No	10.33 (1.78-20.98)	

[α] Low expression defined as score < median and high expression defined as score >= median for each biomarker listed

* Largest value censored

[+] Bold font denotes statistical significance (alpha <.05)

Abbreviations: LN (lymph node), LVI (lymphovascular invasion), PNI (perineural invasion), R0 (negative surgical margins), R1/R2 (microscopic/ macroscopic positive surgical margins)

Table 5. Univariate Association of Covariates with HIF1a Expression (n=98)

		Hif1a <2 N=45		Hif1a >=2 n=53		p-value[α]
		n	**%**	**n**	**%**	
Patient Demographics						
Age (years)[a]						**0.02**
Ethnicity						0.52
	Black	8	17.8	13	24.5	
	White	28	62.2	34	64.2	
	Other	4	8.9	2	3.8	
Clinical Characteristics						
Tumor Grade:						0.22
	I	3	6.7	1	1.9	
	II	37	82.2	40	75.5	
	III	5	11.1	12	22.6	
R0 Resection		32	72.7	39	73.6	0.64
R1/R2 Resection		12	27.3	14	24.5	
LN Positive		36	80.0	34	64.2	0.08
LN Negative		9	20.0	19	35.8	
Tumor Size (mm)						0.41
	<3.5	24	54.5	23	45.1	
	>=3.5 cm	20	45.5	28	54.9	
LVI Positive		24	53.3	31	58.5	0.61
LVI Negative		21	46.7	22	41.5	
PNI Positive		39	86.7	44	83.0	0.62
PNI Negative		6	13.3	9	17.0	
Treatment Characteristics						
Adjuvant Chemotherapy						0.85
	No	6	14.3	8	15.7	
	Yes	36	85.7	43	84.3	

***** Fisher's exact test used where appropriate

[a] Age is treated as a continuous variable, per year

Abbreviations: LN (lymph node), LVI (lymphovascular invasion), PNI (perineural invasion), R0 (negative surgical margins), R1/R2 (microscopic/ macroscopic positive surgical margins)

Table 6. Multivariate Adjusted Logistic Regression Model for Local Recurrence Alone in All Patients (n=98)				
Covariate	**OR**	**95 % CI**	**Chi-square**	**p-value**
Adjuvant Chemotherapy	2.23	0.89-1.03	1.379	0.24
Tumor Size	1.00	0.52-1.88	0.0002	0.99
Margin Status	1.82	0.28-9.62	3.689	0.05
Nodal Status	0.67	0.13-4.10	0.218	0.64
HIF1a Score (High)	0.50	0.22-0.95	3.689	**0.05**

Table 7. Multivariate Adjusted Logistic Regression Model for Distant Recurrence in All Patients (n=98)				
Covariate	**OR**	**95 % CI**	**Chi-square**	**p-value**
Adjuvant Chemotherapy	1.04	0.29-4.11	0.004	0.95
Tumor Size	0.96	0.67-1.34	0.051	0.82
Margin Status	1.21	0.44-3.24	0.121	0.73
Nodal Status	1.45	0.53-4.16	0.442	0.51
HIF1a Score (High)	1.12	0.79-1.60	0.424	0.52
Age (per year)	0.96	0.92-1.00	4.129	**0.04**

Table 8. Multivariate Adjusted Logistic Regression Model for Distant Recurrence in All Patients with Recurrence (n=41)				
Covariate	**OR**	**95 % CI**	**Chi-square**	**p-value**
Adjuvant Chemotherapy	0.24	0.94-1.12	0.504	0.48
Tumor Size	1.04	0.51-2.34	0.009	0.93
Margin Status	0.65	0.09-6.03	0.171	0.68
Nodal Status	1.28	0.17-8.28	0.093	0.76
HIF1a Score (High)	2.35	1.08-6.18	3.819	**0.04**
Age (per year)	1.03	0.94-1.12	0.388	0.53

Figure 1. HIF1a is differentially expressed via immunohistochemistical analysis. Low intensity of staining and low percentage of staining (score 0) are demonstrated in (1a) and high intensity of staining ad high percentage of staining (score 5.3) are demonstrated in (1b). Inset images are TMA core at x100 magnification and large images are at x400 magnification. Figure (1b) demonstrates nuclear positivity of HIF1a.

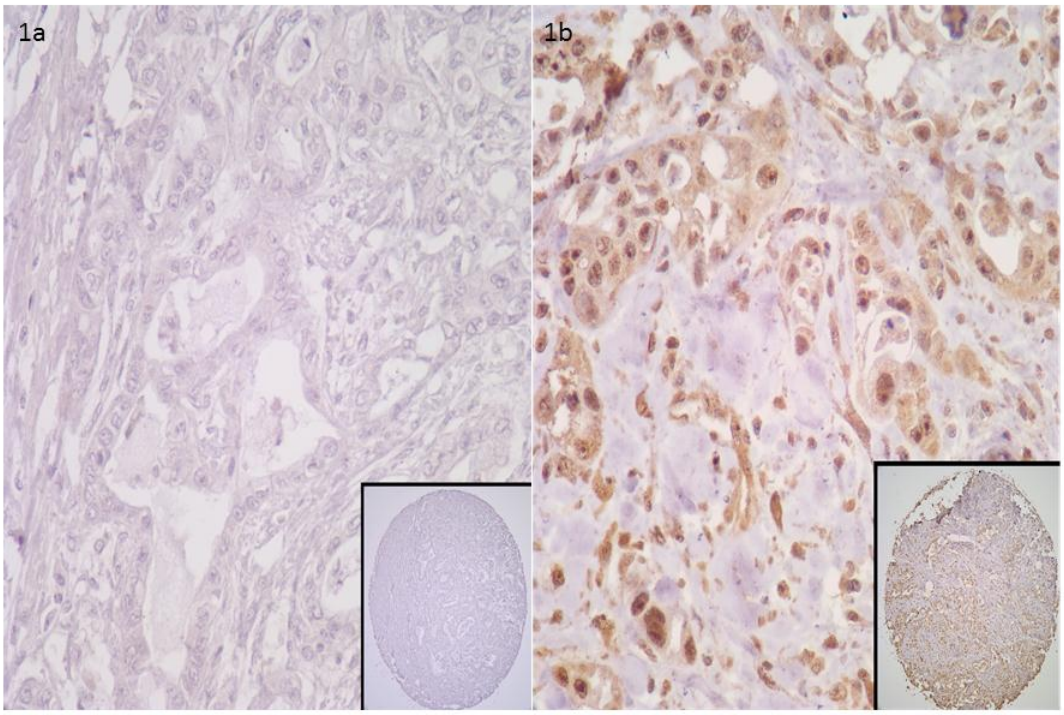

Figure 2.
Distribution of Wilcoxon scores for HIF1a score by site of recurrence (1a) demonstrates statistically significant difference between mean HIF1a score for isolated locoregional versus distant recurrence. Both predicted probability curve (1c) and Receiver operating characteristic curve (1d) for HIF1a score as a predictor of distant recurrence demonstrate high specificity for distant recurrence with high HIF1a score in all patients with recurrence. The percentage of each failure type by HIF1a score (1b), with darkest shading representing lowest scores and lightest shading representing higher scores, demonstrates the local recurrence group composed primarily of low HIF1a scores, distant/local recurrence group composed of a broad range of scores and the distant recurrence group composed of predominantly high scores.

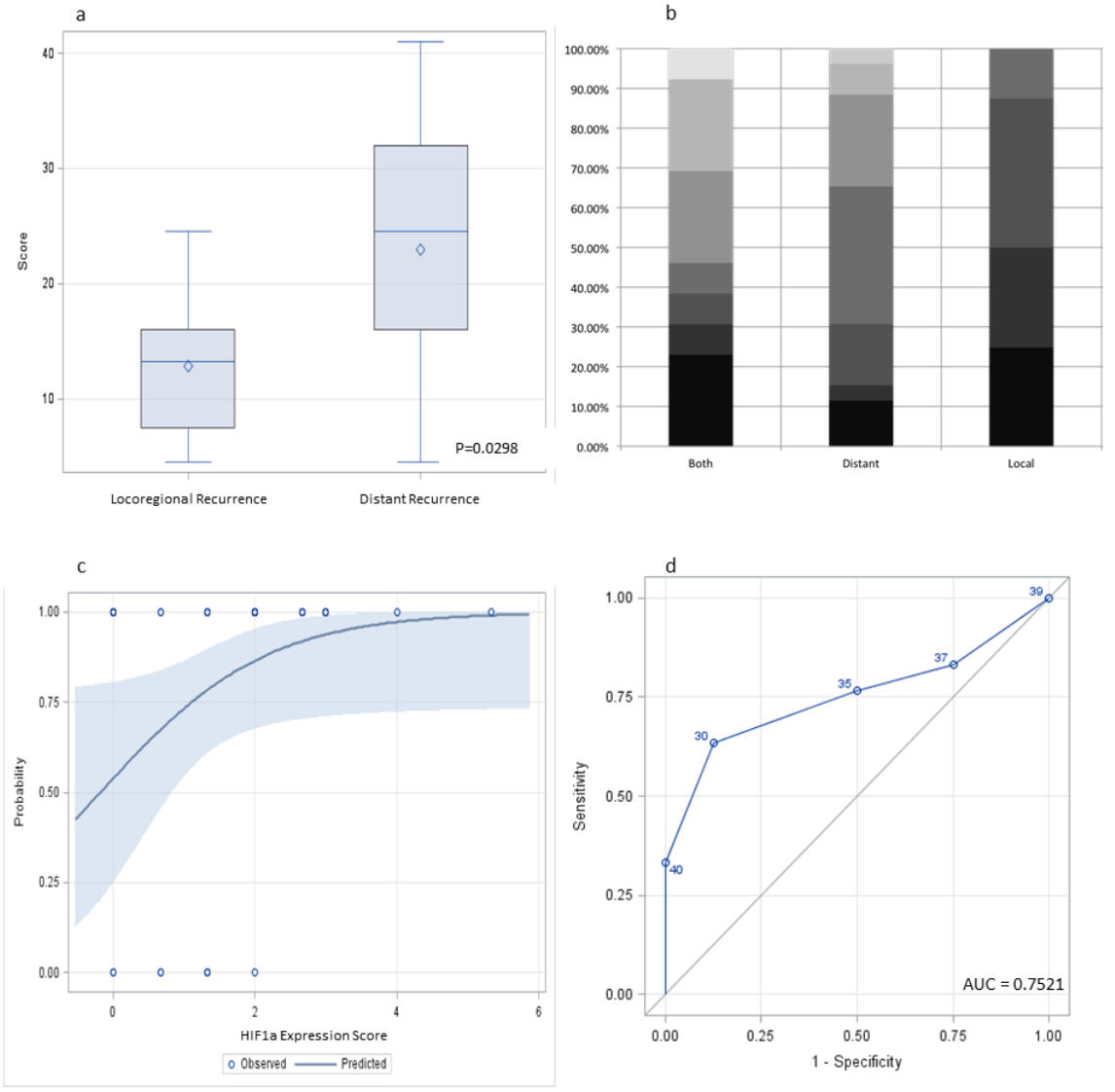

Figure 3. Correlation of HIF1a score with iNOS score (1a), HSP90 score (1b) and CAIX score (1c).