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ORIGINAL ARTICLE

Hypoxia inducible factor-1 alpha and carbonic anhydrase IX overexpression are associated with poor survival in breast cancer patients

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Summary

Purpose: *Hypoxia is common in many solid tumors such as breast, head-neck, and soft tissue malignancies. Hypoxia causes overexpression of hypoxia inducible factor-1 alpha (HIF-1α) and carbonic anhydrase IX (CA IX) which are associated with unfavorable prognosis in breast cancer. In our study, we evaluated HIF-1α and CA IX expression in patients with breast cancer.*

Methods: *Between June 1996 and June 2008, 111 women with breast cancer were evaluated. Estrogen receptor (ER) and progesterone receptor (PR) status and Her2/neu expression were evaluated by immunohistochemical methods. Her-2/neu expression was also assessed by FISH method when needed. Two groups were created: ER and PR positive, Her-2/neu negative (group 1, n=56); and ER and PR negative, Her-2/neu positive (group 2, n=55). HIF-1α and CA IX expressions were investigated in both groups and results were compared. In addition, we investigated the association between HIF-1α and CA IX expressions with stage, grade, lymph node metastasis, tumor size, menopause status and survival.*

Results: *Median patient age in group 1 was 52 years (range 34-77), and in group 2 47 years (range 27-83). HIF-1α expression was detected in 26 (46.4%) of group 1 and in 46 (83.6%) of group 2 patients (p=0.0001). CA IX expression was detected in 25 (46.4%) of group 1 and in 37 (67.3%) of group 2 patients (p=0.013). In group 1, median disease free survival (DFS) was 97 months and in group 2 46 months (p=0.0308). In group 1, median overall survival (OS) was 108 months and in group 2 75 months (p=0.0339).*

Conclusion: *HIF-1α and CA IX overexpressions are observed more often in ER and PR negative, Her-2/neu positive breast cancer and are associated with poor survival.*

Key words: breast cancer, tumor hypoxia, HIF-1α, CA IX, prognosis

Introduction

Breast cancer is the commonest malignancy in females and constitutes 32% of all cancers in women and 16% of cancer-related mortality in United States of America [1]. Lymph node status and tumor size are the most important prognostic factors of breast cancer recurrence and survival [2,3]. Other common prognostic factors are tumor grade, lymphatic and vascular invasion, patient age, ER and PR, and Her-2/neu (c-erbB2) status [4].

Tumor hypoxia is related to poor response to chemotherapy and radiotherapy, genetic instability, resistance to apoptosis, and increased rate of invasion and metastasis [5]. In addition, hypoxia has prognostic significance in breast, head-neck and soft tissue malignancies [6].

Stabilization of HIF-1α is a primary response to hypoxia [7]. HIF-1 protein is a heterodimeric complex which consists of α and β proteins. HIF-1α is not detected in cells with normal oxygen concentration but it is rapidly activated by hypoxia. Although HIF-1α was first described as an erythropoietin regulator in 1995, it is known that HIF-1α regulates the genes that are related to tumor progression including metastasis, angiogenesis, resistance to apoptosis and metabolic adaptation [8].

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CA IX is a gene related to tumor hypoxia which encodes a transmembrane glycoprotein with N-terminal proteoglycan domain in the extracellular region [9]. CA IX expression increases in tumor cell lines by a HIF-1α-dependent pathway. This enzyme regulates tissue pH [9,10]. In several studies, CA IX is shown to be an intrinsic hypoxic marker. CA IX contributes to tumor growth and invasion by acidification of the tumor microenvironment in hypoxic condition [4].

In this study, we evaluated HIF-1α and CA IX expression in patients with breast cancer and their prognostic significance.

Methods

Patients

Between June 1996 and June 2008, 111 females with breast cancer were evaluated. For all patients, ER and PR were evaluated by immunohistochemistry (IHC) and Her-2/neu expression by IHC or FISH method. Two groups were created: ER and PR positive, Her-2/neu negative (group 1; n=56) and ER and PR negative, Her-2/neu positive (group 2; n=55). HIF-1α and CA IX expressions were investigated in both groups and the results were compared. In addition, the association between HIF-1α and CA IX expressions with stage, grade, lymph node metastasis, tumor size and menopausal status was investigated.

Immunohistochemical examination

Sections of 4 μm in width were deparaffinized in an oven at 56° C. They were incubated in 3% hydrogen peroxide for 10 min in order to block endogenous peroxidase activity. After washing with PBS, antigen retrieval process was carried out with 10 mM EDTA, pH 8 for 15 min in microwave oven at 600 Watt. IHC was carried out with streptavidin-biotin-peroxidase method. Sections were incubated with mouse monoclonal HIF-1α antibody (clone ESEE122, 1:50 dilution; Santa Cruz, CA, USA) and rabbit monoclonal CA IX antibody (clone H-120, 1:100 dilution, Santa Cruz, CA, USA) at room temperature for 2 h and incubated with 3,3' diaminobenzidine (DAB) (LabVision, NeoMarkers, CA, USA) for 10 min in order to provide color visualization. Sections were closed by applying background staining using Mayers hematoxylin. In addition, negative control staining was done without using primary antibody. For positive tissue control, lung carcinoma was used for HIF-1α antibody and normal intestinal mucosa was used for CA IX.

HIF-1α and CA IX expressions were semi-quantitatively evaluated in breast cancer cells by using of immunohistochemical method. HIF-1 expression was estimated by comparing nuclear positive stained cell ratios and CA IX expression by comparing membranous stained cells surrounding the tumor cells. Values of HIF-1α expression >2% were considered as positive and < 2% (nuclear staining ratio) as negative. For CA IX expression any membranous staining was accepted as positive and if there was no staining the samples were accepted as negative [11].

Statistical analysis

OS was calculated as the period from diagnosis until death from any cause or until the date of the last follow-up. Based on the intention to treat principle, data on all enrolled patients were used in statistical analysis. For the comparison of HIF-1α and CA IX expressions and other clinicopathologic parameters (nodal status, grade, tumor size, menopause, stage) in groups, x^2 test and Fisher's exact test were used. OS and DFS were estimated by the Kaplan-Meier method. Survival curves were compared with the log-rank test. P values < 0.05 were considered as significant.

Results

One hundred and eleven patients with invasive breast cancer were included in this study. There were 56 patients in the ER and PR positive and Her-2/neu negative group (group 1) and 55 patients in the ER and PR negative and Her-2/neu positive group (group 2). The median patient age in group 1 was 52 years (range 34-77), and in group 2 it was 47 (range 27-83; p=0.0448). Twenty-three (41.1%) patients in group 1 and 33 (60%) in group 2 were premenopausal (p=0.035). Clinical and pathological characteristics of both groups are displayed in Table 1.

All 56 patients in group 1 had invasive ductal carcinoma. However, 53 (96.4%) of 55 patients in group 2 had invasive ductal carcinoma and 2 (3.6%) had invasive lobular carcinoma. In group 1, there were one grade 1 (1.8%), 43 grade 2 (76.8%) and 12 grade 3 (21.4%) tumors. In group 2, there were 24 (43.6%) grade 2 and 31 grade 3 (56.4%) tumors. The difference in tumor grades between groups was statistically significant (p=0.001). Comparison of the two groups for tumor size and lymph node status showed no statistically significant difference (p=0.383 and p=0.703, respectively).

HIF-1α expression

Archival paraffin blocks were stained using HIF 1-α and CA IX antibodies and evaluated for HIF-1α and CA IX expression by two experienced pathologists.

In group 1, positive nuclear HIF-1α expression was detected in 26 (46.4%) samples, while 30 samples (53.6%) had negative expression. In group 2, positive nuclear HIF-1α expression was detected in 46 (83.6%) samples and in 9 samples (16.4%) nuclear staining was negative (p<0.0001).

CA IX expression

Positive membranous CA IX expression was detected in 25 (46.4%) of 56 group 1 patients. In group 2, membranous CA IX expression was positive in 37 (67.3%) patients (p<0.013).

Survival

In group 1, median DFS was 97 months (95%

Table 1. Patient and disease characteristics

Characteristics	*Group 1 (ER and PR positive, Her-2/neu negative)*		*Group 2 (ER and PR negative, Her-2/neu positive)*		*p-value*
	N	*%*	*N*	*%*	
Median age, years (range)	52 (34-77)		47 (27-83)		0.449
Menopausal status					0.035
Pre-	23	41.1	33	60	
Post-	33	58.9	22	40	
Pathology					0.150
Invasive ductal	56	100	53	96.4	
Invasive lobular	0		2	3.6	
Tumor grade					0.001
1	1	1.8	0		
2	43	76.8	24	43.6	
3	12	21.4	31	56.4	
Tumor size					0.383
T1	12	21.4	8	14.5	
T2	35	62.5	39	70.9	
T3	5	8.9	8	14.5	
T4	4	7.1	0		
Lymph node status					0.703
N0	23	41.1	21	38.2	
N1	22	39.3	18	32.7	
N2	7	12.5	10	18.2	
N3	4	7.1	6	10.9	
TNM stage					0.168
T1 N0M0	12	21.4	8	14.5	
T2 N0M0	11	19.6	13	23.6	
T2 N1M0	18	32.1	16	29	
T2 N2M0	6	10.9	10	18.2	
T3 N1M0	–	–	2	3.8	
T3 N2M0	1	1.8	–	–	
T3 N3M0	4	7.1	6	10.9	
T4 N1M0	4	7.1	–	–	
HIF-1 α expression					< 0.0001
Positive	26	46.4	46	83.6	
Negative	30	53.6	9	16.4	
CA IX expression					0.013
Positive	25	44.6	37	67.3	
Negative	31	55.4	18	32.7	

CI: 75.16-101.54) and in group 2 46 months (95% CI: 42.98-49.02; p=0.0308; Figure 1). In group 1, median OS was 108 months (95% CI: 90.65-125.35) vs. 75 months (95% CI: 64.94-85.06) in group 2 (p= 0.0339; Figure 2). The median follow-up time for all patients was 110 months (range 14-136).

Evaluation of HIF-1α and CA IX expressions, with respect to axillary node involvement, ER and PR status, tumor grade, tumor size and menopausal status

Irrespective of patient groups, HIF-1α and CA IX expressions were compared according to prognostic subgroups such as axillary node involvement, tumor size, ER and PR status, tumor grade and menopausal status. The results of these comparisons are shown in Table 2.

HIF-1α and CA IX expressions were collectively compared for DFS and OS. In HIF-1α positive patients the median DFS was 46 months (95% CI: 17.26-74.74), whereas the median DFS in HIF-1a negative patients was 85 months (95% CI: 64.33-93.00; p=0.0126) (Figure 3). In HIF-1α positive and negative patients, the median OS was 85 months (95% CI: 69.79-98.21) and 95 months (95% CI: 91.64-105.36), respectively (p=0.374) (Figure 4). The median DFS was 42 months (95% CI: 35.12-48.88) and 66 months (95% CI: 34-98) in CA IX expression positive and negative patients, respectively (p=0.344; Figure 5). Although in CA IX positive patients the median OS was shorter (78 months; 95% CI: 65.50-90.50 vs. 102 months; 95% CI: 88.09-115.91), this difference was not statistically significant (p=0.109) (Figure 6).

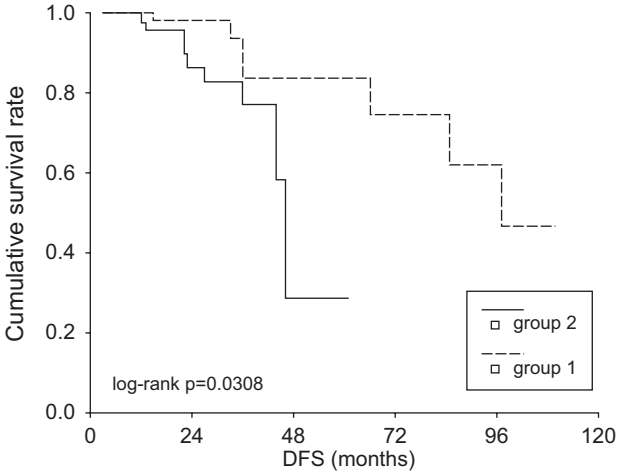

Figure 1. In group 1, median disease free survival was 97 months (95%CI: 75.16-101.54). In group 2, median disease free survival was 46 months (95% CI: 42.98-49.02; p= 0.0308).

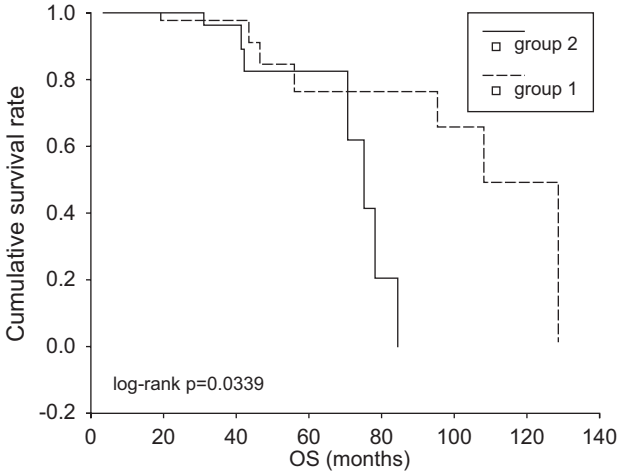

Figure 2. In group 1, median overall survival was 108 months (95% CI: 90.65-125.35). In group 2, median overall survival was 75 months (95% CI: 64.94-85.06) (p= 0.0339).

Table 2. Comparison of HIF-1 α and CA IX expression for prognostic factors

Characteristics	*HIF-1 α*			*CA IX*		
	N (+) (%)	*N (–) (%)*	*p-value*	*N (+) (%)*	*N (–) (%)*	*p-value*
Median age, years (range)	52 (34-75)			46 (27-83)		
Menopausal status			0.002			0.023
Pre-	44 (78.6)	12 (21.4)		37 (66.1)	19 (33.9)	
Post-	28 (50.9)	27 (49.1)		25 (45.5)	30 (54.5)	
Tumor grade			0.045			0.086
1	0	1 (100)		0	1 (100)	
2	39 (58.2)	28 (41.8)		34 (50.7)	33 (49.3)	
3	33 (76.7)	10 (23.3)		28 (65.1)	15 (34.9)	
Lymph node status			0.492			0.513
Axillary (–)	28 (63.6)	16 (36.4)		25 (56.8)	19 (43.2)	
Axillary (+)	44 (65.7)	23 (34.3)		37 (55.2)	30 (44.8)	
Tumor size (cm) in node negative patients			0.443			0.027
≤ 2	9 (69.2)	4 (30.8)		4 (30.8)	9 (69.2)	
> 2	19 (61.3)	12 (38.7)		21 (67.7)	10 (32.3)	
Node positive patients			0.387			0.331
1-3 node (+)	54 (63.5)	31 (36.5)		46 (54.1)	39 (45.9)	
> 3 node (+)	18 (69.2)	8 (30.8)		16 (61.5)	10 (38.5)	

In group 1, HIF-1α was positive in 26 (46.4%) patients and negative in 30 (53.6%). In group 2, HIF-1α was positive in 46 (83.6%) patients and negative in 9 (16.4%; p=0.0001). In group 1, CA IX was positive in 25 (44.6) patients and negative in 31 (55.4%) patients. In group 2, CA IX was positive in 37 (67.3%) patients and negative in 18 (32.7; p=0.013).

Discussion

Tumor cells express hypoxic markers such as HIF-1α and CA IX in hypoxic conditions. Tumors that express hypoxic markers are more aggressive than tumors without such an expression. On the other hand, it was shown that aggressive tumors express hypoxic markers due to rapid growth and irregular increase in vascularization [11,12]. HIF-1α is an important marker and widely investigated in tumor hypoxia and angiogenesis studies. Overexpression of HIF-1α is observed in many cancers. Moreover, strong relation between HIF-1α overexpression and mortality has been demonstrated in specific cancer types including brain, breast, oropharynx, ovary and endometrial cancers. Several recent studies reported that HIF-1α also has a role in chemotherapy and radiotherapy resistance [13-15]. These findings imply that HIF-1α has a critical role in cancer progression.

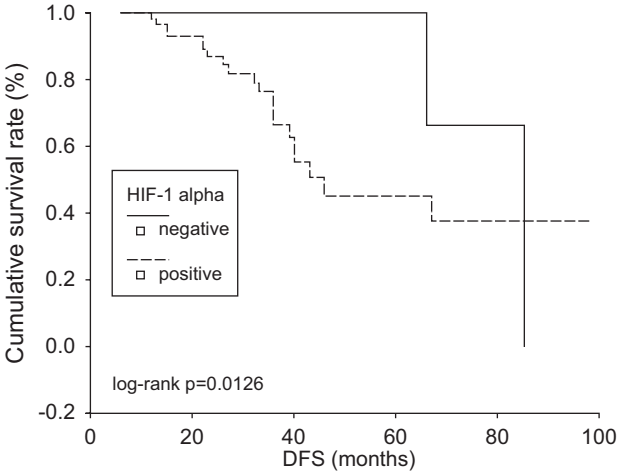

Figure 3. In HIF-1α positive patients, the median disease free survival was 46 months (95% CI: 17.26-74.74), whereas in HIF-1α negative patients, the median disease free survival was 85 months (95%CI: 64.33-93.00; p=0.0126).

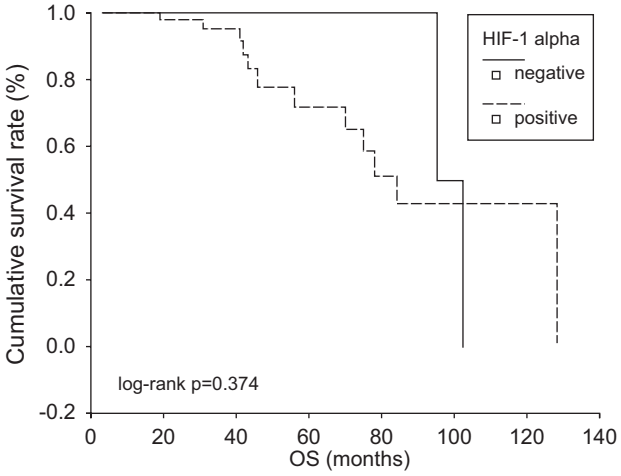

Figure 4. In HIF-1α positive and negative patients the median overall survival was 85 months (95%CI: 69.79-98.21) and 95 months (95% CI: 91.64-105.36), respectively (p=0.374).

In our study, we investigated HIF-1α and CA IX expression in hormone-responsive and hormone-unresponsive breast cancer. We found higher HIF-1α and CA IX expression in ER and PR negative and Her2 positive patients. Moreover, in these patients we observed shorter DFS and OS. However, several recent studies report that HIF-1α and/or CA IX positivity is higher in ER and PR negative tumors [11,16,18].

In a recent study in premenopausal patients with 1-3 axillary lymph node metastasis, CA IX expression was statistically higher in patients with negative ER and PR, grade 3, tumor diameter > 2 cm, and positive HIF-1α expression. In the same study, no correlation of CA IX expression with Her-2/neu positivity (p=0.57) and axillary lymph node status (p=0.99) was proved [16]. In this study, when patients who were administered tamoxifen for 2 years were compared with patients without tamoxifen administration, it was shown that CA IX was an indicator independent from other prognostic factors including axillary lymph node involvement, PR and Her-2/neu status, tumor diameter, tumor grade, Ki-67 and cyclin E. In addition, in the groups with and without tamoxifen for 2 years, no relation between CA IX expression and response to therapy was identified (p=0.345). It was found that DFS, OS and breast cancer specific survival were shorter for the patients with positive CA IX expression (p=0.032, p=0.022, p= 0.005, respectively) [16].

In a recent study, Trastour et al. [17] could not find statistical correlation of HIF-1α and CA IX expression with nodal status, tumor diameter and age, but they

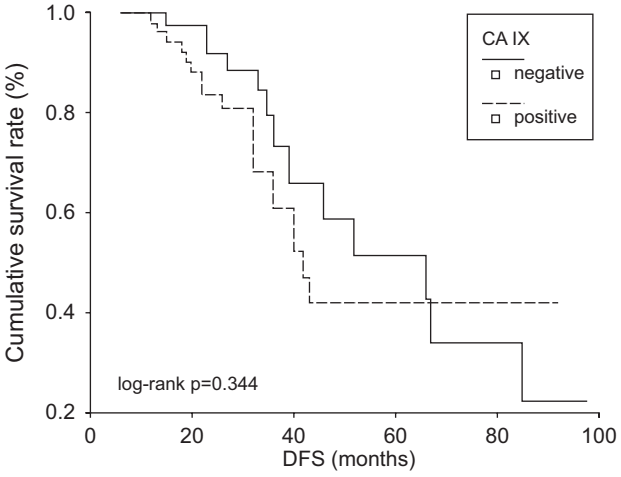

Figure 5. CA IX positive and negative patients. The median disease free survival was 42 months (95% CI: 35.12-48.88) and 66 months (95% CI: 34-98), respectively (p=0.344).

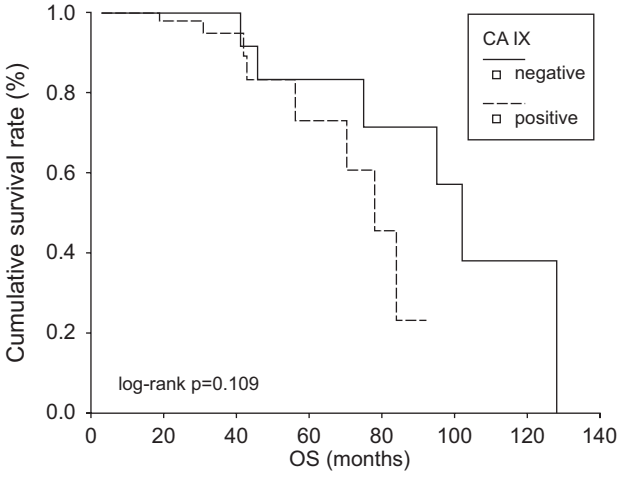

Figure 6. CA IX positive and negative patients. The median overall survival was 78 months (95% CI: 65.50-90.50) and 102 months (95% CI: 88.09-115.91), respectively (p=0.109).

found significant overexpression in grade 3, poorly differentiated, ER negative, and PR negative tumors. The results of this study are similar with our study. In the same study, DFS was shorter in patients with HIF-1α and CA IX overexpression, similar to our study. In addition, shorter OS was reported for patients having both HIF-1α and CA IX overexpression [17].

In a recent study including 183 breast cancer patients, Generali et al. reported significant CA IX overexpression in patients with negative ER and PR, and overexpression of Her2, p53 Ki67, but reported that CA IX expression was unrelated to tumor size and lymph node status [18]. In our study, we identified significant HIF-1α (p=0.0001) and CA IX (p=0.041) overexpressions in ER and PR negative patients. The common finding of these and our study was the higher expression of HIF-1α and CA IX in ER and PR negative breast cancer. In addition, OS was shorter in patients in whom significant HIF-1α and CA IX overexpression was observed. Similar to our study, no significant correlation of axillary nodal metastasis with HIF-1α and CA IX expression was detected [16-18].

In 377 premenopausal women with breast cancer, Kronblad et al. [11] investigated the relation between survival without recurrence and HIF-1α expression. They detected HIF-1α nuclear staining in 24% of the patients. HIF-1α was positively correlated with tumor size, tumor grade and Ki-67 proliferation index and reported significant HIF-1α overexpression in ER and PR negative tumors. Moreover, they reported higher HIF-1α expression in Her-2/neu positive patients compared to Her-2/neu negative ones, similar to our results. In our study, both HIF-1α and CA IX expressions were higher in Her2/neu positive tumors than in Her2/neu negative tumors. In the study reported by Kronblad et al., HIF-1α overexpression was associated with shorter survival without recurrence and breast cancer specific survival, but no relation was reported for OS [11].

In conclusion, hypoxic tumor markers HIF-1α and CA IX expressions are observed more in ER and PR negative, Her-2/neu positive breast cancer. Poor prognosis observed in these hormone-unresponsive patients might be, in part, due to expression of these hypoxic markers.

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