

# No amplifications of hypoxia-inducible factor-1 $\alpha$ gene in invasive breast cancer: A tissue microarray study<sup>1</sup>

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**Abstract.** *Objective:* Hypoxia Inducible Factor-1 (HIF-1) is an important transcription factor that stimulates tumour growth and metastases via several pathways, including angiogenesis and altered metabolism. Activation of HIF-1 depends on the levels of its  $\alpha$ -subunit, which increase during hypoxia. Recent studies showed that the HIF-1 $\alpha$  gene was amplified in prostate cancer, leading to overexpression of HIF-1 $\alpha$  at normoxia. The aim of this study was to evaluate the presence of HIF-1 $\alpha$  gene amplifications in invasive breast cancer as an explanation for HIF-1 $\alpha$  protein overexpression. *Methods:* Protein and gene expression of HIF-1 $\alpha$  were analyzed on a tissue microarray of 94 breast cancers by immunohistochemistry and fluorescent *in situ* hybridization (FISH), respectively. *Results:* Overexpression of HIF-1 $\alpha$  protein was found in 58/94 (62%) of patients. No amplifications of the HIF-1 $\alpha$  gene were detected. *Conclusion:* Increased protein levels of HIF-1 $\alpha$  are not associated with amplification of the HIF-1 $\alpha$  gene in human breast cancer. Therefore, other mechanisms than gene amplification must be responsible for HIF- $\alpha$  overexpression at normoxia.

**Keywords:** HIF-1 $\alpha$ , gene expression, amplification, immunohistochemistry, breast cancer, FISH

## 1. Introduction

The master regulator of cellular oxygen homeostasis in the cell, the hypoxia-inducible factor-1 (HIF-1), was shown to be induced by hypoxia [9,14,16]. Activation of HIF-1 by hypoxia depends on the levels of the oxygen-sensitive  $\alpha$  subunit of HIF-1 [7,10,19,21]. The other subunit of HIF-1, HIF-1 $\beta$ , is constitutively expressed and will bind to HIF-1 $\alpha$  when levels of HIF-1 $\alpha$  are upregulated [9,19].

During normoxia, HIF-1 $\alpha$  is hydroxylated by prolyl hydroxylases. The Von Hippau Lindau (VHL) protein recognizes hydroxylated HIF, leading to continuous

degradation by VHL mediated ubiquitination. Under hypoxic conditions, the rate of prolyl hydroxylation decreases. As VHL cannot recognize unhydroxylated HIF-1 $\alpha$ , hypoxia leads to stabilization and overexpression of the HIF-1 $\alpha$  protein [10].

Upon stabilization, the HIF-1 $\alpha$  protein is transported into the nucleus where it heterodimerizes with HIF-1 $\beta$ . The formed HIF-1 complex binds to its DNA recognition sequence 5'-RCGTG-3' present within the hypoxia responsive element of various target genes [15]. Hypoxia-induced HIF-1 activation leads to activation of many genes stimulating tumour processes such as angiogenesis, glycolysis, erythropoiesis and, if these all fail, apoptosis [4,14].

Overexpression of protein levels of HIF-1 $\alpha$  has been found in several tumours. High levels of the HIF-1 $\alpha$  protein have influence on the growth rate [3] and metastatic potential of these cancers [20]. By immunohistochemical investigation, overexpression of HIF-1 $\alpha$

<sup>1</sup>This work was supported by the unrestricted AEGON First International Scholarship in Oncology.

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was found to be associated with breast carcinogenesis [3] and a poor prognosis in invasive breast cancer [2,13].

Protein overexpression may, apart from posttranscriptional mechanisms, be caused by gene amplification. For example, the HER-2/*neu* oncogene is amplified and overexpressed in about 30% of breast cancer cases [17]. A strong correlation between HER-2/*neu* protein expression and gene copy number is usually present.

It was recently shown that 37% of clinical prostate cancer specimens contain additional copies (3–4 copies) of the HIF-1 $\alpha$  gene encoding HIF-1 $\alpha$ . Furthermore, the HIF-1 $\alpha$  gene was found to be highly amplified in a prostate cancer cell line that overexpressed the HIF-1 $\alpha$  protein even under normoxic conditions [12].

In this study, amplification of HIF-1 $\alpha$  as an explanation for HIF-1 $\alpha$  protein overexpression was, for the first time, investigated in a tissue microarray of invasive breast cancers in relation with protein expression of HIF-1 $\alpha$ .

## 2. Materials and methods

### 2.1. Patients

A group of 94 primary invasive breast cancer patients were selected from the archives of the Gerhard-Domagk-Institute of Pathology (University of Münster, Germany) as described before [11]. Tissue microarray blocks were constructed from the original neutral buffered formaldehyde-fixed paraffin-embedded tumour blocks using published guidelines [11]. Tumours were graded following the criteria of Elston [5] and patients were TNM staged.

Histologically, 81 were ductal invasive, 7 lobular invasive, 4 mucinous invasive and 2 were medullary invasive cancers. Tumours were graded as grade I ( $n = 7$ ), II ( $n = 58$ ) or III ( $n = 29$ ). According to the TNM system the group contained 25 T<sub>1</sub>, 44 T<sub>2</sub>, 8 T<sub>3</sub> and 17 T<sub>4</sub> tumours and 44 lymph node-negative and 50 lymph node-positive patients.

To gain an overview about the intratumoural heterogeneity of putative HIF-1 $\alpha$  amplifications, three spots per tumour were taken according to standard procedures using an H&E-stained section to guarantee representative tumour spots on the array. The spot diameter was 0.6 mm, and the distance between the spots was 1 mm. One spot originated from the center of the tumour, the two others were punched out of the infil-

trative border of the tumour. Five micrometer sections from the tissue arrays were cut onto SuperFrost Plus slides (Menzel-Gläser, Braunschweig, Germany) and baked overnight for immunohistochemistry and fluorescence *in situ* hybridization (FISH).

### 2.2. Immunohistochemistry

Immunohistochemistry for HIF-1 $\alpha$  was performed with the Catalyzed Signal Amplification System (DAKO, Glostrup, Denmark) as described before [3]. After deparaffination and rehydration, target retrieval solution (DAKO) was used for antigen retrieval with the slides placed in a water bath for 45 minutes at 97°C. A cooling off period of 20 minutes preceded the incubation of the HIF-1 $\alpha$  mouse monoclonal HIF-1 $\alpha$  67, (BD Transduction Laboratories, Lexington, KY) [22], at a dilution of 1:500. The primary antibodies were detected using a biotinylated rabbit anti-mouse antibody (DAKO CSA kit). The signal was amplified by avidin-biotin complex formation (DAKO CSA kit) and developed with diaminobenzidine followed by haematoxylin counterstaining. Before the slides were mounted, all sections were dehydrated in alcohol and xylene.

### 2.3. FISH analysis

For FISH a digoxigenin labeled PAC probe specific for the HIF1 $\alpha$  gene was used for dual color analyses with a biotin-labeled locus-specific P1 control probe RMC14P005 (RMC, Berkley, CA, USA) as previously described [8,12]. The tissue microarray slide was deparaffinized in xylene prior to hybridization. Subsequently, the slide was pre-treated with 1 M NaSCN for 10 min at +80°C and digested for 5 min with 4 mg/ml pepsin (Sigma P-7012, in 0.9% NaCl, pH 1.5) at +37°C. The probes and sample were co-denatured in a hybridization mix on a waterbath at +80°C for 8 min, and hybridized in a humid chamber at +37°C for 2–3 days. Next, the slide was washed, stained with anti-digoxigenin-rhodamine (Roche Diagnostics, Mannheim, Germany), and avidin-fluorescein-isothiocyanate (Vector Laboratories Inc. Burlingame, CA, USA), and subsequently counterstained with 0.1 M 4,6-diamidino-2-phenylindole (DAPI) in a Vectashield antifade solution (Vector Laboratories Inc.) as previously described [8].

## 2.4. Quantification

The percentage of nuclei with HIF-1 $\alpha$  protein overexpression in the tumour cores was scored using brightfield microscopy, only regarding homogeneously and darkly stained nuclei as positive, as described previously [3]. *HIF-1 $\alpha$*  gene copy number was assessed using fluorescence microscopy in non-overlapping malignant nuclei in each tissue core. The amplification index was calculated by dividing the # *HIF-1 $\alpha$*  signals by the # P1 control probe signals. A two-fold increase was used as a criterion to verify the presence of *HIF-1 $\alpha$*  gene amplification. Quantifications were done simultaneously by two independent observers (P.J.v.D. and M.M.V.).

## 3. Results

### 3.1. Immunohistochemistry

By immunohistochemical investigation, 58/94 (62%) of breast cancer cores showed nuclear HIF-1 $\alpha$  protein overexpression ( $\geq 1\%$  of nuclei) (Fig. 1).

### 3.2. FISH analysis

To test the specificity of the *HIF-1 $\alpha$*  specific probe, FISH analysis was performed on a normal metaphase chromosome preparation. Two copies of the *HIF-1 $\alpha$*  gene were present on chromosome 14 (Fig. 2A) as expected.

FISH analysis on the micro tissue array showed no *HIF-1 $\alpha$*  gene amplification for 89 tumours (Fig. 2B).

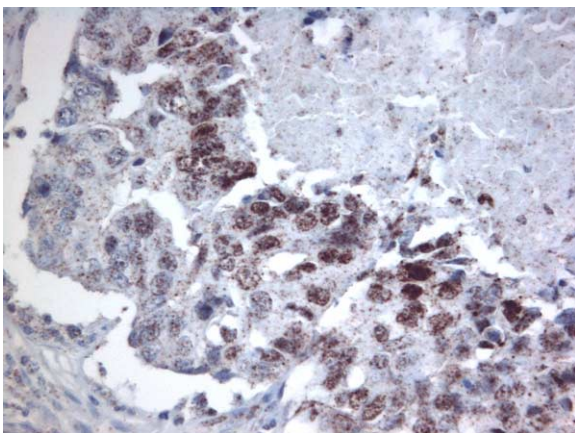


Fig. 1. Example of perinecrotic HIF-1 $\alpha$  immunohistochemical staining in an invasive breast cancer.

Only 5 cases showed consistently 3 or 4 nuclear signals in the presence of 3 or 4 signals of the chromosome 14 control probe pointing to chromosome 14 copy number increase and not to gene amplification (Table 1). From

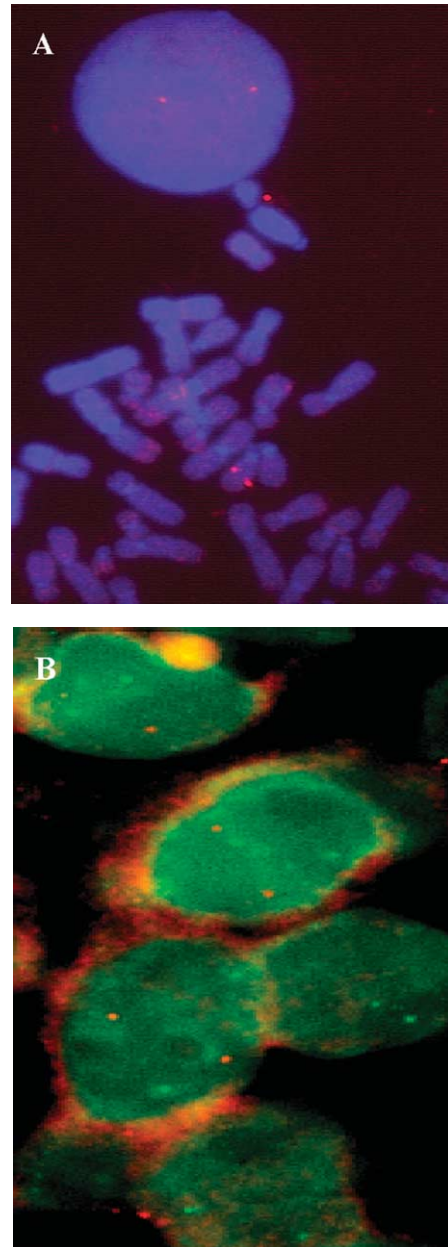


Fig. 2. Fluorescence *in situ* hybridization for *HIF-1 $\alpha$*  (A) FISH analysis on normal metaphase chromosome preparation shows that *HIF-1 $\alpha$*  is present in two copies on chromosome 14. (B) Two color FISH analysis of an invasive breast cancer core on a tissue microarray shows the presence of two copies of *HIF-1 $\alpha$*  (red) and the locus specific control probe for chromosome 14 (green) respectively (Magnification  $\times 1000$ ).

Table 1

Gene copy amplification analysis for HIF-1 $\alpha$ by FISH		
HIF-1 $\alpha$ signals	Amplification index	No of cases
/P1 control probe signals		
2/2	1	89
3/3	1	2
4/4	1	3

these five cases, two cases showed overexpression of HIF-1 $\alpha$  at the protein level.

#### 4. Discussion

HIF-1 $\alpha$  overexpression has been described in various tumours, including breast cancer. Two HIF-1 $\alpha$  overexpression patterns are present in breast cancer: perinecrotic HIF-1 $\alpha$  overexpression caused by (severe) hypoxia and diffuse HIF- $\alpha$  overexpression at (relative) normoxia possibly induced by oncogenes or loss of tumour suppressor genes [18]. However, aneusomy of the HIF-1 $\alpha$  gene as recently observed in a prostate cancer cell line and clinical prostate cancer samples [12] might also be an explanation for this latter phenomenon. To this end we performed FISH analysis for the HIF-1 $\alpha$  gene to see whether HIF-1 $\alpha$  amplifications occur in breast cancer. Other methods to detect a gene amplification like CGH are less suitable to detect specific gene amplification of HIF-1 $\alpha$  in view of the lower resolution of the CGH technique. The results of FISH analysis were compared with HIF-1 $\alpha$  protein overexpression assessed by immunohistochemistry.

HIF-1 $\alpha$  overexpression was found in 62% of breast cancers. These findings are comparable to the HIF-1 $\alpha$  levels we found earlier in other invasive breast cancer groups [2,3]. FISH analysis of the breast cancer tissue microarray revealed however no amplification of the HIF-1 $\alpha$  gene, and only 5 tumors showed aneusomy (3/4 copies) of the gene in the presence of 3/4 copies of the control chromosome 14 probe, indicating chromosome copy number increase rather than gene amplification.

These results indicate that high level HIF-1 $\alpha$  gene amplification or additional HIF-1 $\alpha$  gene copies are not present (or are at least exceedingly rare) in invasive breast cancers. Consequently, other mechanisms than gene amplification must be responsible for diffuse HIF-1 $\alpha$  protein overexpression in invasive breast cancer.

Oxygen independent HIF-1 $\alpha$  expression can be induced by oncogene signalling and involvement of tran-

scription factors like AP-1 [6]. These pathways deserve to be further studied with regard to HIF-1 $\alpha$  expression patterns. Although we have not found any amplifications of the HIF-1 $\alpha$  gene, genetic alterations may still play a role as Anastasiadis et al. recently showed that mutations in the oxygen dependent degradation domains of HIF-1 $\alpha$  may occur. Mutations in this region prevent HIF-1 $\alpha$  hydroxylation by prolyl hydroxylases and thereby ubiquitination by VHL [1]. Therefore, mutations in these domains may explain HIF-1 $\alpha$  expression at normoxia. If these mutations occur in invasive breast cancers will be further investigated.

In conclusion, in contrast to prostate cancer, we could not detect amplifications of the HIF-1 $\alpha$  gene in human breast cancers. Therefore, other mechanisms than gene amplification must be responsible for HIF- $\alpha$  protein overexpression at (relative) normoxia.

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