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The Association between HIF-1α Activity and Time to Breast Cancer Recurrence in the Danish Breast Cancer Cooperative Group

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Abstract

The Association between HIF-1α Activity and Time to Breast Cancer Recurrence in the Danish Breast Cancer Cooperative Group

By Joshua D. Chang

Background. HIF-1α has shown to be a biomarker in initial breast cancer biopsies to be associated with the incidence of recurrence after initial treatment. Multiple biochemical pathways have been hypothesized to give credence to these observed clinical outcomes. The focus of the study was the timing of the recurrence amongst patients who were registered to have had a recurrence and had available recurrent breast tissue sections to associate with HIF-1α's activity.

Methods. Data utilized were from the Danish Breast Cancer Group (DBCG), a subample of a cohort that included 193 recurrent ER/TAM+ subjects and 116 ER/TAM- subjects, which were stratified for all ensuing analysis. Their tissue sections were stained and quantified utilizing immunohistochemical methods for HIF-1α activity and then considered as the main form of exposure in a multivariate logistic regression.

Results. Within the ER/TAM+ group it was observed that those with HIF-1α nuclear activity would have a recurrence within 3 to 10 years 0.87 as many as times those without HIF-1α nuclear activity rather than within 1 to 3 years after initial treatment, 95% confidence interval (0.45, 1.70). Similarly, the ER/TAM- arm of the study observed that those with HIF-1α nuclear activity would have a recurrence within 3 to 10 years 0.60 as many times as those without HIF-1α nuclear activity rather than within 1 to 3 years after initial treatment, 95% confidence interval (0.17, 2.04).

Conclusion. The observations shown herein demonstrate that among those who will have a recurrence of breast cancer, HIF-1α activity is more likely to be associated with an early recurrence of breast cancer rather than a later recurrence of breast cancer.

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Introduction

HIF Molecular Structure

Hypoxia is a state whereby cells experience a reduced availability of oxygen. Since hypoxic conditions may lead to necrosis, cells have evolutionarily developed several mechanisms by which to survive for longer periods of time. One such mechanism is the expression of the protein hypoxia-inducible factor (HIF). HIF exists as a heterodimer: a hypoxia-activated α subunit and a constitutively expressed β subunit, also known as aryl hydrocarbon nuclear receptor translocator (ARNT) (1).

HIF has three isoforms of its α subunit named HIF-1α, HIF-2α, and HIF-3α. HIF-1α and HIF-2α regulate very similar genes and mechanisms and as such, have been the focus of a large portion of research. HIF-3α has been known to be a negative regulator of these genes and so has not been given nearly as much research attention (2, 3).

Various Mechanisms of HIF-α

HIF and Cellular Activity

When the HIF complex has entered a cell's nucleus it acts as a transcriptional factor for hundreds of genes including those involved in angiogenesis such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and angiopoeitin-1 (ANGPT1) (4). HIF-1 will also trigger the construction of enzymes that aid in anaerobic metabolism essential for cell survival in a hypoxic environment (5).

Methods of Regulation

HIF-1α functions via a post-translational modification. Under normoxic conditions, prolyl hydroxylase domain (PHD) proteins will hydroxylate proline residues on HIF-1α, which leads to pVHL, the protein product of the von Hippel Lindau tumor suppressor gene, binding and thus ubiquitinating HIF-1α. The ubiquitin marked protein is then targeted by

proteasomes for degradation due to its ubiquination (6). However, in order for the prolyl hydroxylase domain proteins to function, there must be adequate amounts of iron and 2-oxoglutarate present.

Oxygen gradients will also impact HIF-1α activity by factor-inhibiting HIF (FIH). Also under normoxic conditions, FIH will hydroxylate HIF-1α at asparagine residues at its C-terminus blocking specific coactivators and rendering HIF-1α transcriptionally inactive (7). When a cell experiences hypoxic conditions, both FIH and PHD will be suppressed, allowing the HIF-1α subunit concentration to rise and enter the nucleus to dimerize with HIF-1β. This heterodimer will then bind to hypoxia response elements of target genes and thus activate subsequent biochemical cascades allowing a cell to better survive hypoxic conditions.

Lastly, HIF-1α can also be regulated independent of oxygen concentrations. Multiple pathways have been demonstrated to activate HIF-1α via hormones and/or inflammatory cytokines. Such pathways utilize protein kinases, interleukins, micro RNAs, and/or intracellular reactive oxygen species to increase or decrease HIF-1α transcription activity (1).

HIF-1α and Cancer

As HIF-1α plays a significant role in cell survival in hypoxic conditions, there has been growing evidence suggesting how it impacts the pathogenesis of cancer where poorly vascularized solid tumors can be abundant. Consistent HIF activity can lead to changes in glycolysis, nutrient uptake, waste secretion, angiogenesis, apoptosis, and cell migration in promoting tumor survival and metastasis. Alongside the above mentioned oxygen-independent regulators, HIF activity may also be stabilized through mutations in the von Hippel-Lindau gene and oncogenic genes leading to overexpression of growth factors that

highly impact angiogenesis (8-10). HIF-1α expression will also be upregulated in acute onset hypoxia, such as due to rapid tumor growth, whereas HIF-2α and HIF-3α will be overexpressed in chronic hypoxia, illustrating HIF-1α as a potentially significant marker in assessing tumor activity.

Induction of the HIF pathway has been associated with tumor aggressiveness, invasion, and metastasis through different mechanisms. In a study of breast cancer cells in normoxic, hypoxic, and anaerobic environments, the rate of cell proliferation as well as invasion and migration activities of cancer cells were significantly higher in hypoxic and anaerobic conditions than normoxic and this was associated with an upregulation of HIF-1α mRNA in the groups (4). This activity has been shown to independently predict prognosis in patients with specific types of breast cancer and has been the focus of much research interest in predicting patient outcomes (11, 12). HIF-1α has also been found to predict early recurrence of breast cancer patients, illustrating a poor overall survival and high risk for metastasis when active (13).

HIF-1α and Metastasis

When a cancer cell metastasizes, it must undergo an epithelial-to-mesenchymal transition whereby the cells change most notably by losing cell-cell contact and gain motility. Once the cell is mobile, it must penetrate a blood vessel to circulate in the body, survive, exit the bloodstream, and form a niche at the metastatic site that is favorable for cancer cell growth. HIF-1 plays many roles in breast cancer including angiogenesis, EMT, invasion, metastasis, and resistance to radiation therapy and chemotherapy (14). A high level of HIF-1α activity at diagnosis has been shown to be predictive of early relapse and metastasis and correlates with poor clinical outcomes in breast cancer (15).

Forms of Breast Cancer

Breast cancer is the most commonly diagnosed cancer among women worldwide and includes three broad classifications for treatment: the hormonal receptor positive group (HR positive) characterized by estrogen receptor (ER) and/or progesterone receptor (PR) expression, the human epidermal growth factor receptor 2 (HER2) amplified group, and the triple negative breast cancer (TN) group which lacks any of the prior mentioned receptors. As tumors grow and cells proliferate there is an increased demand for O2, but a decrease in O2 availability due to the abnormal blood vessels that form in the tumor leading to an increase in HIF-1 activity.

Research Focus

As HIF-1α's link to metastasis and early recurrence have been established, the study aimed to estimate HIF-1α's possible association with recurrence and the proportion of recurrence that occur within specific bounds of time. Exploration of breast cancer in the patient population will utilize a retrospective, within case study design while controlling for major confounders of interest to explore the pattern of time to recurrence.

Methods

Study Population

Data for the study were requested from the Danish Breast Cancer Cooperative Group (DBCG). Established in 1975 by the Danish Surgical Society, the DBCG seeks to establish a nation-wide standardization of breast cancer treatment and captures all incident cases of primary invasive breast cancer as well as recurrences of such cancers for research purposes (16).

Inclusion criteria included those who were registered in the database, were diagnosed between 1985 and 2001, were age 35-69 at diagnosis, and were diagnosed with stage 1, 2, or 3 breast cancer. From this cohort, those excluded were those who received an unknown treatment, had an unknown ER-status, had an unknown tamoxifen protocol, and did not survive more than 1 year after the first breast cancer diagnosis. Figure 1 illustrates these criteria graphically and describes the cohort of this study in part.

From this cohort, 1,826 ER-positive and tamoxifen-treated patients (ER+/TAM+) and 1,808 ER-negative and tamoxifen-untreated patients (ER-/TAM-) were selected for analysis.

Cases were denoted as those women who experienced a recurrence within 10 years of the first breast cancer diagnosis or by December 31, 2006. The DBCG organizes follow-up visits every 3 to 6 months for the first 5 years after initial remission and then visits every 12 months after 5 years till 10 years after first breast cancer diagnosis. Recurrence was then defined as any type of local, contralateral or distant recurrence.

Cases were also selected based on the availability of tissue core sample availability of the recurrent cancer biopsy. This led to 193 recurrent ER+/TAM+ cases and 116 recurrent ER-/TAM- cases.

Immunohistochemistry

Tissue biopsies from the cases were evaluated for HIF-1 expression using tissue microarrays (TMAs). Formalin-fixed, paraffin-embedded diagnostic primary breast

cancer tumor specimens were collected from the pathology archives of participating hospitals by a medical research technician.

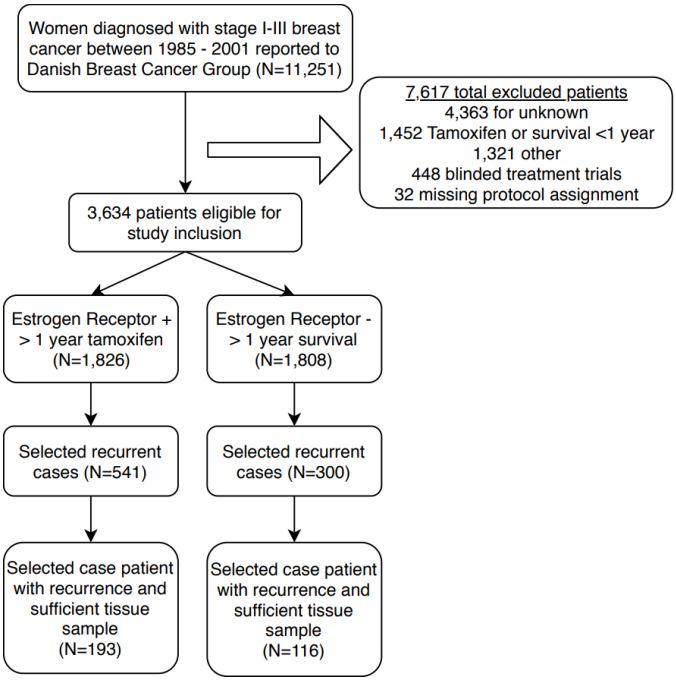

Figure 1. The workflow of study eligibility and resulting sample size used for study analysis.

New tissue sections were cut and used by experienced pathologists to identify blocks with invasive carcinoma. Tissue microarrays were then constructed using a TMA Master (3Dhistech Ltd., Budapest, Hungary), with one to three 1-mm tissue core samples from each tumor specimen. Cores from individual tumors were included in duplicate TMAs. Where possible, up to three representative tumor cores and one nonneoplastic core from marginal or normal tissues were sampled for analysis. 2.5 μm tissue sections were then stained by the pathology laboratory at the Rollins School of Public Health at Emory University / Pathology department at Aarhus University Hospital for HIF-1α using standard immunohistochemical staining protocols outlined below.

TMA slides were deparaffinized in xylene, hydrated in graded alcohols, and blocked for endogenous peroxidase for 5 min in UltraVision hydrogen peroxidase blocks (Thermo Fisher Scientific, ref. TA-125H202Q). Heat-induced epitope retrieval (HIER) was performed in a decloaking chamber (PT Link, Agilent, Santa Clara, CA 95051) in citrate buffer pH 6.0 (Thermo Fisher Scientific, ref. TA-250-PM1X). The slides were rinsed in distillated water, and immersed in wash buffer with tween 20 (Cell Marge, ref. 935B-09) for 5 min to eliminate surface tension.

Staining was carried out at room temperature in an automated instrument, Dako AutostainerPlus, and the slides washed with wash buffer between all steps. The slides were incubated with UltraV block (Thermo Fisher Scientific, ref. TA-125-UB) for 5 min, followed by primary antibody for 30 min; rabbit monoclonal anti-HIF-1 (EP1215Y) (Abcam, Cat. #ab51608) at concentration of 1:1500, UltraVision Goat Polyvalent Secondary (Thermo Fisher Scientific, ref. TL-125-BN) for 15 min, UltraVision Streptavidin Horseradish Peroxidase (Thermo Fisher Scientific, ref. TL-125-HR) for 15 min, and finally diaminobenzidine (DAB, Thermo Fisher Scientific, ref. TA-125-HDX) for 5 min. The slides were counterstained with hematoxylin (Thermo Fisher Scientific, ref. 7211), dehydrated, and coverslipped using Cytoseal XYL (Thermo Fisher Scientific, ref. 8312-4) in a Leica AutoStainer XL and Leica CV5030 instruments. Slides were dried for at least 24 hours before being digitalized using a whole slide image scanner, Pannoranmic Scan 150 (3D HisTech, Budapest, Hungary).

HIF-1α expression was then evaluated at Emory University (Atlanta, GA USA) on digitized images by one of us (JC).

An H-score was implemented in quantifying both nuclear staining and cytoplasmic staining. Activity level was graded using a 4 point scale (0 = no staining, 1 = light, 2 = moderate, 3 = strong) and proportion was graded on a 5 point scale (0 = no staining, 1 = < 1% stained, 2 = 1%-25% stained, 3 = 26%-50% stained, 4 = >50% stained). Scores for both nuclear activity and cytoplasmic activity ranged from 0-12.

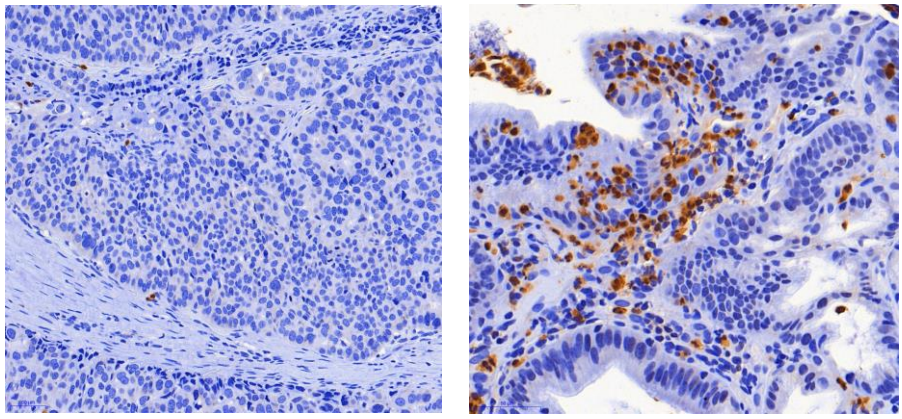

Figure 2. Initial breast cancer biopsy tissue imaging reviewed for scoring. Left, light nuclear staining of HIF-1α, such an area would be given an activity score of 3 and a proportion score of 1. Right, heavy nuclear staining, which would be assigned an activity score of 3 and a proportion score of 4.

Analytic Variables

Exposure to HIF-1A

From the immunohistochemistry analysis, a dichotomized activity score was assigned if within a patient cluster any sample had a total score higher than 4 in any tissue sample. This metric was utilized for both nuclear activity as well as cytoplasmic activity.

Breast Cancer Recurrence

The outcome of breast cancer recurrence was obtained from the DBCG database and for the purposes of this study are limited to those occurring within 1 to 10 years of follow-up after initial treatment therapy. This continuous time was then categorized into groups from 1 to < 3 years and 3 to 10 years.

Covariates of Interest

Other characteristics of interest included UICC stage (I, II, III), menopausal status at diagnosis (pre/post), receipt of chemotherapy (yes/no), receipt of radiotherapy (yes/no), surgery type (mastectomy/breast conserving surgery), age at diagnosis, and county of residence.

	ER+/TAM+ Breast Cancer patients, n (%)		ER-/TAM- Breast Cancer patients, n (%)	
	HIF-1α + Activity	HIF-1α - Activity	HIF-1α + Activity	HIF-1α - Activity
Total Number	68 (22.0)	125 (40.5)	30 (9.7)	86 (27.8)
Age at Diagnosis, Years				
35-44	0 (0)	7 (5.6)	9 (30.0)	12 (14.0)
45-54	10 (14.7)	30 (24.0)	14 (46.7)	37 (43.0)
55-64	38 (55.9)	70 (56.0)	5 (16.7)	22 (25.6)
65-69	20 (29.4)	18 (14.4)	2 (6.7)	15 (17.4)
Menopausal Status at Diagnosis				
Premenopausal	3 (4.41)	11 (8.8)	14 (46.7)	29 (33.7)
Postmenopausal	65 (95.6)	114 (91.2)	16 (53.3)	57 (66.3)
UICC tumor stage at diagnosis				
I	0 (0)	2 (1.6)	2 (6.7)	8 (9.3)
II	35 (51.5)	53 (42.4)	17 (56.7)	42 (48.8)
III	33 (48.5)	70 (56.0)	11 (36.7)	36 (41.9)
Type of surgery				
Breast conserving surgery	5 (7.4)	11 (8.8)	6 (20.0)	5 (5.8)
Mastectomy	63 (92.6)	114 (91.2)	23 (76.7)	81 (94.2)
Missing	0 (0)	0 (0)	1 (3.3)	0 (0)
Receipt of chemotherapy				
Yes	4 (5.9)	15 (12.0)	23 (76.7)	65 (75.6)
No	64 (94.1)	110 (88.0)	7 (23.3)	21 (24.4)
Missing	0 (0)	0 (0)	0 (0)	0 (0)
Receipt of radiation therapy				
Yes	19 (27.9)	38 (30.4)	14 (46.7)	31 (36.1)
No	49 (72.1)	87 (69.6)	14 (46.7)	54 (62.8)
Missing	0 (0)	0 (0)	2 (6.7)	1 (1.2)
Recurrence Time Category				
Within 1 to < 3 years	27 (39.7)	49 (39.2)	19 (63.3)	54 (62.8)
Within 3 to 10 years	41 (60.3)	76 (60.8)	11 (36.7)	32 (37.2)

Table 1. Descriptive statistics of the final cohort used in the association study of HIF-1α and breast cancer recurrence.

Statistical Analysis

Data were analyzed using SAS version 9.4. Two sets of unconditional logistic regressions were modeled to calculate the odds ratios and corresponding 95% confidence intervals utilizing a multivariate analysis. Two models were run so that analysis could be stratified by ER/TAM status and can be seen as thus in Table 1.

The initial unadjusted regression only controlled for the dichotomous HIF-1α nuclear activity to give a crude estimate, stratified by ER/TAM status. Due to the small sample size, the outcome of recurrence time was categorized into within 1 to < 3 years of initial breast cancer treatment, and within 3 to 10 years after initial breast cancer treatment.

The adjusted multivariate regression included the factors of the type of surgery received, the patient's menopausal status at diagnosis, UICC tumor stage, county of residence, receipt of chemotherapy, receipt of radiation therapy, a patient's Charlson comorbidity index score, a patient's year of diagnosis, categorical age at diagnosis, and, for those who are ER+, the duration of Tamoxifen treatment.

Results

The unadjusted model of ER/TAM+ subjects had observed an odds ratio of 0.98 (0.54, 1.79), illustrating that subjects categorized with a positive HIF-1α activity tissue sample had 2% less risk of having a recurrence from 3 to 10 years compared to those subjects who had a negative HIF-1α activity tissue sample. The unadjusted model of ER/TAM- subjects had an observed odds ratio of 0.98 (0.41, 2.31) signifying the same association.

Following the multivariate adjustment, the odds ratios of ER/TAM+ and ER/TAM- subjects were again reviewed. Within the ER/TAM+ group it was observed that those with HIF-1α nuclear activity would have a recurrence within 3 to 10 years 0.87 as many as times those without HIF-1α nuclear activity, 95% confidence interval (0.45, 1.70). The ER/TAM- arm of the study observed that those with HIF-1α nuclear activity would have a recurrence within 3 to 10 years 0.60 as many times as those without HIF-1α nuclear activity, 95% confidence interval (0.17, 2.04).

These trends seem consistent between the initial unadjusted estimates and the wide confidence intervals are likely due to the small sample size leading to large standard errors of estimates, especially in the ER/TAM- arm.

Discussion

From such results it would appear that if HIF-1α has an effect on the timing of recurrence, it would exacerbate and hasten its incidence rather than postpone it. The large confidence intervals around each estimate also demonstrate that there may be no effect of HIF-1α on the timing of recurrence among a population that will have a recurrence.

Past studies referenced earlier have long demonstrated an association between HIF-1α activity and the early recurrence of breast cancer. This study sought to better categorize the timing of when that recurrence occurs within the population of DBCG. Estimates obtained from the study demonstrated an effect ranging from a 13% to 40% of a later recurrence while taking into account multiple treatment and geographic factors and the initial genetic categorization of the subject. However, these conclusions are not without their limitations.

Limitations of the study include the small sample size in order to complete the within-case comparison. As a consequence, not nearly as many covariates could be accounted for as would be preferable, but even amongst those selected for analysis there was still a large standard error associated with each regression. This sample size also contributed to the need to dichotomize recurrence time at the 3 year mark to have a large enough sample of outcomes in each category. Other limitations include the use of a dichotomous HIF-1α nuclear score when on a microbiological level there is a gradient of activity. Lastly, the absence of a true control group only allows for a comparison of HIF-1α's activity in the role

of those who will assuredly have a recurrence rather than aiding in describing the association of HIF-1α's activity amongst all possible scenarios.

Results of the study at hand should be taken critically due to its numerous drawbacks, but it should be noted that even amongst only breast cancer recurrence patients there does appear to be some amount of temporal association with HIF-1α activity. With a larger histologic sample and data, it should be possible to evaluate such associations with a much greater confidence than was observed here. However, the overall goal needs to be considered as well: to better predict a subject's risk of recurrence based upon some quantitation of biomarker activity.

Naturally, future directions would seek to resolve each of these limitations and better describe HIF-1α's role in the timing and risk of recurrence amongst all post-operative breast cancer patients. The most likely first step would be in completing an analysis with both recurrence and non-recurrence data in order to directly inform the association of risk of recurrence during specific time intervals based on the magnitude of HIF-1α activity alongside other controlling covariates. Given promising results of a large observational study within the DBCG's population it may be beneficial to expand to a larger population.

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