# Hypoxia inducible factor-1alpha is a prognostic marker in premenopausal patients with intermediate to highly differentiated breast cancer but not a predictive marker for tamoxifen response

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**Abstract**

Hypoxia is common in many solid tumors including breast cancer. Hypoxia triggers the expression of hypoxia inducible factor-1 alpha (HIF-1α) and HIF-1α has been associated with an impaired prognosis in breast cancer and to down-regulation of the estrogen receptor (ER), potentially affecting the treatment efficiency of anti-estrogens. The role of HIF-1α regarding prognostic and treatment predictive information in breast cancer has not been established and we therefore analyzed HIF-1α using immunohistochemistry in a cohort of 377 premenopausal stage II breast cancers arranged in a tissue microarray. The patients were included in a randomized trial with either 2 years of tamoxifen or no adjuvant treatment. The tamoxifen treatment effect could be studied in subgroups of breast cancer and pure prognostic information could be scrutinized for untreated control patients. HIF-1α was scored as positive in 24% of the tumors and correlated positively to tumor size, Nottingham histological grade (NHG), Ki-67, Her2 and cyclin E expression and negatively to lymph node status, cyclin D1, ER and PR expression. Surprisingly, there was no difference in tamoxifen response for patients with high or low HIF-1α expressing tumors. In lymph node-positive patients as well as NHG 1/2 tumors, high HIF-1α protein expression was significantly associated with an impaired RFS (p=0.014, 0.018). When analyzing the subgroup of NHG 1/2 tumors a high HIF-1α expression was the only independent significant prognostic marker in multivariate analysis, including standard prognostic markers suggesting that HIF-1α might be a useful prognostic marker in this subgroup of breast cancer with a rather good but diverse prognosis.

## Introduction

Hypoxic regions are common in solid tumours such as breast cancer as well as in metastases.[1, 2] Hypoxic areas arise because tumour cells grow rapidly and outpace the formation of new blood vessels.[3] Even mild anaemia in breast cancer patients is linked to the formation of hypoxic tumour regions.[4] One of the key factors adapting tumour cells to hypoxic conditions is HIF-1α (hypoxia inducible factor 1α).[5] At normal oxygen tension, HIF-1α is continuously degraded through the proteasomal system via binding to the von Hippel-Lindow (VHL)-protein, but during hypoxia VHL can no longer bind to HIF-1α causing an accumulation of HIF-1α.[6] HIF-1α is active as a transcription factor in a heterodimeric complex together with HIF-1β or ARNT and binds to DNA sequences called HRE´s (hypoxia responsive elements).[7] HIF-1α has the potential to activate a number of genes known to be involved in tumour progression, invasion and neoangiogenesis, for example VEGF, glucose transporters, carbonic anhydrases, Met receptor, insulin growth factor-1, UPAR, and transforming growth factor-α.[8] HIF-1α expression is not only restricted to hypoxic cells, but is also upregulated by growth factors, such as insulin growth factor 1, insulin,[9] heregulin[10] and oncogenic mutations in RAS,[11] p53,[12] PTEN,[13] SCR, ERBB2[14] or VHL.[15, 16] In general, hypoxia negatively influences tumour prognostic features and causes resistance to many standard therapies and promotes a more malignant phenotype.[17] HIF-1α expression is not detected in normal breast tissue or hyperplastic lesions but is present in well-differentiated ductal carcinoma in situ and in all more malignant forms of breast cancer.[18] In poorly differentiated ductal carcinoma in situ, HIF-1α expression has been correlated to loss of estrogen receptor (ER) expression as well as a more undifferentiated phenotype.[19] From studies of breast cancer cell lines it appears that hypoxia also correlates to loss of ER expression[20-23] and that this may induce tamoxifen resistant growth.[24] Hormone independent growth is frequently caused by abnormalities in growth factor signaling pathways such as EGFR, HER2, MAPK-singalling via ERK1/2, or

IGFR via PI3K,[25] all of which may be influenced by HIF-1α. HIF-1α has also been implicated as an independent prognostic marker in both lymph node negative[26] as well as lymph node positive breast cancers.[27, 28] Also, high histological grade, ER and PR (progesterone receptor) negativity and the presence of necrotic regions have been linked to the presence of HIF-1α,[29] but the relation between histological grade, ER status and HIF-1α expression differs between reports.[14, 26, 27] This difference could potentially be explained by the fact that the role of HIF-1α as a prognostic marker was investigated in small cohorts of pre and postmenopausal patients with both early and advanced breast cancer, receiving different forms of treatment.

We therefore investigated the prognostic information of HIF-1α expression in tumours from patients participating in a randomised trial of two years adjuvant tamoxifen versus no treatment, including only premenopausal patients with stage II invasive breast cancer. The follow-up time was measured as recurrence free survival (RFS). The tumours in the cohort have been arranged in a tissue microarray facilitating stringent analysis of prospective prognostic and predictive markers.

## Methods and materials

### Study design

Between 1984 and 1991, 564 premenopausal women with primary breast cancer in the South and Southeast region of Sweden were enrolled in a clinical trial[30] and randomised to adjuvant tamoxifen treatment (20 or 40 mg daily), *n=276*, for 2 years vs control, *n=288*. The aim of the study was to compare the effect of tamoxifen on RFS (primary outcome). OS (overall survival) is used as secondary outcome since patients with recurrent disease (distant or local) were treated with tamoxifen if the tumour was ER-positive, regardless if they had been randomised to control initially. RFS considered local, regional, distant recurrences and breast-cancer specific death, but not contralateral breast cancer. The inclusion criteria were premenopausal patients, or patients less than 50 years of age, with stage II (pT2 N0 M0, pT1-2 N1 M0) invasive breast cancer operated by modified mastectomy or breast conserving surgery with axillary lymph node dissection. Postoperative radiotherapy and was given after breast conservative surgery and lymph node positive patients received loco-regional radiotherapy. In <2% of the patients, polychemotherapy was given. The median follow-up time for patients without breast cancer events was 13.9 years. The clinical and tumour pathological characteristics did not differ between the treatment arms.

### Tissue microarray

500 paraffin embedded tumours specimens were available for construction of the tissue microarrays. From each tumour two representative cores (0.6 mm) were assembled using a robotised tissue array machine (ATA-27, Beecher Inc, WI, USA). For immunohistochemistry, 4 μm paraffin sections were de-paraffinised using xylen and rehydrated using descending

concentrations of ethanol. Antigen retrieval was achieved by microwave treatment for 2*5 min in a citrate buffer before being processed either in the Ventana Benchmark (Ventana Medical Systems Inc, AZ, USA) using prediluted antibodies to ER (Anti-ER, clone 6F11) and PR (Anti-PgR, clone 16) Her2 (Pathway CB-USA, 760-2694) or in the Dako Techmate 500 (Dako, Glostrup, Denmark) for HIF-1α (1:500, NB100-123H2, Abcam, US), Ki-67 (1:200, M7240, Dako), VEGF-A (1:400, A20, Santa-Cruz, CA, USA), cyclin E (1:50, HE12, Santa-Cruz), cyclin A (1:200, H432, Santa-Cruz), cyclin D1 (1:100, M7155, Dako). Nottingham histological grade (NHG) according to Elston Ellis scoring system had been evaluated in 491 primary tumours. All breast carcinomas were classified according to the WHO criteria as one of the following: ductal (n =411), lobular (n=43), mucinous (n=3), tubular (n=5), medullary (n=25), lobular and ductal (n=1), DCIS/microinvasive (n=5), other or not classified (n=7). The study has been approved by the ethical committees at Linköping and Lund Universities.

Immunohistochemical evaluation

ER and PR-expression was defined as the fraction of positive nuclei subdivided into 4 groups (0-10, 11-50, 51-75, 76-100%).[30] ER and PR were scored as positive if the fraction of positive tumour cells was >10%, which is in line with current clinical recommended determines. HIF-1α protein was evaluated by two investigators scoring the tissue array samples according to the fraction of positively stained nuclei, since active HIF-1α is located only in the nucleus cytoplasmic staining was discarded. Fractions were divided into 3 groups (0-1, 2-10, 11-100%), see table 1. There was a high concordance between the investigators and in the few cases of differing results, biopsies were re-evaluated and discussed to reach consensus. Most cases had no or below 2% positively stained nuclei. HIF-1α 2-10% and 11-100% constituted one group in survival analysis and $\chi^2$ tests, owing to the relatively small number of patients in these groups. Cyclin D1 and cyclin E were scored according to the nuclear fraction (0, 1-25,

26-50, 51-75, 76-100%). Ki-67 and cyclin A2 were also scored according to nuclear fraction (0-1, 2-10, 11-25, 26-50, 51-100%). These scorings were divided by the median value into 2 groups, see table 2. Cyclin D1, cyclin A2, Ki-67 (Jirström et al 2005, submitted) and cyclin E (Stighall et al, 2005, submitted) in correlation to prognosis and clinico-patological parameters will be described in detail elsewhere. Her2 staining has been evaluated according to a standard protocol (HercepTest) and scored into 4 groups,[31] these scorings are divided into two groups with normal/weak (0-2) Her2 expression and overexpression (3+). VEGF-A intensities are scored as positive (3) or negative (0-2) cytoplasmic staining.[31] By using the tissue microarray approach, staining variation between samples were minimised and positive samples could be used as supplements for internal controls. Her2 gene amplification was determined using FISH (fluorescent *in situ* hybridisation) according to standard protocols (Ventana Medi-manufacturer's Systems Ind.,AZ, USA) and described elsewhere.[31]

Statistics

Differences in distribution between HIF-1α negative/low and HIF-1α high tumours, regarding clinical data and tumour characteristics were evaluated by the $\chi^2$ test. Kaplan-Meier's plot and log rank test were used for illustrating and calculating RFS. The Cox multivariate proportional hazards model was fitted to explore the effects on RFS of HIF-1α, tumour size, Her2, lymph node status, NHG, Ki-67 in the untreated patients; the analysis was also preformed after NHG 3 exclusion. All calculations were performed in SPSS version 11.0 (SPSS Inc, Chicago, IL, USA).

Results

*Distribution of HIF-1α*

Immunohistochemical tumor specific HIF-1α expression could be evaluated in 377 (67%) of the tumours (table 1). Nuclear staining of HIF-1α was scored as positive in 24% of the evaluated breast carcinomas. Examples of immunohistochemical staining of HIF-1α and verification of the antibody specificity are shown in figure 1. Distribution according to clinico-pathological parameters is further delineated in table 2. HIF-1α correlated positively to tumour size, NHG, Ki-67, Her2 and cyclin E expression. HIF-1α was also correlated to lymph node-negativity, cyclin D1-negativity, ER-and PR-negativity. There was further a trend towards an association between HIF-1α and Her2 amplification (0.069) but not to patient age. Surprisingly, there was no significant association between HIF-1α and VEGF-A expression (p=0.68). Baseline clinicopathological characteristics according to cases with HIF-1α evaluated tumours and missing cases was examined for selection-bias and found non-significant except in the NHG distribution (data not shown). Due to an overrepresentation of NHG 1/2 tumours among the missing tumours the evaluated tumours had significantly more NHG 3 tumours than expected; despite this the distribution between NGH1/2 versus NHG 3 among the HIF-1α evaluated tumours was even (182 vr 183).

*HIF-1α and tamoxifen treatment response*

In the treated patient cohort, only patients with ER-positive tumours responded to tamoxifen treatment[30] and this subgroup was therefore selected for further studies of a potential link between HIF-1α expression and tamoxifen response. By comparing tamoxifen treated patients with untreated patients within subgroups of ER-positive tumours defined by HIF-1α expression, the tamoxifen response in relation to HIF-1α could be defined. As illustrated in figure 2 there was no obvious difference in tamoxifen response between high and low HIF-1α

expressing tumours in terms of RFS. In the ER-positive and HIF-1α negative/low tumours there was a nearly significant tamoxifen effect (p=0.093) whereas there was a significant tamoxifen effect in the smaller group of HIF-1α positive tumours (p=0.011). The fact that HIF-1α expression was negatively associated with ER contributes to the low number of patients in panel B, figure 2. It can nevertheless be concluded that HIF-1α and hormone receptor positive breast cancer seem to be able to respond to tamoxifen treatment despite obvious links between hypoxia and ER-modulations. Ki-67 and ER-status in relation to HIF-1 expression in the two study groups (control versus tamoxifen treatment) are presented in table 3. HIF-1 high tumours were frequently more ER negative and Ki-67 positive, as shown by sequential immunohistochemical stainings for the different markers from the same representative tumour core, shown in figure 2, panel C.

*HIF-1α and survival*

In the entire cohort of patients (n=377) there was a significantly worse RFS for patients with HIF-1α high tumour (p=0.048) compared to negative or low expression (figure 3A). There was further a trend towards a worse overall survival for patients with HIF-1α high tumours (p=0.12) (figure 3B), as well as significant difference in breast cancer specific survival (p=0.028) (figure 3C). We next restricted the recurrence free survival analysis to the 288 untreated patients in order to investigate pure prognostic information in relation to HIF-1α staining. Interestingly, there was no significant difference in RFS in relation to HIF-1α in the untreated patients even though there was a non-significant trend towards an impaired RFS for patients with HIF-1α high tumours (figure 4A). This trend was slightly more apparent in the subgroup analysis including only ductal breast cancers (figure 4B). In the tamoxifen treated cohort, HIF-1α showed a similar trend towards worse RFS (p=0.14). Earlier reports have observed independent prognostic values for HIF-1α in lymph node-negative breast cancer[26] as

well as in lymph node-positive breast cancer patients,[27] and we therefore analysed the subgroups of lymph node-negative (n=50) and lymph node-positive (n=145) untreated control cases separately (figure 4 C,D). There was no significant association to RFS in the subgroup of lymph node-negative patients but there was a significant association between a shorter RFS and HIF-1α expression in the lymph node-positive subgroup. To further investigate the prognostic information of HIF-1α we analyzed the subgroups of NHG 1/2 tumours versus NHG 3 tumours. In NHG 1/2 tumours HIF-1α high expression was significantly linked to a shorter RFS (figure 4 E) whereas there was no significant association between HIF-1α and RFS in the NHG 3 tumours (figure 4 F). In the treated cohort, there were no significant associations between HIF-1α and RFS in either lymph node-negative (p=0.088) or lymph node-positive patients (p=0.46) or NHG1/2 tumours (p=0.70) or in NHG 3 tumours (p=0.24). Table 4 shows the results of multivariate analysis of RFS in the untreated cohort of patients. When including all untreated patients, HIF-1α was not an independent prognostic factor in contrast to node status and NHG. However, when taking the above-presented results into account and excluding the NHG 3 tumours from the multivariate analysis, only HIF-1α remained an independent prognostic marker for RFS (table 5).

## Discussion

The aim of this study was to investigate the role of HIF-1α as a prognostic marker in stage II premenopausal breast cancer patients in relation to RFS using a randomised study cohort. The prognostic impact of HIF-1α in breast cancer has neither been explored in an untreated patient cohort and neither with regards to tamoxifen response in a randomised treatment trial cohort. In the whole material, 24% of the tumours showed nuclear HIF-1α staining, which is in concordance with other articles even if the patient cohorts differ between the reported studies.[27, 28, 32]

ER down-regulation in breast cancer cell lines as well as in primary breast cancer has been linked to HIF-1α induction.[14, 19, 20, 26] In contrast, ER positivity has also been reported to be associated with HIF-1α expression[18] whereas others have not observed any significant link between HIF-1α and ER expression.[27, 28] Decreased ER expression and its downstream target PR in tumour cells surrounding necrotic zones are nevertheless obvious[19, 22, 29] and despite some inconsistencies between studies, there seems to be an inverse link between HIF-1α expression and ER in both experimental models as well as in *in vivo* tumors. The data presented in this study further supports a link between HIF-1α and the presence of ER in breast cancer but surprisingly not to the tamoxifen response as further deliberated below.

HIF-1α is considered to support tumour growth and the significant association between tumour size and HIF-1α observed in this study fits well with this theory. For some reason this correlation appears to be lost when analysing T1, T2 and T3 tumours together[14] or T1, T2 versus T3, T4.[28] Our data suggest that HIF-1α and tumour size is correlated in tumours up to 5 cm in size, but in larger tumours other factors might be more important in regulating tumour size or growth. Further, HIF-1α was associated with proliferation in this study indicating the

presence of HIF-1α in actively proliferating cells, which is in line with other publications.[18, 26] Proliferating cells are under the control of proteins in the cell cycle and HIF-1α appears to be positively associated to proteins involved in $S/G_2$-phase such as cyclin E and cyclin A2, but negatively associated to cyclin D1, which, mainly functions in early $G_1$-phase. This is in concordance with earlier published data[33] but apart from the association to the cell cycle this could also indicate that HIF-1α expression is correlated to a certain type of breast cancer with proliferative features and frequent overexpression of cyclin E.[34, 35]

HIF-1α has not previously been explored in relation to treatment prediction in a randomised treatment trial, but one breast cancer cell line study has demonstrated that hypoxia induces tamoxifen resistant growth.[24] In our study we could not confirm this link between hypoxia and tamoxifen response and both HIF-1α low and high tumour appeared to respond to tamoxifen treatment. Since there is a negative association between ER and HIF-1α expression, the amount of ER-positive and HIF-1α high tumours was rather small in this study, which might have affected the results, but despite few tumours there was a significant tamoxifen effect in the subgroup of HIF-1α high tumours, clearly suggesting that these tumours indeed have a functional tamoxifen response. Nevertheless, cell line studies indicate that ER positive breast cell lines downregulate the ER under hypoxic conditions, which is in contrast to the existence of tumours with a high ER and HIF-1α expression as observed in this study.[19] It is possible that the tumours with a high HIF-1α expression and ER-positivity represent a subgroup of tumours where HIF-1α expression is under the control of growth factor signaling and perhaps not hypoxia, which could affect the results concerning ER-content and tamoxifen response. HIF-1α has been correlated to VEGF-expression in progressive breast cancer stages and in a subgroup of lymph node- and ER-negative cancer.[18, 26, 33] The relation of VEGF to HIF-1α expression has not been investigated in lymph node positive patients. Surprisingly, we did not

observe any association between HIF-1α and VEGF-A expression in the whole cohort nor in the ER positive or negative cohort, nor in lymph node positive or negative disease (data not shown). Since hormonal stimulation (estrogen) regulate VEGF secretion in breast cancer[36, 37] we speculate that this might influence the outcome of correlation analysis in the present tumour material. EGF signaling has also been shown to regulate the VEGF-levels,[38] potentially further influencing the analysis. Recently it was shown that there is an inverse balance between the amount of VEGFR1 and VEGF levels in breast cancer, and the balance is partly dependent on hormonal stimulation.[39]

In general, our outcome data including breast cancer recurrence, overall survival and breast cancer specific survival strengthen and clarify the findings of others,[14, 26-28] suggesting that HIF-1α is linked to aggressive tumour features and a bad prognosis for breast cancer patients. The patient group where this correlation is of prognostic value is nevertheless a matter of debate and different reports show different results. In lymph node-negative patients it has previously been shown that HIF-1α correlated to a worse prognosis whereas in the lymph node-positive cohort there were no such association.[26]
Others have shown that HIF-1α was indeed prognostic in a lymph node-positive cohort[27] of T1 and T2 tumours.[28] HIF-1α expression has also been observed to increase with an increasing occurrence of lymph node metastasis,[14] which is in direct disagreement with our data where HIF-1α expression is inversely correlated to lymph node status. In an attempt to clarify the prognostic information in HIF-1α expression and lymph node status we used the untreated patient cohort where interference of different treatment regimes on survival can be minimised. We found that HIF-1α was not associated with outcome (RFS) in lymph node-negative cancer in contrast to lymph node-positive cancer. In the entire cohort of lymph node-negative tumours, HIF-1α showed a trend (p=0.12) towards a shorter survival that possibly

could be an indirect result of treatment since this trend completely disappeared in the control cohort. Speculatively, HIF-1α high tumours do not seem to commonly metastasize, but if they do, HIF-1α expression indeed seem to be linked to aggressive features and a bad prognosis. In this study we observed a positive correlation between HIF-1α and NHG, which is supported by the fact that a high histological grade and the presence of necrotic regions in breast tumours are closely associated.[29] Further, this association was also observed in a study cohort of 153 stage I/II invasive breast cancers[26] but not in studies where only lymph node-positive patients were included.[27, 28] In the present cohort, the correlation between NHG and HIF-1α expression persisted, also when analysing the lymph node-positive control cohort only ($p=0.003$). Even though there was a positive correlation between HIF-1α and NHG, HIF-1α only influenced RFS in the control cohort of NHG 1/2 tumours and not in NHG 3. NHG 3 tumours seem to have passed the point where HIF-1α is linked to prognostic values. When analysing the untreated control cohort in a multivariate analysis, HIF-1α was not independently associated to RFS, whereas both lymph node status and NHG were. However, after exclusion of the NHG 3 tumours, HIF-1α expression was the only significant independent factor for RFS, whereas lymph node status lost its significance, as did the difference between NHG 1/2. By excluding confounding mechanisms due to treatment and pre/post menopausal patients we are convinced that these data represent valid prognostic information of HIF-1α.

Direct associations between HIF-1α and Her2 expression/gene amplifications have earlier been reported in breast cancer.[26] A recently published combination analysis showed that tumours with a high HIF-1α expression and Her2 gene amplification had a poor survival outcome.[14] In premenopausal stage II breast cancer with long follow-up, it is nevertheless

obvious that high HIF-1α and Her2 in combination does not contribute to poor outcome (data not shown).

In summary, our results suggest that HIF-1α is linked to, and possibly directs the tumours towards a worse RFS in premenopausal patients with stage II tumours with special emphasis in lymph node positive patients. The prognostic information of HIF-1α further appears to be restricted to NHG 1/2 tumours where HIF-1α is the only significant prognostic marker in a multivariate analysis. Surpringly, HIF-1α did not have a predictive value regarding tamoxifen treatment response. Further, HIF-1α was associated with markers of aggressive breast cancer such as histological grade, tumour size, Ki-67, cyclin E and A, Her2 expression and amplification, loss of ER and PR. Therefore HIF-1α may improve the clinical decision regarding NHG 1/2 tumours and their adjuvant treatment.

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References


1. Vaupel P, Schlenger K, Knoop C, Hockel M. Oxygenation of human tumors: evaluation of tissue oxygen distribution in breast cancers by computerized O2 tension measurements. Cancer Res 1991;51(12):3316-22.
2. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, Simons JW. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 1999;59(22):5830-5.
3. Dang CV, Semenza GL. Oncogenic alterations of metabolism. Trends Biochem Sci 1999;24(2):68-72.
4. Vaupel P, Mayer A, Briest S, Hockel M. Oxygenation gain factor: a novel parameter characterizing the association between hemoglobin level and the oxygenation status of breast cancers. Cancer Res 2003;63(22):7634-7.
5. Semenza GL. Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 1999;15:551-78.
6. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 1999;399(6733):271-5.
7. Dachs GU, Patterson AV, Firth JD, Ratcliffe PJ, Townsend KM, Stratford IJ, Harris AL. Targeting gene expression to hypoxic tumor cells. Nat Med 1997;3(5):515-20.
8. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3(10):721-32.
9. Zelzer E, Levy Y, Kahana C, Shilo BZ, Rubinstein M, Cohen B. Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT. Embo J 1998;17(17):5085-94.
10. Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol Cell Biol 2001;21(12):3995-4004.
11. Blancher C, Moore JW, Robertson N, Harris AL. Effects of ras and von Hippel-Lindau (VHL) gene mutations on hypoxia-inducible factor (HIF)-1alpha, HIF-2alpha, and vascular endothelial growth factor expression and their regulation by the phosphatidylinositol 3'-kinase/Akt signaling pathway. Cancer Res 2001;61(19):7349-55.
12. Blagosklonny MV, An WG, Romanova LY, Trepel J, Fojo T, Neckers L. p53 inhibits hypoxia-inducible factor-stimulated transcription. J Biol Chem 1998;273(20):11995-8.
13. Zundel W, Schindler C, Haas-Kogan D, Koong A, Kaper F, Chen E, Gottschalk AR, Ryan HE, Johnson RS, Jefferson AB, Stokoe D, Giaccia AJ. Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev 2000;14(4):391-6.
14. Giatromanolaki A, Koukourakis MI, Simopoulos C, Polychronidis A, Gatter KC, Harris AL, Sivridis E. c-erbB-2 related aggressiveness in breast cancer is hypoxia inducible factor-1alpha dependent. Clin Cancer Res 2004;10(23):7972-7.
15. Kim WY, Kaelin WG. Role of VHL gene mutation in human cancer. J Clin Oncol 2004;22(24):4991-5004.
16. Dery MA, Michaud MD, Richard DE. Hypoxia-inducible factor 1: regulation by hypoxic and non-hypoxic activators. Int J Biochem Cell Biol 2005;37(3):535-40.
17. Brown JM, Wilson WR. Exploiting tumour hypoxia in cancer treatment. Nat Rev Cancer 2004;4(6):437-47.

18. Bos R, Zhong H, Hanrahan CF, Mommers EC, Semenza GL, Pinedo HM, Abeloff MD, Simons JW, van Diest PJ, van der Wall E. Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. J Natl Cancer Inst 2001;93(4):309-14.
19. Helczynska K, Kronblad A, Jogi A, Nilsson E, Beckman S, Landberg G, Pahlman S. Hypoxia promotes a dedifferentiated phenotype in ductal breast carcinoma in situ. Cancer Res 2003;63(7):1441-4.
20. Stoner M, Saville B, Wormke M, Dean D, Burghardt R, Safe S. Hypoxia induces proteasome-dependent degradation of estrogen receptor alpha in ZR-75 breast cancer cells. Mol Endocrinol 2002;16(10):2231-42.
21. Kronblad A, Helczynska K, Nielsen NH, Pahlman E, Emdin S, Pahlman S, Landberg G. Regional cyclin D1 overexpression or hypoxia correlate inversely with heterogeneous oestrogen receptor-alpha expression in human breast cancer. In Vivo 2003;17(4):311-8.
22. Cooper C, Liu GY, Niu YL, Santos S, Murphy LC, Watson PH. Intermittent hypoxia induces proteasome-dependent down-regulation of estrogen receptor alpha in human breast carcinoma. Clin Cancer Res 2004;10(24):8720-7.
23. Cho J, Kim D, Lee S, Lee Y. Cobalt Chloride-Induced Estrogen Receptor Alpha Downregulation Involves Hypoxia-Inducible Factor-1{alpha} in MCF-7 Human Breast Cancer Cells. Mol Endocrinol 2005.
24. Kurebayashi J, Otsuki T, Moriya T, Sonoo H. Hypoxia reduces hormone responsiveness of human breast cancer cells. Jpn J Cancer Res 2001;92(10):1093-101.
25. Johnston SR, Head J, Pancholi S, Detre S, Martin LA, Smith IE, Dowsett M. Integration of signal transduction inhibitors with endocrine therapy: an approach to overcoming hormone resistance in breast cancer. Clin Cancer Res 2003;9(1 Pt 2):524S-32S.
26. Bos R, van der Groep P, Greijer AE, Shvarts A, Meijer S, Pinedo HM, Semenza GL, van Diest PJ, van der Wall E. Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. Cancer 2003;97(6):1573-81.
27. Schindl M, Schoppmann SF, Samonigg H, Hausmaninger H, Kwasny W, Gnant M, Jakesz R, Kubista E, Birner P, Oberhuber G. Overexpression of hypoxia-inducible factor 1alpha is associated with an unfavorable prognosis in lymph node-positive breast cancer. Clin Cancer Res 2002;8(6):1831-7.
28. Gruber G, Greiner RH, Hlushchuk R, Aebersold DM, Altermatt HJ, Berclaz G, Djonov V. Hypoxia-inducible factor 1 alpha in high-risk breast cancer: an independent prognostic parameter? Breast Cancer Res 2004;6(3):R191-8.
29. Tomes L, Emberley E, Niu Y, Troup S, Pastorek J, Strange K, Harris A, Watson PH. Necrosis and hypoxia in invasive breast carcinoma. Breast Cancer Res Treat 2003;81(1):61-9.
30. Ryden L, Jonsson PE, Chebil G, Dufmats M, Ferno M, Jirstrom K, Kallstrom AC, Landberg G, Stal O, Thorstenson S, Nordenskjold B. Two years of adjuvant tamoxifen in premenopausal patients with breast cancer: a randomised, controlled trial with long-term follow-up. Eur J Cancer 2005;41(2):256-64.
31. Ryden L, Jirstrom K, Bendahl P-O, Ferno M, Nordenskjold B, Stal O, Thorstenson S, Jonsson P-E, Landberg G. Tumor-specific expression of VEGFR2is associated with impaired tamoxifen response in premenopausal breast cancer. Journal of clinical oncology 2005;In Press, Corrected Proof.
32. Chakrabarti J, Turley H, Campo L, Han C, Harris AL, Gatter KC, Fox SB. The transcription factor DEC1 (stra13, SHARP2) is associated with the hypoxic response and high tumour grade in human breast cancers. Br J Cancer 2004;91(5):954-8.

33. Bos R, van Diest PJ, van der Groep P, Shvarts A, Greijer AE, van der Wall E. Expression of hypoxia-inducible factor-1alpha and cell cycle proteins in invasive breast cancer are estrogen receptor related. Breast Cancer Res 2004;6(4):R450-9.
34. Nielsen NH, Emdin SO, Cajander J, Landberg G. Deregulation of cyclin E and D1 in breast cancer is associated with inactivation of the retinoblastoma protein. Oncogene 1997;14(3):295-304.
35. Loden M, Stighall M, Nielsen NH, Roos G, Emdin SO, Ostlund H, Landberg G. The cyclin D1 high and cyclin E high subgroups of breast cancer: separate pathways in tumorogenesis based on pattern of genetic aberrations and inactivation of the pRb node. Oncogene 2002;21(30):4680-90.
36. Stoner M, Wormke M, Saville B, Samudio I, Qin C, Abdelrahim M, Safe S. Estrogen regulation of vascular endothelial growth factor gene expression in ZR-75 breast cancer cells through interaction of estrogen receptor alpha and SP proteins. Oncogene 2004;23(5):1052-63.
37. Dabrosin C, Margetts PJ, Gauldie J. Estradiol increases extracellular levels of vascular endothelial growth factor in vivo in murine mammary cancer. Int J Cancer 2003;107(4):535-40.
38. Pietras RJ. Interactions between estrogen and growth factor receptors in human breast cancers and the tumor-associated vasculature. Breast J 2003;9(5):361-73.
39. Elkin M, Orgel A, Kleinman HK. An angiogenic switch in breast cancer involves estrogen and soluble vascular endothelial growth factor receptor 1. J Natl Cancer Inst 2004;96(11):875-8.

Table 1

HIF-1α distitribution in the material.

| HIF-1α | N | HIF-1α in 2 groups |
|---|---|---|
| 0-1% | 286 | 286 (negative/low) |
| 2-10% | 61 | 91 (positive) |
| 11-100% | 30 | |
| Total | 377 | |
| Missing | 187* | |

* = 64 due to no available paraffin blocks, 123 due to no tumour material in the core or loss during the staining process.

Table 2

Clinical data and tumour characteristics according to HIF-1α in two groups.

| Variable | HIF-1α (negative/low) | HIF-1α (positive) | *P*-value ($\chi^2$-test) |
|---|---|---|---|
| *Age* | | | |
| < median (25-45) | 164 | 52 | 0.97 |
| > median (46-57) | 122 | 39 | |
| *Node status* | | | |
| N0 | 68 | 34 | 0.012 |
| N1+ | 216 | 57 | |
| Unknown | 2 | | |
| *Tumour size* | | | |
| 0-10 mm | 21 | 3 | 0.009 |
| 11-20 mm | 88 | 16 | |
| 21+ mm | 176 | 72 | |
| Unknown | 1 | | |
| *NHG** | | | |
| 1 | 36 | 5 | <0.0001 |
| 2 | 123 | 19 | |
| 3 | 118 | 64 | |
| Unknown | 9 | 3 | |
| *Ki-67* | | | |
| 0-10% | 124 | 27 | 0.005 |
| >10% | 135 | 61 | |
| Unknown | 27 | 3 | |
| *ER status* | | | |
| ER – | 69 | 53 | <0.0001 |
| ER + | 211 | 36 | |
| Unknown | 6 | 2 | |
| *PR status* | | | |
| PR – | 69 | 56 | <0.0001 |

| | | | |
|---|---|---|---|
| PR + | 211 | 32 | |
| Unknown | 6 | 3 | |
| *Her2 status* | | | |
| Normal/Weak (0-2+) | 222 | 65 | 0.015 |
| Overexpressed (3+) | 34 | 21 | |
| Unknown | 30 | 5 | |
| *Her2 FISH* | | | |
| Non amplified | 204 | 60 | 0.069 |
| Amplified | 27 | 15 | |
| Unknown | 55 | 16 | |
| *VEGF-A* | | | |
| Negative (0-2) | 213 | 66 | 0.68 |
| Positive (3) | 63 | 22 | |
| Unknown | 10 | 3 | |
| *Cyclin A2* | | | |
| 0-10% | 134 | 36 | 0.056 |
| >10% | 115 | 50 | |
| Unknown | 37 | 5 | |
| *Cyclin E* | | | |
| 0-25% | 115 | 14 | <0.0001 |
| >25% | 69 | 44 | |
| Unknown | 102 | 33 | |
| *Cyclin D1* | | | |
| 0-25% | 149 | 65 | <0.0001 |
| >25% | 127 | 21 | |
| Unknown | 10 | 5 | |
| *Randomization* | | | |
| Control | 151 | 45 | 0.58 |
| Tamoxifen | 135 | 46 | |

Abbreviations: N0= node negative, N1+ = node positive, NHG= Nottingham histological grade, ER=oestrogen receptor, PR= progesterone receptor

Table 3

Clinical data and tumour characteristics according to treatment arm

| | Control | | Tamoxifen treated | |
|---|---|---|---|---|
| Variable | *HIF-1α positive* | *HIF-1α negative/low* | *HIF-1α positive* | *HIF-1α negative/low* |
| *Ki-67* | | | | |
| 0-10% | 13 | 66 | 14 | 54 |
| >10% | 31 | 70 | 30 | 69 |
| *p*-Value | 0.011 | | 0.16 | |
| *ER-status* | | | | |
| ER+ | 20 | 114 | 16 | 97 |
| ER– | 24 | 36 | 29 | 33 |
| *p*-Value | <0.0001 | | <0.0001 | |

Table 4

Recurrence free survival with Cox-multivariate analysis for untreated patients.

| | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|
| Variable | HR | 95% CI | p-value | HR | 95% CI | *p*-value |
| *HIF-1α (neg vr pos)* | 1.3 | 0.9-2.1 | 0.19 | 1.4 | 0.9-2.3 | 0.18 |
| *Her2 (0-2+ vr 3+)* | 1.4 | 0.9-2.3 | 0.15 | 0.9 | 0.5-1.6 | 0.73 |
| *Node status (N0 vr N1+)* | 2.0 | 1.3-3.0 | 0.002 | 3.3 | 1.8-6.0 | <0.0001 |
| *Tumour size (0-20 vr 21+mm)* | 1.2 | 0.9-1.7 | 0.21 | 1.1 | 0.7-1.8 | 0.67 |
| *NHG (1,2 vr 3)* | 1.8 | 1.3-2.5 | <0.0001 | 1.7 | 1.0-2.8 | 0.028 |
| *Ki-67 (0-10% vr 11%+)* | 1.4 | 1.0-2.0 | 0.073 | 1.3 | 0.8-2.2 | 0.26 |

Abbreviation: HR = hazard ratio, CI = confidence interval.

Table 5

Recurrence free survival with Cox-multivariate analysis for untreated patients, NHG 1/2.

| | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|
| Variable | HR | 95% CI | *p*-Value | HR | 95% CI | *p*-Value |
| *HIF-1α (neg vr pos)* | 2.3 | 1.1-4.6 | 0.022 | 2.3 | 1.1-4.9 | 0.033 |
| *Node status (N0 vr N1+)* | 1.2 | 0.6-2.2 | 0.58 | 1.2 | 0.5-2.7 | 0.68 |
| *Tumour size(0-20 vr 21+mm)* | 1.2 | 0.7-1.9 | 0.45 | 1.0 | 0.5-1.9 | 0.99 |
| *NHG (1 vr 2)* | 1.6 | 0.8-3.0 | 0.17 | 1.3 | 0.6-3.0 | 0.49 |
| *Ki-67 (0-10% vr 11%+)* | 1.8 | 1.0-3.1 | 0.036 | 1.5 | 0.8-2.9 | 0.19 |

Abbreviation: HR = hazard ratio, CI = confidence interval.

Figure legends.

Figure 1.

A) Immunohistochemical HIF-1α staining of breast cancer array samples illustrating HIF-1α positive and HIF-1α negative examples. B) Immunohistochemical evaluation of the antibody, MCF-7 cells were exposed to 21% oxygen compared to hypoxic conditions for 72 hours. C) MCF-7 cells transfected with empty vector or with HIF-1α expression-vector.

Figure 2.

Kaplan-Meier estimate for patients according to treatment arm in patients with ER (oestrogen receptor)-positive tumours of recurrence free survival for patients with A) HIF-1α negative or low tumours or B) HIF-1α positive tumours. C) Immunohistochemical stainings of HIF-1α positive and negative/low tumours, with the corresponding tissue microarray core stained for ER and Ki-67, illustrating examples from both control and tamoxifen treated patients. The log-rank test was used to calculate the p-values.

Figure 3.

Kaplan-Meier curves for negative/low (N=286, black line) contra high (N=91, grey line) HIF-1α expression for A) recurrence free survival, B) overall survival and C) breast cancer specific survival. The log-rank test was used when calculating the p-values.

Figure 4.

Kaplan-Meiers estimate of recurrence free survival for patients with negative/low contra high HIF-1α expression for A) Control cohort, B) Control cohort resticted to ductal tumours only or C) lymph node negative or D) lymph node positive patients or E) NHG 1/2 tumours or F) NHG 3 tumours only. The log-rank test was used to calculate the p-values.