HYPOXIA INDUCIBLE FACTOR-1α

IN CARCINOGENESIS AND PROGRESSION OF

BREAST CANCER

REINHARD BOS

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Italian (fashion)photographer who visualizes women in an erotic and elegant way. By computerassisted manipulation one breast has disappeared without loss of sensuality…

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Design and DTP cover: Doroté Evers, SvH + ROB Rotterdam

Lay-out: Reinhard Bos

Printed by: OPTIMA grafische communicatie, Rotterdam

ISBN 90-393-3858-2

Hypoxia-Inducible Factor-1α in carcinogenesis and progression of breast cancer

Hypoxia-Inducible Factor-1α in carcinogenese en progressie van borstkanker
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de Rector Magnificus, prof.dr. W.H. Gispen,
in gevolge van het besluit van het College voor Promoties
in het openbaar te verdedigen
op dinsdag 23 november 2004 des middags te 12.45 uur

door
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geboren op 1 februari 1975 te Rotterdam

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This thesis is the first to result from the 1ST AEGON international Scholarship in Oncology, a joint research program between the Breast Cancer Research Group Utrecht (Department of Pathology, and the Division of Internal Medicine and Dermatology), the VU Oncology School (Departments of Pathology and Oncology, VU University Medical Center, Amsterdam, The Netherlands), and the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins School of Medicine, Baltimore, Maryland, USA. This joint research program is coordinated by Prof. dr. E. van der Wall and Prof M.A. Carducci. It is funded by an unrestricted educational grant from AEGON Inc. (thanks to the kind support of Mr drs. K.J. Storm, previous CEO and Mr D.J. Sheppard, present CEO of AEGON Inc).

Financial support for printing this thesis was kindly provided by: Amgen B.V., Ortho Biotech, Roche Nederland B.V., Aventis Oncologie, AstraZeneca B.V.

Leef je dromen

voor Nannie

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13 September 2004

Dear Reinhard:

Congratulations on passing the MD exam and completing your thesis, which is an exceptional body of work. I present data from your landmark studies every time I lecture about the role of HIF-1 in breast cancer. It was a great pleasure for me to participate in your project and to see our monoclonal antibody put to such good use for immunohistochemistry at your skilled hands. Thanks very much for your thoughtfulness in sending me a copy of your thesis. Our graduate students are very good, but few of them are productive enough to have six publications. I wish you continued success and I am confident that you will be an outstanding physician-scientist.

Regards,

Gregg

Gregg L. Semenza, M.D., Ph.D.
Professor of Pediatrics, Medicine, Oncology, Radiation Oncology, and Institute of Genetic Medicine

Contents

ABBREVIATIONS

AI	apoptotic index
AKT	protein kinase B
BCT	breast conserving treatment
bFGF	basic fibroblast growth factor (also known as FGF-2)
BRCA	breast cancer related gene (1 or 2)
BSA	bovine serum albumin
CA IX	carbonic anhydrase IX
CD	cluster of differentiation
CGH	Comparative genomic hybridization
DAB	3,3'diaminobenzidine tetrahydrochlodride
DCIS	ductal carcinoma in situ
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	estrogen receptor
FDG	Fluorodeoxyglucose
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FGF-BP	fibroblast growth factor binding protein
FISH	fluorescent in situ hybridization
Flk-1	vascular endothelial growth factor receptor II
Flt-1	vascular endothelial growth factor receptor I
FRAP	FKBP-rapamycin-associated protein
Glut-1	glucose transporter-1
H&E	haematoxylin and eosin
HIF-1	hypoxia inducible factor-1
Ig	immunoglobulin
IGF	insulin like growth factor
KDR	kinase domain receptor
Ki-67	proliferation marker
MAI	mitotic activity index
Mab	monoclonal antibody
mRNA	messenger ribonucleic acid
MVD	microvessel density
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PDGF-R	platelet derived growth factor receptor
PET	positron emission tomography
PI(3)K	phosphatidylinositol-3-OH kinase
PR	progesterone receptor
pRb	retinoblastoma protein
PTEN	phosphatase and tensin homolog deleted on chromosome ten
Rb	retinoblastoma gene

SPSS	statistical products and service solution
TBS	tris-buffered saline
TGFα	transforming growth factor alpha
TGFβ	transforming growth factor beta
TGFβR	transforming growth factor beta receptor
TNF-α	tumor necrosis factor-alpha
VEGF	vascular endothelial growth factor
VHL	Von Hippel Lindau tumor suppressor gene
WHO	World Health Organization

Chapter 1

General introduction

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I BREAST CANCER:

Physiology
Breast carcinogenesis
Breast cancer
Treatment of breast cancer

II SOLID TUMOR BIOLOGY:

Proliferation
Tumor metabolism, pH and Warburg effect
Hypoxia
Angiogenesis
Positron emission tomography

III AIMS OF THIS THESIS

All over the world, more than 1 million women per year will be confronted with the diagnosis breast cancer. With over 11000 women that are yearly affected, breast cancer is the most prevalent form of cancer in the Netherlands. The incidence is still increasing whereas breast cancer related mortality seems to decline over the last years. This is thought to be due to earlier detection and improvements in treatment efficacy and toxicity. However, understanding the exact pathogenesis of breast cancer still requires improved insight into the fundamental biological processes that underlie carcinogenesis Ultimately, new prognostic markers and diagnostic tools reflecting innovative knowledge will guide the development of new preventive and treatment strategies.

It is known that hypoxia (lack of cellular oxygen) often occurs during carcinogenesis and tumor progression at the time that tumors outgrow their vasculature. Hypoxia has been described to predict poor prognosis in different solid tumors and hematological malignancies and results in resistance to chemotherapy and radiotherapy. However, little has been known about the biological effects of hypoxia in breast cancer. In 1995, Greg Semenza discovered the Hypoxia-inducible Factor-1 (HIF-1) gene which led to an ever since increasing interest in hypoxia, including its role in carcinogenesis. HIF-1 is a basic helix-loop-helix transcription factor and an important regulator of the hypoxia response of mammalian cells. During hypoxia, its two subunits HIF-1α and HIF-1β will form an active HIF-1 complex, which in turn activates many target genes (such as vascular endothelial growth factor (VEGF), erythropoietin, glucose transporter-1) all involved in cellular survival. These survival mechanisms are also beneficial to tumor cells, because of the resulting angiogenic, glycolytic, and metastatic effects that turn tumor cells into warriors.

In 1999, Zhong et al. showed that protein levels of HIF-1α, that determine HIF-1 activity, were detectable in many human cancers. This thesis aimed to unravel the role of HIF-1α in breast carcinogenesis and progression. Ultimately, this may lead to a rationale for clinical targeting of HIF-1 in breast cancer.

I BREAST CANCER:

Physiology

Until puberty the development and function of the breast is the same in both sexes. Unlike most organs, maturation of the breast takes place during puberty in females, when hormonal regulation changes and the immature breast tissue starts to grow and differentiate into functional breast tissue. This process is mainly due to the activity of the sex-steroid hormones estrogen and progesteron. These steroids are able to bind intracellular steroid receptors present in the adeno-epithelium of the breast. Upon binding they stimulate growth and differentiation. Mature breasts harbor the capacity to produce and secrete milk when stimulated by lactational hormones, which themselves are secreted during pregnancy. Milk is produced by the glandular epithelium of the acini of the breast and is secreted into the lumina of the glands that form ducts, which connect to form larger ducts that transport the milk to the nipple. During the menstrual cycle, breast glandular cells undergo cyclic proliferation and apoptosis. In the menopause, when steroid production is decreased, involution leads to definite non-lactating breasts.

Breast Carcinogenesis

Normal breast tissue and invasive breast cancer are two extreme ends of the histologic spectrum of the breast. In between, a wide spectrum of breast lesions exists (Table 1). The development of breast cancer (breast carcinogenesis) is thought to occur via progression of increasingly aberrant benign and pre-invasive breast lesions. Putative precursor lesions of breast cancer are (atypical) ductal hyperplasia and ductal/lobular carcinoma *in situ* [1]. This gradual development from normal breast

TABLE 1. List of breast lesions with incidence, preferential age of presentation, and relative risk (RR) of development of invasive cancer

Lesion	Incidence	preferential age	RR
Fibrocystic change*	common	35-50	1
Fibroadenoma^	common	20-30	2-4#
Hamartoma	uncommon	50-60	1
Phyllodes tumor	uncommon	40-70	unknown
Sclerosing adenosis	common	20-70	unknown
Duct papilloma^	uncommon	35-55	1 or ↑
Usual ductal hyperplasia	common	unknown	2
Atypical ductal hyperplasia	less common	unknown	5
Ductal carcinoma *in situ*	less common	unknown	11§

*including apocrine metaplasia, duct adenosis, cysts
^probably no direct precursor, but subsequent risk for breast cancer development
#two-fold for common lesions, three-fold for complicated lesions, and four-fold for familial predisposition
↑, increased when multiple papillomas are present
§Especially patients with poorly differentiated lesions develop breast cancer (20-30%) after 15-20 years

to invasive cancer via precursor lesions fits well with the concept that cancer is a multistep process where the increasingly aberrant genetic make up is reflected also by morphological changes [2]. Evidence supporting this progression model are increased risk for development of breast cancer in people bearing these preinvasive lesions [3,4,5] (Table 1), the common finding of these lesions in the direct vicinity of invasive cancers, and the fact that the preinvasive and invasive lesions share many

alterations on the genetic and protein levels [6].

However, only a minority of these precursor lesions will actually progress into breast cancer. Several biologic, morphologic, and clinical features have been studied for their potential predictive value as to development of invasive breast cancer in case of a precursor lesion. The following factors are considered to indicate a higher risk for cancer development: familial predisposition, low age, loss of estrogen receptor (ER) or progesterone receptor (PR), amplification/overexpression of HER-2/*neu* [7], p53 [8], cyclin D_1 [9], EGFR [10], necrosis, increased proliferation, and marked nuclear morphologic changes [11]. Although some of these genetic changes play a relatively early or late role in cancer development and are especially frequent in breast cancer, a clearly defined stepwise progression model is largely lacking, and progression towards malignancy should be considered to follow the "bingo principle": the order of genetic changes does not seem to be of major importance, but rather the random combination of a certain number of major and minor genetic events will eventually lead to cancer [12].

Breast Cancer

Incidence & Risk factors

Breast cancer is the most common carcinoma in women. Breast cancer is not limited to women, but yearly also affects several thousands of men worldwide. Breast cancer incidence increases with age and is especially high in Europe, North America and Australia.

Risk factors include, beside age, duration of exposure to estrogen [13] (early menarche, late menopause, late first pregnancy or never having been pregnant, or prolonged use of oral estrogen-based contraceptives), and a history of either benign breast lesions (Table 1), ductal/lobular carcinoma in situ or earlier treated breast cancer [3]. In 8%-15% of breast cancers a familiar predisposition can be observed; in around 5% of patients breast cancer seems to be hereditary. Genetic aberrations involved in hereditary breast cancer so far discovered are germline mutations in breast cancer related gene 1 [14] (BRCA1) or BRCA2 [15], phosphatase and tensin homolog deleted on chromosome ten (PTEN) [16,17] (Cowden syndrome), p53 [18,19] (Li Fraumeni syndrome), and CHEK2 [20].

Prognostic & Predictive factors

Prognostic factors predict the risk of recurrence of (or death related to) breast cancer after primary surgery. The most important single prognostic indicators in breast cancer are lymph node status [21,22,23] tumor size [24,25] and proliferation rate [26]. Multivariate internationally established classification systems include TNM staging, and tumor grading systems [27,28]. Some emerging prognostic factors are bone marrow status [29], intratumoral microvessel density [30], Urokinase-type plasminogen activator (uPA) and its inhibitor (PAI-1) [31,32], and gene expression patterns by microarray [33,34], but all these need to be further confirmed and do also not provide optimal prognostic power.

Predictive factors predict the chance of response to therapy. ER and PR are predictors of response to hormonal therapy [35]. Two other important predictive factors in breast cancer are HER-2/*neu* [36,37] and epidermal growth factor (EGFR) receptors

[38,39] that predict response to the respective antibody based therapies.

There still is a paucity of new and simple markers that are able to recognize primary breast cancer patients who harbor an increased risk to develop recurrent or metastastic disease. This applies especially to lymph node negative patients where accurate high-risk indicators are urgently needed to select patients for adjuvant therapy, but also for low risk lymph node positive patients that do not require therapy. Recently, a 70-gene prognosis microarray profile addressed this issue to the best ever [40,41], however, this technique should further be simplified before daily clinical implementation can be achieved. In addition, reliable markers that can predict response to newly developed therapies are necessary.

Treatment of breast cancer

The ultimate treatment of breast cancer would be its prevention. However, there are no realistic possibilities for primary prevention except for proven BRCA1/2 germline mutation carriers at a certain age. Also in the Netherlands, mammography based population screening has proven to be useful to detect breast cancer in an earlier, better operable phase [42].

Treatment of breast cancer can be grossly divided into local treatment and systemic treatment.

Surgery is the preferred primary mode of local treatment of the breast. Two common surgical approaches are breast conserving treatment (BCT) or mastectomy. Resection of axillary lymph nodes is important to detect lymph node metastases for staging. The introduction of sentinel node mapping is an important improvement in the approach of the axilla, allowing to select a more limited group of patients that should undergo axillary dissection (the sentinel node positives) while sparing the sentinel node negatives from further surgical treatment of the axilla.

Local radiotherapy is always applied as part of the BCT. Further, radiotherapy is indicated following irradical tumor resection, invasion of the thoracic wall, lymph node positivity, locally advanced carcinomas, local recurrences or in case of –mainly bone-metastases.

Systemic treatment of breast cancer is applied as adjuvant therapy following high-risk primary breast cancer or to treat metastatic disease and consists mainly of chemo- and hormonal therapy. Adjuvant chemo and/or hormonal therapy is indicated in early stage lymph node positive breast cancer, leading to a relative reduction in the risk of recurrence of 25% following each form of systemic treatment. Neoadjuvant systemic therapy is used in locally advanced breast cancer. A trend towards implementation of neoadjuvant systemic therapy in early stage breast cancer is however ongoing. As it is assumed that the majority of breast cancer patients have (microscopic) disseminated disease, it is argued that a subgroup of patients with lymph node negative primary breast cancer will benefit from adjuvant chemotherapy. It is still difficult to distinguish the patients that belong to that high-risk subgroup [43]. As mentioned before, mitotic index is already being used to this end but does not have perfect sensitivity and specificity, and microarray analysis of breast cancer tissue that provides data on overexpressing or silenced genes might be useful but is still in the experimental stage [40,41].

Improvement in the quality and length of live of breast cancer patients so far

primarily depends on optimal selection for the best treatment based on reliable and reproducible prognostic and predictive factors. Further, some new treatment strategies are quite promising. However, both the factors and the treatments definitely need improvement.

New treatment strategies focus on tumor targeted approaches with anti-angiogenesis agents (e.g. anti-VEGF antibody, VEGF receptor inhibitors, anti-HER-2/*neu* antibody, and EGFR blockers). These new therapies reflect progress in the treatment of cancer based on a better understanding of tumor biology. Further improvements in the more classical chemo- and hormonal treatments are also to be expected from unraveling the fundamentals of carcinogenesis.

II SOLID TUMOR BIOLOGY:

A hallmark of cancer is increased proliferation. Increased proliferation will both cause an altered metabolism and often leads to hypoxia. Hypoxia will occur at the time that the tumor has grown beyond a size that can be fed by the surrounding microvessels. Hypoxia itself will lead to the formation of new vessels, a process called angiogenesis. Angiogenesis on its turn will allow further proliferation and will facilitate the systemic spread of tumor cells. These cells must however first gain invasion-, motility- and adhesion capabilities, and will have to escape from the immune system. Thus, in studying one process, like hypoxia, it must be remembered that cancer is a complex interplay of many molecular and cellular processes.

In the following, we will not try to give a full overview of these complex processes but rather focus on those that are relevant within the scope of this thesis: proliferation, hypoxia and tumor metabolism.

Proliferation

Cell replication is orderly regulated via a cascade of events, called the cell cycle. During different phases of the cell cycle, division into two daughter cells is prepared. The first phase (G_1) allows the cell to grow. At a certain size it enters the phase of DNA-synthesis (S) where the chromosomes are duplicated. Subsequently, in the next gap phase (G_2) the cell prepares for the actual division. During mitosis (M) chromosomes are separated and segregated into the daughter cells [44]. The two resulting cells are then back in G_1 and the cell cycle is completed. A whole machinery of positive and negative regulatory proteins is involved to control this essential process. Especially the switch (transition) from one cell cycle phase to another is extremely controlled to overcome replication errors and are, therefore, called transition checkpoints [45]. Most dominant is the regulatory capacity of the cyclins, whose activity is counteracted by inhibitors of the cyclin-dependent kinases (such as p16, p21, and p27). The cyclins differ in expression of subtypes during the subsequent stages of the cell cycle, and can be upregulated by several mitogenic stimuli. For instance, in breast cancer the subtype cyclin D_1 can be upregulated by estrogen [46]. Loss of checkpoint control due to loss of function or abnormal expression of any involved protein may lead to dysregulation of the cell cycle and, consequently, an altered proliferation rate [47]. It was shown that several cell cycle regulated proteins were abnormally expressed during human breast carcinogenesis [48,49]. In invasive breast

cancer, even more alterations of cell cycle regulators have been described.

For breast cancer, cyclin D_1 is the most thoroughly studied cell cycle-related protein. Cyclin D_1 is overexpressed in the majority and amplified (chromosome 11q13) in 10-20% of breast cancers [9,50,51]. Cyclin D_1 is a growth factor responsive cyclin that plays an important role in regulating the G_1/S checkpoint. Upregulated cyclin D_1 can allow transit of the checkpoint even in the absence of growth factors. This was shown for transgenic mice that overexpressed cyclin D_1, and consequently developed mammary carcinomas [52]. Activity of cyclin D_1 (cell cycle progression) can be blocked by p21. p21 plays a key role in growth arrest, and is, besides other mechanisms, especially p53 mediated. p21 can also inhibit the activity of cyclin E. Further, it is interesting to point at the potential impact of the recent finding of Shvarts et al, who reported a new mechanism for upregulation of cyclin D_1 via BCL-6 [53]. The BCL-6 gene encodes a protein which harbors transcriptional repressor activity, whose function is still uncertain [54]. However, the protein encoded by this gene is homologous to zinc finger transcription factors and may be involved in activating certain growth-promoting genes. Until recently, BCL-6 aberrations were thought to be unique for non-Hodgkin lymphomas where rearrangement of the BCL-6 gene (chromosome 3q27) is present in approximately one-third of diffuse large B cell lymphomas. After the study of Shvarts et al, a role of BCL-6 in other diseases might be postulated.

Among several tumor suppressor genes p53, the retinoblastoma (Rb) gene and PTEN might be most relevant for the scope of this thesis. These factors render blockade of cell cycle progression based on different stimuli. In fact, ‘wild type’ p53 protein levels are increased in damaged cells to gain time and allow repair of DNA. If necessary, ‘wild type’ p53 can cause apoptosis (programmed cell death) when the damage is too severe. A somatic p53 mutation is the most frequent found mutation in human cancer, hereby its is strongly associated with carcinogenesis because mutated p53 does not regulate DNA damage repair and thus causes chromosome instability. Overexpression of mutated p53 occurs in approximately 25-45% of primary breast cancers [55]. As mentioned above, an inheritable genetic defect (germ-line mutation) in p53 leads to the Li Fraumeni syndrome [56] with a high frequency of breast and other cancers occur in young affected individuals.

In 1971 Knudson postulated that retinoblastoma might be caused by two mutational events [57]. In 1993 the Rb gene was mapped on chromosome 13 [58]. Rb is constitutively expressed and constrains cells from progressing through the G_1 phase [59], and the hypophospholylated protein product of Rb (pRb) complexes with many cellular proteins including the E2F transcription factors (nuclear factors involved in S phase replication). pRb is inactivated in less than 20% of breast cancers [60,61]. During hypoxia, cells may undergo a p53-independent, reversible G_1/S cell cycle arrest due to hypophosphorylation of pRb [62,63] sequestering E2F and thus preventing initiation of DNA synthesis.

The phophatidylinositol-3-kinase (PI(3)K) pathway is activated in 30-40% of human cancers and stimulate key downstream effectors such as Akt, transcription nuclear factor κB tumor and mammalian target of rapamycin. In this way the PI(3)K pathway stimulate cell survival, proliferation and migration. PTEN is a tumor suppressor gene because it suppress cell survival and proliferation by dephosphorylating

phosphoinositides to prevent their activation of the AKT signal transduction kinases [64]. Therefore, loss of function of this tumor suppressor gene is associated with increased (and uncontrolled) intracellular signaling via the PI(3)K pathway.

Hypoxia

Hypoxic conditions (defined as an oxygen tension below the physiological level, where it has to be noted that oxygen tension in tissues is far below 20%) frequently occur in tumors, especially in solid tumors. A developing solid tumor will outgrow its own vasculature beyond the size of several mm^3 [65] resulting in hypoxia. *In vivo*, the tissue oxygen tension decreases with increasing distance of a tumor cell from the center of a capillary vessel. Because the oxygen diffusion distance is 100 to 200 μm, tumor cells that lie beyond these distances from a capillary vessel become anoxic [66]. The resulting hypoxic levels affect all viable cells, since normal oxygen levels (normoxia) is essential for maintaining cellular homeostasis. In a reaction to these circumstances, cells will alter their metabolism and activate certain survival genes as described below. Intratumoral necrotic areas will be formed if these mechanisms fail. In 1970 Tannock showed that the proliferation rate decreased with increasing distance from functional blood vessels [67]. However, a general feature of tumors is the presence of blood vessels that fail to function properly so that individual tumor cells nearby these blood vessels also do become hypoxic. Distinct from chronic hypoxia is the acute form of hypoxia, which results from a sudden closure of the vasculature or from an acute decrease in blood flow. This form of hypoxia has been shown to be present in experimental tumors [68,69]. In general, acute responses are thought to be modulated by posttranslational modification of proteins which can take place in seconds to minutes. Chronic responses represent changes in gene transcription and take place in minutes to hours. Both chronic and acute hypoxia do coexist in the same tumor [70].

Direct measurement of hypoxia via polarographic oxygen electrodes showed, indeed, that hypoxia was present in head and neck, brain, cervical and breast cancers [71]. And, as expected, a wide range of oxygen levels could be measured within the same tumor. Besides, not all tumors showed hypoxia, and almost all tumors harbored normoxic tumor parts.

Chronically hypoxic cells are, due to the lack of oxygen and nutrients, non-rapidly proliferating cells and therefore resistant to many anti-cancer therapies [72]. As a consequence, tumor specific treatment strategies are currently tested to overcome treatment resistance of hypoxic cells. Some examples are use of oxygen; chemical agents that selectively sensitize hypoxic cells; and bioreductive drugs that specifically kill hypoxic cells.

Hypoxia Inducible Factor-1

Hypoxic conditions that occur during embryogenesis, cardiovascular disease, and tumor development induce the activity of hypoxia-inducible factor 1 (HIF-1), the master regulator of cellular O_2 homeostasis [73]. Special interest in the role of hypoxia in cancer was stimulated by the discovery of HIF-1 by Semenza [74], and the data of Zhong et al who showed the overexpression of HIF-1α in many human cancers [75]. HIF-1 is a transcription factor consisting of two subunits called HIF-1α and HIF-1β,

which both contain basic helix-loop-helix and PAS (PER-aryl hydrocarbon nuclear translocator-SIM) domains to bind to DNA [76,77]. Combination of these two subunits forms the active HIF-1 complex which binds the consensus sequence 5'-RCGTG-3' in the hypoxia-response elements of various target genes [78]. Hereby, HIF-1 activates the transcription of many genes controlling glucose transporters, glycolytic enzymes, gluconeogenesis, high-energy phosphate metabolism, growth factors, erythropoiesis, haem metabolism, iron transport, vasomotor regulation and nitric oxide synthesis [78,79]. Protein products of these downstream genes function to help the cell to survive the hypoxic stress by increasing O_2 delivery, by activating alternate metabolic pathways that do not require O_2 or by stimulating pro-apoptotic proteins. In this way HIF-1 elicits systemic, local and intracellular homeostatic responses.

The level of HIF-1α in cells is dependent on the intracellular oxygen concentration [80,81]. HIF-1α protein has a very short half-life under normoxia due to its continuous ubiquitination and proteasome-mediated degradation. This process is inhibited by hypoxia [80,82] or by mutated p53 [83] and von Hippel–Lindau (VHL) [84] tumor-suppressor gene defects, leading to overexpression of the HIF-1α protein. A nuclear localization signal at the C-terminal end of HIF-1α allows its transport from the cytoplasm to the nucleus, where it forms an active HIF-1 complex by binding to HIF-1β. HIF-1β can form heterodimers with other proteins, such as the aryl hydrocarbon receptor [85], and is constitutively and abundantly present [80,86]. Thus, the amount of HIF-1α protein in the nucleus is rate limiting and determines the functional activity of the HIF-1 complex [78]. This was further confirmed by studies who showed that the level of the HIF-1α protein is inversely related to the oxygen tension both in cultured cells [81] and *in vivo* [80].

Besides HIF-1α, also HIF-2α (EPAS-1) and HIF-3α have been described [87,88]. They have been discovered on basis of their cDNA sequence similarity. Most studies focus on HIF-1α, as for most tumors HIF-2α seems to have only a minor functional role in addition to HIF-1α [89]. HIF-3α is largely unstudied, but seems to inhibit HIF-1α activity [90].

During the episode of the research described in this thesis, the exact mechanism of oxygen sensing was elucidated. Earlier on it was believed that cells sense their oxygen concentration through reactive oxygen species, so that stabilization of the HIF-1α protein was thought to be redox induced [91]. Then, it was found that the von Hippel-Lindau tumor-suppressor protein (pVHL), as a subunit of a multiprotein complex that consists E3 ubiquitin ligase activity, is responsible for the proteasomal degradation of HIF-1α [84]. Subsequently, it was shown that pVHL binds to the oxygen dependent domain (ODD) of HIF-1α [92,93,94]. Indeed, either a mutation of pVHL or lack of (part of) the ODD domain in the HIF-1α protein will lead to normoxic overexpression of HIF-1α and HIF-1 activation. However, hypoxia does not disturb binding of pVHL to HIF-1α, thus another mechanism is responsible for oxygen sensing [84]. More recently, prolyl hydroxylase activity emerged as the basic cellular device for oxygen-sensing. In the presence of oxygen, HIF-1α is hydroxylated at the proline residues 402 and/or 564 [95] of the ODD domain, by specific HIF-1α-proline hydroxylases [96,97]. Hereby, also the presence of Fe++ as a co-factor and dioxygen and 2-otoglutarate as co-substrates are required for the prolyl hydroxylation, which facilitates the specific interaction of pVHL to HIF-1α, resulting in proteasomal degradation.

Hypoxia and prognosis

In a large study involving 1539 invasive breast cancer patients, 60% of cases showed some degree of necrosis [98]. In the same study, a marked degree of the non-comedo type of necrosis was positively correlated with increased rates of treatment failure.

Hypoxia influences the proliferation of tumor cells [99], the rate of apoptotic cell death [100,101] and metastasis [102,103,104,105]. HIF-1α expression induced by hypoxia have been demonstrated in tumors *in vivo* and is associated with a poor clinical outcome [102,106,107] (for an overview, see Hockel and Vaupel [108]). In addition, by activating transcription of the vascular endothelial growth factor (VEGF) gene, HIF-1 is considered to be a central initiator of angiogenic activity in tumors [109,110,111,112]. Thus, HIF-1 is a regulator of many processes, which all seem to be correlated to prognosis of cancer patients.

Further, it can be hypothesized that together with the oxygen levels also the concentration of chemotherapeutics diminishes in hypoxic areas, as it cannot be supplied by an adequate blood supply. The proliferation rate in these areas may, as a result of hypoxia, also be lowered. Thus, chemotherapeutic agents may not reach the hypoxic areas in sufficient concentrations and may thereby not adequately affect these tumor cells because of their low proliferation rate. For radiation therapy it is recognized that hypoxia negatively affect its therapeutic effect [113]. This might be due to the lower potential of hypoxic cells to generate radical species.

During the period of this thesis, the prognostic significance of HIF-1 in different human solid tumors was extensively studied as shown in table 2. In general, HIF-1α overexpression is associated with a poor prognosis in many human solid malignancies. These associations are no surprise, and can be explained by the many downstream processes that are activated by HIF-1.

TABLE 2. Tumor types studied for protein expression of HIF-1α, and its relation to prognosis*

Tumor type	No of Patients	Association	Date/Ref
Non-small-cell lung cancer	96	Mortality ↓	2000 [114]
Cervical cancer (early stage)	91	Mortality ↑	2000 [115]
Oligodendroglioma	51	Mortality ↑	2001 [116]
Oropharyngeal squamous cell carcinoma	98	Mortality ↑, radiation resistance	2001 [117]
Non-small-cell lung cancer	108	Mortality ↑ (HIF-2α, HIF-1α)	2001 [118]
Epithelial ovarian cancer	102	Mortality ↑ (only p53 combined)	2001 [119]
Head and neck squamous cell carcinoma	76	Mortality ↓ (surgery)	2002 [120]
Head and neck squamous cell carcinoma	75	Mortality ↑ (chemoradiation)	2002 [121]
Cervical cancer (advanced stage)	42	No significance (radiation therapy)	2002 [122]
Endometrial cancer	81	Mortality ↑	2002 [123]
Breast cancer (lymph node positive)	206	Mortality ↑	2002 [124]
Breast cancer (lymph node negative)	81	Mortality ↑	2003 [125]
Cervical cancer (advanced stage)	78	Mortality ↑ (radiation therapy)	2003 [126]
Colorectal cancer	139	No significance	2003 [127]
Gastrointestinal stromal tumor (stomach)	53	Mortality ↑	2003 [128]
Breast cancer (lymph node positive)	77	Mortality ↑	2004 [129]

* all studies are based upon immunohistochemistry data

Tumor metabolism, pH and the Warburg effect

The physiologic environment of a tumor differs in many ways from the normal tissue. In 1930, Warburg et al [130] discovered that tumors are characterized by the production of lactate (caused by glycolysis) despite the presence of sufficient oxygen,

indicating the importance of cell metabolism in tumor biology. A new impulse was given to this phenomenon by Weber [131], who described the role of key isoenzymes in tumor metabolism. In 1997, Brand et al confirmed the findings of Warburg and proposed that tumors switch to anaerobic glycolysis to overcome the generation of reactive oxygen species, which might otherwise damage DNA replication [132]. Further, it was shown that the switch from oxidative phosphorylation to anaerobic glycolysis indeed parallels the diminishing oxygen gradient [133]. In addition, overexpression of proteins involved in the glycolytic pathway, such as glucose transporter 1 (glut-1) [134,135], phosphoglycerate kinase-1 [136], and pyruvate kinase M were found in several types of cancer. Interestingly, the expression of most of these proteins is regulated by HIF-1 [137].

The above described knowledge cumulated in the use of several metabolic isoenzymes (eg, lactate dehydrogenase) for the prediction of prognosis and monitoring of treatment response [138]. Further, it was shown that the environment of tumors exhibit acidic extracellular pH (pH 6.5-7.0) (the intracellular tumor compartment is kept to nearly neutral) and high levels of lactate dehydrogenase [139]. In combination with hypoxia, this environment promotes angiogenesis, invasion of adjacent tissue and the formation of metastases [140].

Angiogenesis

The normal vasculature is quiescent in healthy adults, with each endothelial cell dividing once about every 10 years. The formation of new blood vessels (angiogenesis) is restricted to embryonic development, wound healing, endometrial proliferation, postlactational mammary gland involution and pregnancy. However, in breast and other types of cancer, angiogenesis is also present and essential for tumor growth and spread of metastases.

Multiple actions together form the process of angiogenesis. After activation of the endothelial cells of an existing mature blood vessel, the surrounding basement membrane is degraded, followed by movement of adjacent endothelial cells into the connective tissue, proliferation of these endothelial cells, formation of tubular structures, and the formation of a network. Ultimately, this will lead to functioning blood vessels. In tumors, however, often leaky, dilated or non-functioning vessels are generated [141].

As postulated by Folkman in 1971 [142], the process of angiogenesis is initiated and regulated by many growth factors and receptors. By binding of growth factors (or ligands) to cognate receptors (large proteins that span the plasma membrane), an intracellular enzymatic function is activated (e.g. tyrosine kinase).

In 1987 basic fibroblast growth factor (bFGF) was the first growth factor which could be linked to angiogenesis [143]. Another important angiogenic factor is VEGF, which was found in 1983 to induce permeability in blood vessels [144], and was shown to be mitogenic for endothelial cells [145]. Also the presence of VEGF receptors 1 and 2 (VEGF-R1, VEGF-R2, formerly known as Flt-1 and Flk-1/KDR, respectively) on activated endothelial cells confirm the importance of VEGF as an angiogenesis stimulating factor. Further, it has been well documented that VEGF itself can be upregulated by HIF-1, because VEGF harbors an HRE located at the transcription start site of the VEGF gene [109,146]. Up to date, many growth factors are known for their angiogenic activity, some are summarized in table 3.

TABLE 3. Some angiogenic growth factors, with mode of activity and receptor couple*

Lesion	Activity	Receptor
Vascular endothelial growth factor (VEGF)	Endothelial mitogen, permeability↑	VEGFR1-2
Placental growth factor (PIGF)	Weak endothelial mitogen	VEGFR1
Basic fibroblast growth factor (bFGF/FGF-2)	Endothelial mitogen, angiogenesis↑	FGFR1-4
Acidic fibroblast growth factor (aFGF/FGF-1)	Endothelial mitogen, angiogenesis↑	FGFR1-4
Fibroblast growth factor 3 (FGF-3/Int-2)	Endothelial mitogen, angiogenesis↑	FGFR1-4
Fibroblast growth factor 4 (FGF-4/Hst/K-FGF)	Endothelial mitogen, angiogenesis↑	FGFR1-4
Transforming growth factor α (TGF-α)	Endothelial mitogen, angiogenesis↑, VEGF↑	EGFR
Epidermal growth factor (EGF)	Weak endothelial mitogen, VEGF↑	EGFR
Hepatocyte growth factor (HGF)	Endothelial mitogen, angiogenesis↑	c-MET
Transforming growth factor β (TGF-β)	angiogenesis↑, VEGF↑	TGF-βR I-III
Tumor necrosis factor α (TNF-α)	Endothelial mitogen, angiogenesis↑, VEGF↑	TNFR-55
Platelet-derived growth factor (PDGF)	Endothelial mitogen and motility, angiogenesis↑	PDGFR
Granulocyte colony-stimulating factor (G-CSF)	Weak endothelial mitogen, angiogenesis↑	G-CSFR
Thymidine phosphorylase (tP)	Angiogenesis↑	Unknown
Angiogenin	Angiogenesis↑	angiogeninR

*Modified from The Basic science of oncology / editors, Tannock and Hill, 3d edition, chapter 9

Most growth factors mentioned in table 2 have, besides angiogenic potential, additional effects. For example, TGF-β exhibits also effects on cell proliferation, differentiation, embryonal development, immune-system modulation, cell-matrix production, and apoptosis [147]. Further, effects of growth factors can be mimicked by oncogenes. In breast cancer this phenomenon has been best studied for HER-2/*neu*, a growth factor receptor of the epidermal growth factor receptor (EGFR) family [148]. Amplifications of the HER-2/*neu* gene (also known as c-*erb*-B2), which are accompanied by protein overexpression, are found in 20%-30% of invasive breast cancers, and in ductal carcinoma *in situ* an even higher proportion of HER-2/*neu* overexpression has been found [149]. HER-2/*neu* constantly stimulates cell growth and angiogenesis via intracellular tyrosine kinase activity, without the necessity of any bound growth factor [150]. Thus, besides many growth factors, also oncogenes can stimulate angiogenesis. In addition to their angiogenic potential, many growth factors (such as bFGF and EGF) also induce increased production of plasminogen activator and collagenases in proliferating endothelial cells [151]. Besides degradation of the local basement membrane for blood vessel growth, such proteases stimulate the process of metastasis.

As described earlier, proliferating tumor cells will outgrow (100-200μm) the original vasculature and, as a result, hypoxia will develop. To sustain tumor growth new blood vessels are generated for delivery of oxygen and supply of nutrients. The beginning of this process is called the angiogenic switch. This switch takes place in an early phase of tumor development and commonly precedes tumor invasion [152]. In breast cancer development, the formation of new blood vessels is witnessed during the preinvasive stage of ductal carcinoma in situ (DCIS) [153]. However, most prominent is the presence of angiogenesis in invasive cancers. In search for quantification methods to assess the rate of intratumoral angiogenesis, different techniques have been developed. Most commonly used is the hotspot (manual) counting method on paraffin embedded tumor sections stained for either CD34, CD31 or Factor VIII. By light microscopy, the observer selects x400 magnification four adjacent fields harboring most microvessels for counting [154]. Alternatively, also fully-automated

methods have been developed to scan and count either selected areas or whole tumor specimens stained for microvessels [155].

In 1991, Weidner et al were the first who demonstrated the prognostic significance of hotspot quantification in breast cancer [156]. Many further studies have since been performed on this topic. The majority of these studies found a positive association between increased microvessel density and poor prognosis, and the negative results of some studies can be attributed to different or poor methodology of counting vessels.

Positron emission tomography

In the 1960s positron emission tomography (PET) came available, and was primarily used for scientific purposes. The basis of PET is the annihilation of a positively charged electron with a negatively charged electron, resulting in the emission of two oppositely directed 511-KeV photons. The positvely charged electron is emitted by intravenously administrated radionuclides. The two antiparallel 511-KeV photons can be detected external to the body by a PET scanner. In this way, (tumor) biologically interesting compounds can be labeled, administrated and visualized. As a result, PET scanning is now widely accepted as both a research and a clinical diagnostic tool [157,158,159].

In oncology, the glucose analogue ^{18}Fluorodeoxyglucose (^{18}FDG) is commonly used as radionuclide. PET scanning, in contrast to conventional imaging, hereby has the ability to image dynamic processes, such as metabolism, *in vivo*. Thus, in the absence of an anatomic substrate, PET scanning may help to differentiate between malignant and benign on basis of differences in metabolic rate. Since ^{18}FDG is a glucose analogue, it follows the same pathway as glucose. It enters the cell via glucose transporters and is phosphorylated by hexokinases in the intracellular compartment. Then, in contrast to glucose, ^{18}FDG-6-phosphate accumulates in the cell because it cannot be further metabolized. Finally, intensity of ^{18}FDG signal reflects the rate of glucose utilization. PET scanning is used for clinical detection and staging of many tumor types, such as brain, head and neck, non-small-cell lung, pancreatic, and colorectal cancer. In these tumor types it is suggested that the rate of signal intensity reflects tumor aggressiveness and parallels clinical behavior.

In breast cancer, PET scanning has been used for tumor detection and staging [160], to obtain long-term prognostic information [161], and to identify tumor response to chemotherapy at an early phase of treatment [162,163,164,165]. Surprisingly, after many years of research, the underlying mechanisms for ^{18}FDG uptake in tumors are still a matter of debate. Further, in breast cancer, the degree of ^{18}FDG uptake seems to be more heterogeneous among different patients in comparison to many other cancers [160].

III AIMS OF THIS THESIS

Despite the improved treatment and survival rates of breast cancer in Western countries, the incidence is still increasing. Therefore, more research is necessary to improve the current understanding of molecular pathogenesis of breast cancer. Ultimately, new prognostic markers and diagnostic tools will help to better indi-

vidualize treatment and guide the development of new therapeutic strategies.

It is known that hypoxia (lack of cellular oxygen) predicts poor prognosis in some cancers and might lead to chemotherapy resistance, but little is known in breast cancer about the role of hypoxia and HIF-1, the master regulator of the cellular hypoxia response, in breast carcinogenesis and clinical behavior of breast cancer.

The aims of this thesis were therefore to explore the presence of HIF-1α during breast carcinogenesis, its prognostic value in breast cancer, and its relationship with known pathobiologic key players of breast cancer and PET scanning results.

In chapter 2, the expression of HIF-1α is described in different stages of breast disease progressing to breast cancer. In chapter 3, we investigated 150 invasive breast cancers for aberrant expression of cell cycle related proteins and their association with levels of HIF-1α. In chapter 4, we studied BCL-6 protein expression in breast cancer in relation with HIF-1α. In chapter 5, we describe the association between HIF-1α and several important growth factors, growth inhibiting factors and their receptors in 45 invasive breast cancers. In chapter 6, we related the protein levels of HIF-1α with long-term survival of 150 breast cancer patients. In chapter 7, we investigated HIF-1α and other proliferation and metabolism related features in relation to signal intensity of ^{18}FDG PET scanning in breast cancer patients.

REFERENCES

1. van Diest PJ. Ductal carcinoma in situ in breast carcinogenesis. J Pathol 1999;187:383-384.
2. Fearon ER and Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990;61:759-767.
3. Dupont WD and Page DL. Risk factors for breast cancer in women with proliferative breast disease. N Engl J Med 1985;312:146-151.
4. Dupont WD, Page DL, Parl FF, Vnencak-Jones CL, Plummer WD, Jr., Rados MS, and Schuyler PA. Long-term risk of breast cancer in women with fibroadenoma. N Engl J Med 1994;331:10-15.
5. Tavassoli FA and Norris HJ. A comparison of the results of long-term follow-up for atypical intraductal hyperplasia and intraductal hyperplasia of the breast. Cancer 1990;65:518-529.
6. Boecker W, Buerger H, Schmitz K, Ellis IA, van Diest PJ, Sinn HP, Geradts J, Diallo R, Poremba C, and Herbst H. Ductal epithelial proliferations of the breast: a biological continuum? Comparative genomic hybridization and high-molecular-weight cytokeratin expression patterns. J Pathol 2001;195:415-421.
7. Wilbur DC and Barrows GH. Estrogen and progesterone receptor and c-erbB-2 oncoprotein analysis in pure in situ breast carcinoma: an immunohistochemical study. Mod Pathol 1993;6:114-120.
8. O'Malley FP, Vnencak-Jones CL, Dupont WD, Parl F, Manning S, and Page DL. p53 mutations are confined to the comedo type ductal carcinoma in situ of the breast. Immunohistochemical and sequencing data. Lab Invest 1994;71:67-72.
9. Weinstat-Saslow D, Merino MJ, Manrow RE, Lawrence JA, Bluth RF, Wittenbel KD, Simpson JF, Page DL, and Steeg PS. Overexpression of cyclin D mRNA distinguishes invasive and in situ breast carcinomas from non-malignant lesions. Nat Med 1995;1:1257-1260.
10. Kim H and Muller WJ. The role of the epidermal growth factor receptor family in mammary tumorigenesis and metastasis. Exp Cell Res 1999;253:78-87.
11. Mommers EC, Page DL, Dupont WD, Schuyler P, Leonhart AM, Baak JP, Meijer CJ, and van Diest PJ. Prognostic value of morphometry in patients with normal breast tissue or usual ductal hyperplasia of the breast. Int J Cancer 2001;95:282-285.
12. Diest PJ and Michalides R. Cell cycle regulators: interactions and their role in diagnosis, prognosis and treatment of cancer. In: Cancer drug discovery and development. Cell cycle inhibitors in cancer therapy. Giordano A, Soprano KJ, eds. Humana Press, Totowa, New Jersey, USA, 2003, p207-252.

13. Henderson BE, Ross RK, Judd HL, Krailo MD, and Pike MC. Do regular ovulatory cycles increase breast cancer risk? Cancer 1985;56:1206-1208.
14. Miki Y, Swensen J, Shattuck-Eidens D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, Ding W, and . A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 1994;266:66-71.
15. Wooster R, Neuhausen SL, Mangion J, Quirk Y, Ford D, Collins N, Nguyen K, Seal S, Tran T, Averill D, and . Localization of a breast cancer susceptibility gene, BRCA2, to chromosome 13q12-13. Science 1994;265:2088-2090.
16. Liaw D, Marsh DJ, Li J, Dahia PL, Wang SI, Zheng Z, Bose S, Call KM, Tsou HC, Peacocke M, Eng C, and Parsons R. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat Genet 1997;16:64-67.
17. Nelen MR, Padberg GW, Peeters EA, Lin AY, van den HB, Frants RR, Coulon V, Goldstein AM, van Reen MM, Easton DF, Eeles RA, Hodgsen S, Mulvihill JJ, Murday VA, Tucker MA, Mariman EC, Starink TM, Ponder BA, Ropers HH, Kremer H, Longy M, and Eng C. Localization of the gene for Cowden disease to chromosome 10q22-23. Nat Genet 1996;13:114-116.
18. Malkin D, Li FP, Strong LC, Fraumeni JF, Jr., Nelson CE, Kim DH, Kassel J, Gryka MA, Bischoff FZ, Tainsky MA, and . Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science 1990;250:1233-1238.
19. Srivastava S, Zou ZQ, Pirollo K, Blattner W, and Chang EH. Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. Nature 1990;348:747-749.
20. Meijers-Heijboer H, van den OA, Klijn J, Wasielewski M, de Snoo A, Oldenburg R, Hollestelle A, Houben M, Crepin E, Veghel-Plandsoen M, Elstrodt F, van Duijn C, Bartels C, Meijers C, Schutte M, McGuffog L, Thompson D, Easton D, Sodha N, Seal S, Barfoot R, Mangion J, Chang-Claude J, Eccles D, Eeles R, Evans DG, Houlston R, Murday V, Narod S, Peretz T, Peto J, Phelan C, Zhang HX, Szabo C, Devilee P, Goldgar D, Futreal PA, Nathanson KL, Weber B, Rahman N, and Stratton MR. Low-penetrance susceptibility to breast cancer due to CHEK2(*)1100delC in noncarriers of BRCA1 or BRCA2 mutations. Nat Genet 2002;31:55-59.
21. Fisher B and Slack NH. Number of lymph nodes examined and the prognosis of breast carcinoma. Surg Gynecol Obstet 1970;131:79-88.
22. Fisher B, Bauer M, Wickerham DL, Redmond CK, Fisher ER, Cruz AB, Foster R, Gardner B, Lerner H, Margolese R, and . Relation of number of positive axillary nodes to the prognosis of patients with primary breast cancer. An NSABP update. Cancer 1983;52:1551-1557.
23. Rosen PP, Saigo PE, Braun DW, Weathers E, Fracchia AA, and Kinne DW. Axillary micro- and macrometastases in breast cancer: prognostic significance of tumor size. Ann Surg 1981;194:585-591.
24. McGuire WL and Clark GM. Prognostic factors and treatment decisions in axillary-node-negative breast cancer. N Engl J Med 1992;326:1756-1761.
25. Rosen PR, Groshen S, Saigo PE, Kinne DW, and Hellman S. A long-term follow-up study of survival in stage I (T1N0M0) and stage II (T1N1M0) breast carcinoma. J Clin Oncol 1989;7:355-366.
26. Sigurdsson H, Baldetorp B, Borg A, Dalberg M, Ferno M, Killander D, and Olsson H. Indicators of prognosis in node-negative breast cancer. N Engl J Med 1990;322:1045-1053.
27. Elston CW and Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991;19:403-410.
28. World Health Organization. Histological typing of breast tumors. Tumori 1982;68:181-198.
29. Wiedswang G, Borgen E, Karesen R, Kvalheim G, Nesland JM, Qvist H, Schlichting E, Sauer T, Janbu J, Harbitz T, and Naume B. Detection of isolated tumor cells in bone marrow is an independent prognostic factor in breast cancer. J Clin Oncol 2003;21:3469-3478.
30. Weidner N, Folkman J, Pozza F, Bevilacqua P, Allred EN, Moore DH, Meli S, and Gasparini G. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. J Natl Cancer Inst 1992;84:1875-1887.
31. Look MP, van Putten WL, Duffy MJ, Harbeck N, Christensen IJ, Thomssen C, Kates R, Spyratos F, Ferno M, Eppenberger-Castori S, Sweep CG, Ulm K, Peyrat JP, Martin PM, Magdelenat H, Brunner N, Duggan C, Lisboa BW, Bendahl PO, Quillien V, Daver A, Ricolleau G, Meijer-van Gelder ME, Manders P, Fiets WE, Blankenstein MA, Broet P, Romain S, Daxenbichler G, Windbichler G, Cufer T, Borstnar S, Kueng W, Beex LV, Klijn JG, O'Higgins N, Eppenberger U, Janicke F, Schmitt M, and Foekens JA. Pooled analysis of prognostic impact of urokinase-type plasminogen activator and its inhibitor PAI-1 in 8377 breast cancer patients. J Natl Cancer Inst 2002;94:116-128.
32. Zemzoum I, Kates RE, Ross JS, Dettmar P, Dutta M, Henrichs C, Yurdseven S, Hofler H, Kiechle M,

Schmitt M, and Harbeck N. Invasion factors uPA/PAI-1 and HER2 status provide independent and complementary information on patient outcome in node-negative breast cancer. J Clin Oncol 2003;21:1022-1028.
33. 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der KK, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, and Friend SH. Gene expression profiling predicts clinical outcome of breast cancer. Nature 2002;415:530-536.
34. Perou CM, Sorlie T, Eisen MB, van de RM, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, and Botstein D. Molecular portraits of human breast tumours. Nature 2000;406:747-752.
35. McGuire WL. Hormone receptors: their role in predicting prognosis and response to endocrine therapy. Semin Oncol 1978;5:428-433.
36. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, and . Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 1989;244:707-712.
37. Toikkanen S, Helin H, Isola J, and Joensuu H. Prognostic significance of HER-2 oncoprotein expression in breast cancer: a 30-year follow-up. J Clin Oncol 1992;10:1044-1048.
38. Harris AL, Nicholson S, Sainsbury JR, Farndon J, and Wright C. Epidermal growth factor receptors in breast cancer: association with early relapse and death, poor response to hormones and interactions with neu. J Steroid Biochem 1989;34:123-131.
39. Pegram MD, Pauletti G, and Slamon DJ. HER-2/neu as a predictive marker of response to breast cancer therapy. Breast Cancer Res Treat 1998;52:65-77.
40. Huang E, Cheng SH, Dressman H, Pittman J, Tsou MH, Horng CF, Bild A, Iversen ES, Liao M, Chen CM, West M, Nevins JR, and Huang AT. Gene expression predictors of breast cancer outcomes. Lancet 2003;361:1590-1596.
41. van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, Witteveen A, Glas A, Delahaye L, van d, V, Bartelink H, Rodenhuis S, Rutgers ET, Friend SH, and Bernards R. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 2002;347:1999-2009.
42. Otto SJ, Fracheboud J, Looman CW, Broeders MJ, Boer R, Hendriks JH, Verbeek AL, and de Koning HJ. Initiation of population-based mammography screening in Dutch municipalities and effect on breast-cancer mortality: a systematic review. Lancet 2003;361:1411-1417.
43. Anon. Polychemotherapy for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. Lancet 1998;352:930-942.
44. Heichman KA and Roberts JM. Rules to replicate by. Cell 1994;79:557-562.
45. Elledge SJ. Cell cycle checkpoints: preventing an identity crisis. Science 1996;274:1664-1672.
46. Altucci L, Addeo R, Cicatiello L, Dauvois S, Parker MG, Truss M, Beato M, Sica V, Bresciani F, and Weisz A. 17beta-Estradiol induces cyclin D1 gene transcription, p36D1-p34cdk4 complex activation and p105Rb phosphorylation during mitogenic stimulation of G(1)-arrested human breast cancer cells. Oncogene 1996;12:2315-2324.
47. Sherr CJ. Cancer cell cycles. Science 1996;274:1672-1677.
48. Mommers EC, van Diest PJ, Leonhart AM, Meijer CJ, and Baak JP. Expression of proliferation and apoptosis-related proteins in usual ductal hyperplasia of the breast. Hum Pathol 1998;29:1539-1545.
49. Mommers EC, Leonhart AM, Falix F, Michalides R, Meijer CJ, Baak JP, and Diest PJ. Similarity in expression of cell cycle proteins between in situ and invasive ductal breast lesions of same differentiation grade. J Pathol 2001;194:327-333.
50. Gillett C, Fantl V, Smith R, Fisher C, Bartek J, Dickson C, Barnes D, and Peters G. Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. Cancer Res 1994;54:1812-1817.
51. Bartkova J, Lukas J, Muller H, Lutzhoft D, Strauss M, and Bartek J. Cyclin D1 protein expression and function in human breast cancer. Int J Cancer 1994;57:353-361.
52. Wang TC, Cardiff RD, Zukerberg L, Lees E, Arnold A, and Schmidt EV. Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. Nature 1994;369:669-671.
53. Shvarts A, Brummelkamp TR, Scheeren F, Koh E, Daley GQ, Spits H, and Bernards R. A senescence rescue screen identifies BCL6 as an inhibitor of anti- proliferative p19(ARF)-p53 signaling. Genes Dev 2002;16:681-686.
54. Chang CC, Ye BH, Chaganti RS, and Dalla-Favera R. BCL-6, a POZ/zinc-finger protein, is a sequence-specific transcriptional repressor. Proc Natl Acad Sci U S A 1996;93:6947-6952.
55. Osborne RJ, Merlo GR, Mitsudomi T, Venesio T, Liscia DS, Cappa AP, Chiba I, Takahashi T, Nau MM,

Callahan R, and . Mutations in the p53 gene in primary human breast cancers. Cancer Res 1991;51:6194-6198.
56. Malkin D. p53 and the Li-Fraumeni syndrome. Biochim Biophys Acta 1994;1198:197-213.
57. Knudson AG, Jr. Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A 1971;68:820-823.
58. Goodrich DW and Lee WH. Molecular characterization of the retinoblastoma susceptibility gene. Biochim Biophys Acta 1993;1155:43-61.
59. Goodrich DW, Wang NP, Qian YW, Lee EY, and Lee WH. The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. Cell 1991;67:293-302.
60. Borg A, Zhang QX, Alm P, Olsson H, and Sellberg G. The retinoblastoma gene in breast cancer: allele loss is not correlated with loss of gene protein expression. Cancer Res 1992;52:2991-2994.
61. Varley JM, Armour J, Swallow JE, Jeffreys AJ, Ponder BA, T'Ang A, Fung YK, Brammar WJ, and Walker RA. The retinoblastoma gene is frequently altered leading to loss of expression in primary breast tumours. Oncogene 1989;4:725-729.
62. Graeber TG, Peterson JF, Tsai M, Monica K, Fornace AJ, Jr., and Giaccia AJ. Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status. Mol Cell Biol 1994;14:6264-6277.
63. Ludlow JW, Howell RL, and Smith HC. Hypoxic stress induces reversible hypophosphorylation of pRB and reduction in cyclin A abundance independent of cell cycle progression. Oncogene 1993;8:331-339.
64. Weng LP, Smith WM, Dahia PL, Ziebold U, Gil E, Lees JA, and Eng C. PTEN suppresses breast cancer cell growth by phosphatase activity-dependent G1 arrest followed by cell death. Cancer Res 1999;59:5808-5814.
65. Fidler IJ and Ellis LM. The implications of angiogenesis for the biology and therapy of cancer metastasis. Cell 1994;79:185-188.
66. Helmlinger G, Yuan F, Dellian M, and Jain RK. Interstitial pH and pO2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. Nat Med 1997;3:177-182.
67. Tannock IF. Population kinetics of carcinoma cells, capillary endothelial cells, and fibroblasts in a transplanted mouse mammary tumor. Cancer Res 1970;30:2470-2476.
68. Chaplin DJ and Hill SA. Temporal heterogeneity in microregional erythrocyte flux in experimental solid tumours. Br J Cancer 1995;71:1210-1213.
69. Trotter MJ, Chaplin DJ, Durand RE, and Olive PL. The use of fluorescent probes to identify regions of transient perfusion in murine tumors. Int J Radiat Oncol Biol Phys 1989;16:931-934.
70. Chaplin DJ, Durand RE, and Olive PL. Acute hypoxia in tumors: implications for modifiers of radiation effects. Int J Radiat Oncol Biol Phys 1986;12:1279-1282.
71. Vaupel P, Schlenger K, Knoop C, and Hockel M. Oxygenation of human tumors: evaluation of tissue oxygen distribution in breast cancers by computerized O2 tension measurements. Cancer Res 1991;51:3316-3322.
72. Teicher BA. Hypoxia and drug resistance. Cancer Metastasis Rev 1994;13:139-168.
73. Semenza GL. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol 2000;88:1474-1480.
74. Semenza GL and Wang GL. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 1992;12:5447-5454.
75. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, and Simons JW. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 1999;59:5830-5835.
76. Jiang BH, Rue E, Wang GL, Roe R, and Semenza GL. Dimerization, DNA binding, and transactivation properties of hypoxia- inducible factor 1. J Biol Chem 1996;271:17771-17778.
77. Wang GL, Jiang BH, Rue EA, and Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A 1995;92:5510-5514.
78. Semenza GL. Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 1999;15:551-578.
79. Ratcliffe PJ, O'Rourke JF, Maxwell PH, and Pugh CW. Oxygen sensing, hypoxia-inducible factor-1 and the regulation of mammalian gene expression. J Exp Biol 1998;201:1153-1162.
80. Huang LE, Arany Z, Livingston DM, and Bunn HF. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. J Biol Chem 1996;271:32253-32259.
81. Jiang BH, Semenza GL, Bauer C, and Marti HH. Hypoxia-inducible factor 1 levels vary exponentially over

a physiologically relevant range of O2 tension. Am J Physiol 1996;271:C1172-C1180.
82. Sutter CH, Laughner E, and Semenza GL. Hypoxia-inducible factor 1alpha protein expression is controlled by oxygen-regulated ubiquitination that is disrupted by deletions and missense mutations. Proc Natl Acad Sci U S A 2000;97:4748-4753.
83. Ravi R, Mookerjee B, Bhujwalla ZM, Sutter CH, Artemov D, Zeng Q, Dillehay LE, Madan A, Semenza GL, and Bedi A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia- inducible factor 1alpha. Genes Dev 2000;14:34-44.
84. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, and Ratcliffe PJ. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 1999;399:271-275.
85. Reyes H, Reisz-Porszasz S, and Hankinson O. Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. Science 1992;256:1193-1195.
86. Martin C, Yu AY, Jiang BH, Davis L, Kimberly D, Hohimer AR, and Semenza GL. Cardiac hypertrophy in chronically anemic fetal sheep: Increased vascularization is associated with increased myocardial expression of vascular endothelial growth factor and hypoxia-inducible factor 1. Am J Obstet Gynecol 1998;178:527-534.
87. Gu YZ, Moran SM, Hogenesch JB, Wartman L, and Bradfield CA. Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. Gene Expr 1998;7:205-213.
88. Tian H, McKnight SL, and Russell DW. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev 1997;11:72-82.
89. Maranchie JK, Vasselli JR, Riss J, Bonifacino JS, Linehan WM, and Klausner RD. The contribution of VHL substrate binding and HIF1-alpha to the phenotype of VHL loss in renal cell carcinoma. Cancer Cell 2002;1:247-255.
90. Makino Y, Kanopka A, Wilson WJ, Tanaka H, and Poellinger L. Inhibitory PAS domain protein (IPAS) is a hypoxia-inducible splicing variant of the hypoxia-inducible factor-3alpha locus. J Biol Chem 2002;277:32405-32408.
91. Salceda S and Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem 1997;272:22642-22647.
92. Cockman ME, Masson N, Mole DR, Jaakkola P, Chang GW, Clifford SC, Maher ER, Pugh CW, Ratcliffe PJ, and Maxwell PH. Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. J Biol Chem 2000;
93. Ohh M, Park CW, Ivan M, Hoffman MA, Kim TY, Huang LE, Pavletich N, Chau V, and Kaelin WG. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. Nat Cell Biol 2000;2:423-427.
94. Tanimoto K, Makino Y, Pereira T, and Poellinger L. Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von Hippel-Lindau tumor suppressor protein. EMBO J 2000;19:4298-4309.
95. Masson N, Willam C, Maxwell PH, Pugh CW, and Ratcliffe PJ. Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. EMBO J 2001;20:5197-5206.
96. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, and Kaelin WG, Jr. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 2001;292:464-468.
97. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, and Ratcliffe PJ. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science 2001;292:468-472.
98. Fisher ER, Palekar AS, Gregorio RM, Redmond C, and Fisher B. Pathological findings from the national surgical adjuvant breast project (Protocol No. 4). IV. Significance of tumor necrosis. Hum Pathol 1978;9:523-530.
99. Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshert E, and Keshet E. Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature 1998;394:485-490.
100. Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, and Giaccia AJ. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 1996;379:88-91.
101. Kim CY, Tsai MH, Osmanian C, Graeber TG, Lee JE, Giffard RG, DiPaolo JA, Peehl DM, and Giaccia AJ.

Selection of human cervical epithelial cells that possess reduced apoptotic potential to low-oxygen conditions. Cancer Res 1997;57:4200-4204.
102. Brizel DM, Scully SP, Harrelson JM, Layfield LJ, Bean JM, Prosnitz LR, and Dewhirst MW. Tumor oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. Cancer Res 1996;56:941-943.
103. De Jaeger K, Kavanagh MC, and Hill RP. Relationship of hypoxia to metastatic ability in rodent tumours. Br J Cancer 2001;84:1280-1285.
104. Rofstad EK and Danielsen T. Hypoxia-induced metastasis of human melanoma cells: involvement of vascular endothelial growth factor-mediated angiogenesis. Br J Cancer 1999;80:1697-1707.
105. Young SD, Marshall RS, and Hill RP. Hypoxia induces DNA overreplication and enhances metastatic potential of murine tumor cells. Proc Natl Acad Sci U S A 1988;85:9533-9537.
106. Nordsmark M, Overgaard M, and Overgaard J. Pretreatment oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. Radiother Oncol 1996;41:31-39.
107. Hockel M, Schlenger K, Knoop C, and Vaupel P. Oxygenation of carcinomas of the uterine cervix: evaluation by computerized O2 tension measurements. Cancer Res 1991;51:6098-6102.
108. Hockel M and Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 2001;93:266-276.
109. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, and Semenza GL. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 1996;16:4604-4613.
110. Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, and Semenza GL. Cellular and developmental control of O2 homeostasis by hypoxia- inducible factor 1 alpha. Genes Dev 1998;12:149-162.
111. Plate KH, Breier G, Weich HA, and Risau W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. Nature 1992;359:845-848.
112. Ryan HE, Lo J, and Johnson RS. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. EMBO J 1998;17:3005-3015.
113. Gatenby RA, Kessler HB, Rosenblum JS, Coia LR, Moldofsky PJ, Hartz WH, and Broder GJ. Oxygen distribution in squamous cell carcinoma metastases and its relationship to outcome of radiation therapy. Int J Radiat Oncol Biol Phys 1988;14:831-838.
114. Volm M and Koomagi R. Hypoxia-inducible factor (HIF-1) and its relationship to apoptosis and proliferation in lung cancer. Anticancer Res 2000;20:1527-1533.
115. Birner P, Schindl M, Obermair A, Plank C, Breitenecker G, and Oberhuber G. Overexpression of hypoxia-inducible factor 1alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. Cancer Res 2000;60:4693-4696.
116. Birner P, Gatterbauer B, Oberhuber G, Schindl M, Rossler K, Prodinger A, Budka H, and Hainfellner JA. Expression of hypoxia-inducible factor-1 alpha in oligodendrogliomas: its impact on prognosis and on neoangiogenesis. Cancer 2001;92:165-171.
117. Aebersold DM, Burri P, Beer KT, Laissue J, Djonov V, Greiner RH, and Semenza GL. Expression of hypoxia-inducible factor-1alpha: a novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer. Cancer Res 2001;61:2911-2916.
118. Giatromanolaki A, Koukourakis MI, Sivridis E, Turley H, Talks K, Pezzella F, Gatter KC, and Harris AL. Relation of hypoxia inducible factor 1 alpha and 2 alpha in operable non-small cell lung cancer to angiogenic/molecular profile of tumours and survival. Br J Cancer 2001;85:881-890.
119. Birner P, Schindl M, Obermair A, Breitenecker G, and Oberhuber G. Expression of hypoxia-inducible factor 1alpha in epithelial ovarian tumors: its impact on prognosis and on response to chemotherapy. Clin Cancer Res 2001;7:1661-1668.
120. Beasley NJ, Leek R, Alam M, Turley H, Cox GJ, Gatter K, Millard P, Fuggle S, and Harris AL. Hypoxia-inducible factors HIF-1alpha and HIF-2alpha in head and neck cancer: relationship to tumor biology and treatment outcome in surgically resected patients. Cancer Res 2002;62:2493-2497.
121. Koukourakis MI, Giatromanolaki A, Sivridis E, Simopoulos C, Turley H, Talks K, Gatter KC, and Harris AL. Hypoxia-inducible factor (HIF1A and HIF2A), angiogenesis, and chemoradiotherapy outcome of squamous cell head-and-neck cancer. Int J Radiat Oncol Biol Phys 2002;53:1192-1202.
122. Haugland HK, Vukovic V, Pintilie M, Fyles AW, Milosevic M, Hill RP, and Hedley DW. Expression of hypoxia-inducible factor-1alpha in cervical carcinomas: correlation with tumor oxygenation. Int J Radiat Oncol Biol Phys 2002;53:854-861.
123. Sivridis E, Giatromanolaki A, Gatter KC, Harris AL, and Koukourakis MI. Association of hypoxia-inducible factors 1alpha and 2alpha with activated angiogenic pathways and prognosis in patients with

endometrial carcinoma. Cancer 2002;95:1055-1063.
124. Schindl M, Schoppmann SF, Samonigg H, Hausmaninger H, Kwasny W, Gnant M, Jakesz R, Kubista E, Birner P, and Oberhuber G. Overexpression of Hypoxia-inducible Factor 1alpha Is Associated with an Unfavorable Prognosis in Lymph Node-positive Breast Cancer. Clin Cancer Res 2002;8:1831-1837.
125. Bos R, van der Groep P, Greijer AE, Shvarts A, Meijer S, Pinedo HM, Semenza GL, van Diest PJ, and van der Wall E. Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. Cancer 2003;97:1573-1581.
126. Burri P, Djonov V, Aebersold DM, Lindel K, Studer U, Altermatt HJ, Mazzucchelli L, Greiner RH, and Gruber G. Significant correlation of hypoxia-inducible factor-1alpha with treatment outcome in cervical cancer treated with radical radiotherapy. Int J Radiat Oncol Biol Phys 2003;56:494-501.
127. Kuwai T, Kitadai Y, Tanaka S, Onogawa S, Matsutani N, Kaio E, Ito M, and Chayama K. Expression of hypoxia-inducible factor-1alpha is associated with tumor vascularization in human colorectal carcinoma. Int J Cancer 2003;105:176-181.
128. Takahashi R, Tanaka S, Hiyama T, Ito M, Kitadai Y, Sumii M, Haruma K, and Chayama K. Hypoxia-inducible factor-1alpha expression and angiogenesis in gastrointestinal stromal tumor of the stomach. Oncol Rep 2003;10:797-802.
129. Gruber G, Greiner RH, Hlushchuk R, Aebersold DM, Altermatt HJ, Berclaz G, and Djonov V. Hypoxia-inducible factor 1 alpha in high-risk breast cancer: an independent prognostic parameter? Breast Cancer Res 2004;6:R191-R198.
130. Warburg O, Wind F, and Negalein E. The metabolism of tumours in the body. J Physiol 1927;8:519-530.
131. Weber G. Enzymology of cancer cells (first of two parts). N Engl J Med 1977;296:486-492.
132. Brand KA and Hermfisse U. Aerobic glycolysis by proliferating cells: a protective strategy against reactive oxygen species. FASEB J 1997;11:388-395.
133. Walenta S, Snyder S, Haroon ZA, Braun RD, Amin K, Brizel D, Mueller-Klieser W, Chance B, and Dewhirst MW. Tissue gradients of energy metabolites mirror oxygen tension gradients in a rat mammary carcinoma model. Int J Radiat Oncol Biol Phys 2001;51:840-848.
134. Hatanaka M. Transport of sugars in tumor cell membranes. Biochim Biophys Acta 1974;355:77-104.
135. Younes M, Lechago LV, Somoano JR, Mosharaf M, and Lechago J. Wide expression of the human erythrocyte glucose transporter Glut1 in human cancers. Cancer Res 1996;56:1164-1167.
136. Firth JD, Ebert BL, Pugh CW, and Ratcliffe PJ. Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: similarities with the erythropoietin 3' enhancer. Proc Natl Acad Sci U S A 1994;91:6496-6500.
137. Semenza GL, Roth PH, Fang HM, and Wang GL. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem 1994;269:23757-23763.
138. Csako G, Magrath IT, and Elin RJ. Serum total and isoenzyme lactate dehydrogenase activity in American Burkitt's lymphoma patients. Am J Clin Pathol 1982;78:712-717.
139. Sagman U, Feld R, Evans WK, Warr D, Shepherd FA, Payne D, Pringle J, Yeoh J, DeBoer G, Malkin A, and . The prognostic significance of pretreatment serum lactate dehydrogenase in patients with small-cell lung cancer. J Clin Oncol 1991;9:954-961.
140. Tannock IF and Rotin D. Acid pH in tumors and its potential for therapeutic exploitation. Cancer Res 1989;49:4373-4384.
141. Kimura H, Braun RD, Ong ET, Hsu R, Secomb TW, Papahadjopoulos D, Hong K, and Dewhirst MW. Fluctuations in red cell flux in tumor microvessels can lead to transient hypoxia and reoxygenation in tumor parenchyma. Cancer Res 1996;56:5522-5528.
142. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971;285:1182-1186.
143. Folkman J and Klagsbrun M. Angiogenic factors. Science 1987;235:442-447.
144. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, and Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 1983;219:983-985.
145. Gospodarowicz D, Abraham JA, and Schilling J. Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. Proc Natl Acad Sci U S A 1989;86:7311-7315.
146. Liu Y, Cox SR, Morita T, and Kourembanas S. Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer. Circ Res 1995;77:638-643.
147. Massague J. The transforming growth factor-beta family. Annu Rev Cell Biol 1990;6:597-641.
148. Menard S, Tagliabue E, Campiglio M, and Pupa SM. Role of HER2 gene overexpression in breast carcinoma. J Cell Physiol 2000;182:150-162.
149. Paik S, Hazan R, Fisher ER, Sass RE, Fisher B, Redmond C, Schlessinger J, Lippman ME, and King CR. Pathologic findings from the National Surgical Adjuvant Breast and Bowel Project: prognostic

significance of erbB-2 protein overexpression in primary breast cancer. J Clin Oncol 1990;8:103-112.
150. Peles E and Yarden Y. Neu and its ligands: from an oncogene to neural factors. Bioessays 1993;15:815-824.
151. Kalebic T, Garbisa S, Glaser B, and Liotta LA. Basement membrane collagen: degradation by migrating endothelial cells. Science 1983;221:281-283.
152. Hanahan D and Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 1996;86:353-364.
153. Guidi AJ, Fischer L, Harris JR, and Schnitt SJ. Microvessel density and distribution in ductal carcinoma in situ of the breast. J Natl Cancer Inst 1994;86:614-619.
154. Vermeulen PB, Gasparini G, Fox SB, Toi M, Martin L, McCulloch P, Pezzella F, Viale G, Weidner N, Harris AL, and Dirix LY. Quantification of angiogenesis in solid human tumours: an international consensus on the methodology and criteria of evaluation. Eur J Cancer 1996;32A:2474-2484.
155. Belien JA, Somi S, De Jong JS, van Diest PJ, and Baak JP. Fully automated microvessel counting and hot spot selection by image processing of whole tumour sections in invasive breast cancer. J Clin Pathol 1999;52:184-192.
156. Weidner N, Semple JP, Welch WR, and Folkman J. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. N Engl J Med 1991;324:1-8.
157. Flanagan FL, Dehdashti F, and Siegel BA. PET in breast cancer. Semin Nucl Med 1998;28:290-302.
158. Fowler JS, Volkow ND, Wang GJ, Ding YS, and Dewey SL. PET and drug research and development. J Nucl Med 1999;40:1154-1163.
159. Oehr P, Ruhlmann J, and Biersack HJ. FDG-PET in clinical oncology: review of the literature and report of one institution's experience. J Investig Med 1999;47:452-461.
160. Avril N, Rose CA, Schelling M, Dose J, Kuhn W, Bense S, Weber W, Ziegler S, Graeff H, and Schwaiger M. Breast imaging with positron emission tomography and fluorine-18 fluorodeoxyglucose: use and limitations. J Clin Oncol 2000;18:3495-3502.
161. Oshida M, Uno K, Suzuki M, Nagashima T, Hashimoto H, Yagata H, Shishikura T, Imazeki K, and Nakajima N. Predicting the prognoses of breast carcinoma patients with positron emission tomography using 2-deoxy-2-fluoro[18F]-D-glucose. Cancer 1998;82:2227-2234.
162. Jansson T, Westlin JE, Ahlstrom H, Lilja A, Langstrom B, and Bergh J. Positron emission tomography studies in patients with locally advanced and/or metastatic breast cancer: a method for early therapy evaluation? J Clin Oncol 1995;13:1470-1477.
163. Schelling M, Avril N, Nahrig J, Kuhn W, Romer W, Sattler D, Werner M, Dose J, Janicke F, Graeff H, and Schwaiger M. Positron emission tomography using 18F-Fluorodeoxyglucose for monitoring primary chemotherapy in breast cancer. J Clin Oncol 2000;18:1689-1695.
164. Smith IC, Welch AE, Hutcheon AW, Miller ID, Payne S, Chilcott F, Waikar S, Whitaker T, Ah-See AK, Eremin O, Heys SD, Gilbert FJ, and Sharp PF. Positron emission tomography using 18F-fluorodeoxy-D-glucose to predict the pathologic response of breast cancer to primary chemotherapy. J Clin Oncol 2000;18:1676-1688.
165. Wahl RL, Zasadny K, Helvie M, Hutchins GD, Weber B, and Cody R. Metabolic monitoring of breast cancer chemohormonotherapy using positron emission tomography: initial evaluation. J Clin Oncol 1993;11:2101-2111.

Chapter 2

Levels of Hypoxia-Inducible Factor-1α During Breast Carcinogenesis

J Natl Cancer Inst 2001;93:309-14

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BACKGROUND. Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that regulates gene expression in critical pathways involved in tumor growth and metastases. In this report, we investigated whether the level of HIF-1α is increased during carcinogenesis in breast tissue and is associated with other tumor biomarkers.

METHODS. Paraffin-embedded clinical specimens from five pathologic stages of breast tumorigenesis and from normal breast tissue were used. HIF-1α protein and the biomarkers vascular endothelial growth factor (VEGF), HER-2/neu, p53, Ki-67, and estrogen receptor (ER) were identified immunohistochemically, and microvessel density (a measure of angiogenesis) was determined. Associations among levels of HIF-1α and these biomarkers were tested statistically. All statistical test are two-sided.

RESULTS. The frequency of HIF-1α positive cells in a specimen increased with the specimen's pathologic stage (P<0.001, χ^2 test for trend) as follows: normal breast tissue (0 specimens with $\geq$1% HIF-1α-positive cells in 10 specimens tested), ductal hyperplastic lesions (0 in 10), well-differentiated ductal carcinomas *in situ* (DCIS) (11 in 20), well-differentiated invasive breast cancers (12 in 20), poorly differentiated DCIS (17 in 20), and poorly differentiated invasive carcinomas (20 in 20). Increased levels of HIF-1α were statistically significantly associated with high proliferation and increased expression of VEGF and ER proteins. In DCIS lesions, increased levels of HIF-1α were statistically significantly associated with increased microvessel density. HIF-1α showed a borderline association with HER-2/neu but no association with p53.

CONCLUSIONS. The level of HIF-1α increases as the pathologic stage increases and is higher in poorly differentiated lesions than in the corresponding type of well-differentiated lesions. Increased levels of HIF-1α are associated with increased proliferation and increased expression of ER and VEGF. Thus, increased levels of HIF-1α are potentially associated with more aggressive tumors.

THE ROLE OF hypoxia-inducible factor-1α (HIF-1α) is under increasing scrutiny by cancer researchers [1]. HIF-1 binds the consensus sequence 5'-RCGTG.3' (where R is any purine) in the hypoxia-response elements of various target genes [2]. HIF-1 activates the transcription of many genes controlling glucose transporters, glycolytic enzymes, gluconeogenesis, high-energy phosphate metabolism, growth factors, erythropoiesis, heme metabolism, iron transport, vasomotor regulation and nitric oxide synthesis [2,3] and, thus, may increase the survival of tumor cells under hypoxic conditions. Hypoxia influences the proliferation of tumor cells [4], the rate of apoptotic cell death [5] and metastasis [6]. In addition, by activating transcription of the vascular endothelial growth factor (VEGF) gene, HIF-1 is considered to be a central initiator of angiogenic activity in tumors [7,8,9,10].

The level of HIF-1α in cells is dependent on the intracellular oxygen concentration [11,12]. When cells have normal concentrations of oxygen, HIF-1α protein is continuously degraded via the ubiquitin pathway. However, under low concentrations of oxygen (hypoxic conditions), the ubiquitination of HIF-1α is blocked, the protein is stabilized [11,13], and thus the protein's intracellular levels increase. Although the exact mechanism of oxygen sensing remains to be elucidated, the cell probably senses its oxygen concentration through reactive oxygen species, so that stabilization of the HIF-1α protein is said to be redox induced [14].

A nuclear localization signal at the C-terminal end of HIF-1α allows its transport from the cytoplasm to the nucleus, where it forms an active HIF-1 complex by binding to HIF-1β. HIF-1β can form heterodimers with other proteins, such as the aryl hydrocarbon receptor [15], and is constitutively and abundantly present [11,16]. Thus, the amount of HIF-1α protein in the nucleus is rate limiting and determines the functional activity of the HIF-1 complex [2].

The level of the HIF-1α protein is inversely related to the oxygen tension in cultured cells [12] and in vivo [11]. Hypoxic oxygen tensions that induce HIF-1α expression have been demonstrated in tumors in vivo and are associated with a poor clinical outcome [6,17,18]. HIF-1 activity also is correlated with tumor progression and angiogenesis in xenograft assays [19,20,21]. These observations and the fact that increased levels of HIF-1α are found in many common human cancers at diagnosis [1,2], suggest that HIF-1 has an important role in cancer progression [22].

Since the discovery of HIF-1 [23], the characteristics of HIF-1 have been explored in many studies many studies using cultured cell lines. In this study, we focused on the role of HIF-1α in human breast carcinogenesis. Because angiogenesis is necessary for hyperplastic epithelial cells to progress to malignant cells [24] and because HIF-1 may induce angiogenesis by activating transcription of the VEGF gene, we propose that HIF-1 plays a role in breast carcinogenesis. This hypothesis is supported by previous findings that VEGF, one of the most potent molecules in angiogenesis, is increased in ductal carcinoma in situ (DCIS) [25]. We tested this hypothesis by evaluating the levels of HIF-1α in normal breast tissue and in different stages of breast cancer development (usual ductal hyperplasia, DCIS and invasive breast cancer) and by determining whether the level of HIF-1α was associated with proliferation (Ki-67), microvessel formation, and/or the expression of VEGF, HER-2/neu, p53 and the estrogen receptor (ER).

MATERIALS AND METHODS

The level of HIF-1 was examined in randomly selected samples of breast tissue from patients. These samples had been deposited in the breast cancer tumor banks of the pathology departments of the Free University Hospital, Amsterdam, The Netherlands, and the Gooi-Noord Hospital, Blaricum, The Netherlands. Normal breast tissue was from reduction mammoplasties performed on patients without proliferative breast disease. Specimens of pure ductal hyperplasias, pure DCIS, and invasive carcinoma were obtained from excision biopsy procedures or mastectomies. None of the patients with invasive breast cancer had received any preoperative therapy. All specimens were fixed in neutral 4% buffered formaldehyde. Informed consent to anonymously use leftover patient material for scientific purposes was a standard item in the treatment contract with the patients.

We examined normal breast tissue (from 10 patients), usual ductal hyperplasia (from 10 patients), well-differentiated DCIS (from 20 patients), poorly-differentiated DCIS (from 20 patients), invasive carcinoma grade 1 (from 20 patients) and invasive carcinoma grade 3 (from 20 patients). Grading of DCIS and invasive cancer was done according to the procedure of Holland et al [26] and Elston and Ellis [27], respectively. The grading pathologist (P.J. van Diest) was blinded in scoring all specimens for HIF-1α with respect to other biomarkers.

Immunohistochemistry

Table 1 presents all antibodies, dilutions, incubation times, and antigen retrieval methods used. Immunohistochemistry was performed on 4-μm-thick slides.

Table 1. Used antibodies, dilution, incubation and detection methods used*

Antibody	Species	Company	Dilution	Incubation time	Antigen-retrieval method	Detection
HIF-1α	Mouse MAb	Abcam	1:1000	30 min, 20°C	WB, TRS, 95 °C, 45 min	CSA
CD31	Mouse MAb	DAKO	1:40	o/n, 4 °C	MW, Citr., 95 °C, 10 min	ABC
VEGF	Goat poly	R&D	1:50	o/n, 4 °C	MW, Citr., 95 °C, 10 min	ABC
Ki-67	Mouse MAb	Immunotech	1:40	o/n, 4 °C	MW, Citr., 95 °C, 10 min	ABC
HER-2/*neu*	Mouse MAb	MvdVijver	1:10.000	o/n, 4 °C	None	ABC
p53	Mouse MAb	DAKO	1:50	o/n, 4 °C	MW, Citr., 95 °C, 10 min	ABC
ER	Mouse MAb	DAKO	1:50	o/n, 4 °C	MW, Citr., 95 °C, 10 min	ABC

*HIF-1α = hypoxia-inducible factor-1α; VEGF = endothelial growth factor; ER = estrogen receptor; MAb = monoclonal antibody; poly = polyclonal antibody; Abcam = Abcam Cambridge, U.K.; DAKO = DAKO, Glostrup, Denmark; R&D = R&D systems, Abingdon, U.K.; Immunotech = Immunotech SA, Marseille, France; MvdVijver = Dr. Marc van de Vijver, Dutch Cancer Institute Amsterdam, The Netherlands; o/n = overnight; WB = waterbath; TRS = target retrieval solution, DAKO; MW = microwave; Citr. = citrate (pH 6.0); CSA = catalyzed signal amplification system, DAKO; ABC = avidin-biotin complex, DAKO.

After deparaffination and rehydration, endogenous peroxidase activity was blocked for 30 minutes in methanol containing 0.3% hydrogen peroxide. After antigen retrieval, a cooling-off period of 20 minutes preceded the incubation of the primary antibody. Thereafter, the catalyzed signal amplification system (DAKO, Glostrup, Denmark) was used for HIF-1α staining according to the manufacturer's instructions. All other antibodies were detected by a standard avidin-biotin complex with a biotinylated rabbit anti-mouse antibody (DAKO) and an avidin-biotin complex (DAKO). All stainings were developed with diaminobenzidine. Before the slides were mounted, all sections were counterstained for 45 seconds with haematoxylin and dehydrated in alcohol and xylene. Appropriate negative controls (obtained by omission of the pri-

mary antibody) and positive controls were used throughout, for HIF-1α-negative and –positive controls, respectively, we used normoxic and hypoxic prostate cancer TSU cells, which were embedded in paraffin and provided by Dr. A. M. DeMarzo (The Johns Hopkins University School of Medicine, Baltimore, MD).

Quantification

For HIF-1α staining, only cells with completely and darkly stained epithelial nuclei were regarded as positive, and this nuclear staining was interpreted as an increased level; cytoplasmic staining, observed occasionally, was ignored because active HIF-1 is located only in the nucleus. The fraction of nuclei with an increased level of HIF-1α or p53 or Ki-67 positivity was estimated visually by two observers (R. Bos and P.J. van Diest). In DCIS specimens, the angiogenic activity was assessed by scoring the presence of a vascular rim around the ducts and by estimating the microvessel density in the surrounding stroma previously described by Guidi et al [28]. In invasive cancers, the microvessels were manually counted by use of an ocular grid at a magnification of 400x, following the criteria of Weidner et al [29,30] as described previously [31]. In short, in four adjacent fields of vision in the most vascularized area ("hotspot," total area = 0.6 mm^2), microvessels were counted and expressed as the microvessel density per millimeters square.

HER-2/*neu* staining was scored as negative or positive (membrane staining). VEGF expression was scored as weakly or strongly positive. ER status was determined by the evaluating of the histoscore as described previously [32], a histoscore of 100 or more was regarded as positive.

Statistical Methods

To evaluate whether frequency of cells with the elevated levels of HIF-1α increased during breast carcinogenesis, we performed a χ^2 test for trend, grouping the results as normal, hyperplasia, DCIS, or invasive cancer. Associations between increased levels of HIF-1α and the other biomarkers were analyzed with Fisher's exact test. The mean percentages of HIF-1α -positive cells in the different histologic groups were compared with the Mann-Whitney test. All analysis were performed with the SPSS package of computer programs for windows (SPSS Inc., Chicago, IL). *P* values of less than 0.05 were regarded as statistically significant. All statistical test were two-sided.

RESULTS

A summary of the HIF-1α nuclear staining in normal breast tissue and tissues from five stages of breast carcinogenesis is provided in Table 2. Normal breast tissue (n = 10) and hyperplastic lesions (n = 10) showed no detectable HIF-1α. In contrast, increased levels of HIF-1α were found in well-differentiated DCIS specimens (11 specimens had ≥1% immunopositive cells of 20 specimens tested), with the number of positive specimens further increasing in well-differentiated invasive breast (12 of 20). This trend continued with poorly differentiated DCIS lesions (17 of 20) and poorly differentiated invasive carcinomas (20 of 20). In 28 of 30 HIF-1α positive specimens that contained necrosis, regional HIF-1α positivity was especially noted around the

areas of necrosis (Figure 1D). Occasionally, a few cells with increased HIF-1α levels were observed in ductal hyperplastic areas adjacent to invasive cancer and in areas of atypical ductal hyperplasia.

TABLE 2. Increased hypoxia-inducible factor-1α level in tissues at different stages during breast carcinogenesis

	N	HIF-1α positive*, N	% HIF-1α** Mean	% HIF-1α** Range
Normal breast tissue	10	0	0	(0-0)
Usual hyperplasia	10	0	0	(0-0)
DCIS well-differentiated	20	11	3.5	(0-15)
DCIS poorly-differentiated	20	17	14.9	(0-50)
Invasive well-differentiated	20	12	11.8	(0-53)
Invasive poorly-differentiated	20	20	15.7	(1-53)

*HIF-1α positive = 1% or higher positive cells (per tissue sample)
**Mean percentage of positive cells (per tissue sample)
DCIS, ductal carcinoma in situ

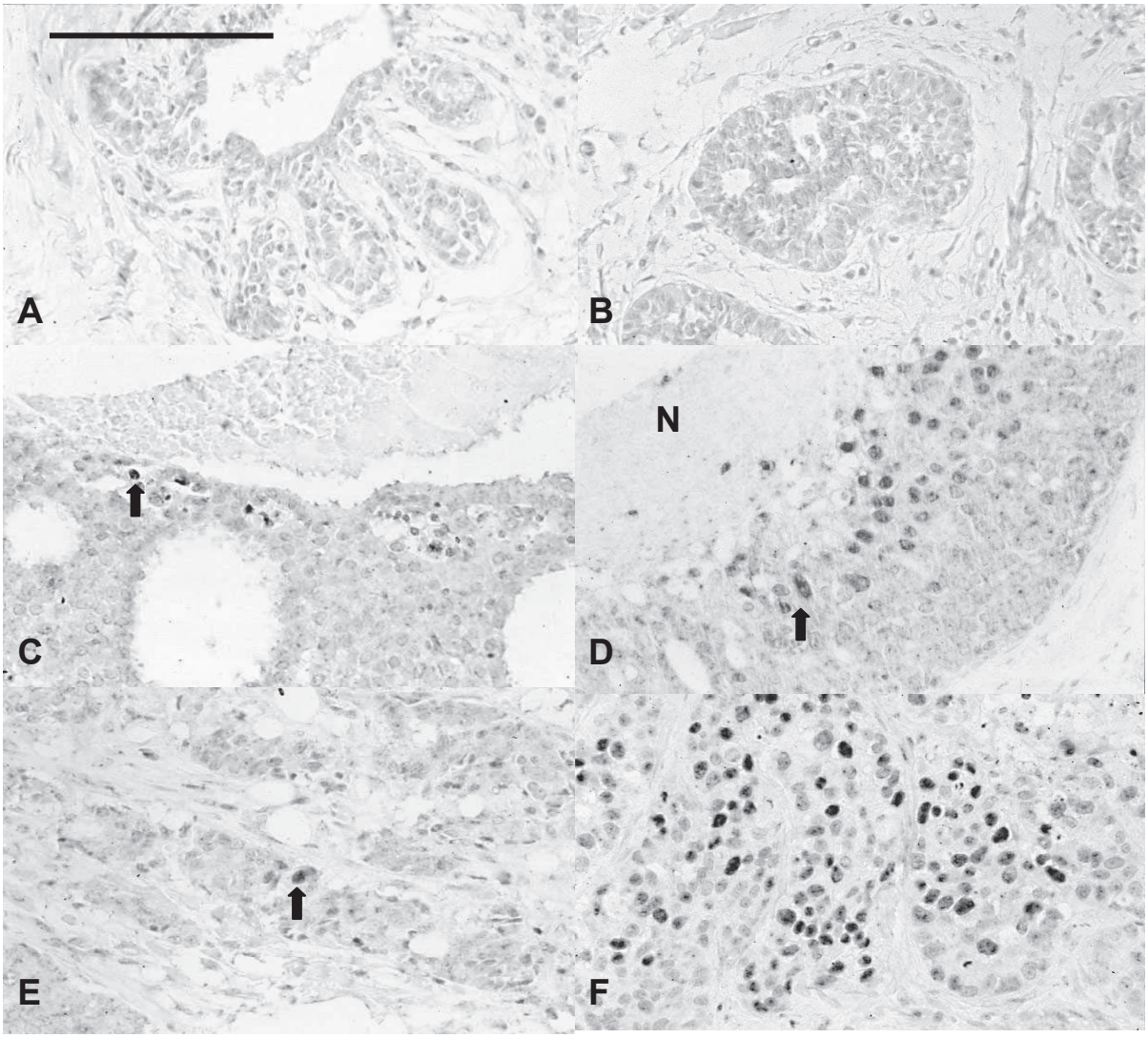

FIGURE 1. Immunohistochemical analysis of hypoxia-inducible factor-1α in normal breast tissue (**A**) and in hyperplasia (**B**) shows no increase in HIF-1α. Well-differentiated ductal carcinoma in situ (DCIS) (**C**) and poorly differentiated DCIS (D) show a striking pattern of increased HIF-1α around necrosis (N). A well-differentiated ductal carcinoma shows HIF-1α positivity (**E**). A poorly-differentiated medullary breast carcinoma shows increased regional levels of HIF-1α (**F**). Scale bar = 100μm
(for **color plate** see **p.126**)

Statistical analysis (χ^2 test) of the HIF-1α data showed a statistically significant increase in the number of cells with increased levels of HIF-1α over the progression spectrum (normal = 0 of 10; hyperplasia = 0 of 10; DCIS = 28 of 40; invasive cancer = 32 of 40; (*P*<0.001). More poorly differentiated (DCIS and invasive cancer) lesions (37 of 40) than the corresponding well-differentiated lesions (23 of 40) showed increased levels of HIF-1α (*P*<0.001). Well-differentiated and poorly differentiated DCIS lesions had statistically different levels of HIF-1α (*P*=0.028), as did well-differentiated and poorly differentiated invasive breast cancers (*P*<0.001). Also, the percentage of cells with increased levels of HIF-1α varied with different pathology, with a statistically significant higher (*P*<0.001) percentage of positive cells in the poorly differentiated specimens (Table 2).

Table 3 shows comparisons of increased levels of HIF-1α with various biomarkers. Even in this small dataset, VEGF expression (*P*=0.001), Ki-67 expression (*P*<0.001) (10% threshold), and ER status (*P*=0.001) were highly positively associated with increased levels of HIF-1α. HER-2/*neu* showed a positive, but borderline, association (*P*=0.053) with HIF-1α. However, p53 expression (5% threshold for positivity) was not associated with increased levels of HIF-1α. In the invasive cancers, a nearly positive association was found between HIF-1α and microvessel density (*P*<0.120). Stromal vascular density in DCIS showed a statistically significant positive correlation with HIF-1α (*P*=0.041), but vascular rim formation did not (*P*=1.00).

TABLE 3 Increased hypoxia-inducible factor-1α level associated with HER-2/*neu*, vascular endothelial growth factor (VEGF), estrogen receptor (ER), p53, Ki-67 and microvessel density (MVD)

	HER-2/*neu*		VEGF		ER		p53 %		Ki-67 %		CD31 DCIS Vascular rim		CD31 DCIS Stromal angiogenesis		MVD invasive cancers, vessels/mm^2	
	-	+	+	++	-	+	≤ 5	> 5	≤10	>10	-	+	-	+	≤100	> 100
HIF-1α -	35	5	16	24	30	10	36	4	38	2	6	6	10	2	6	2
HIF-1α +	42	18	6	54	25	35	49	11	38	22	13	15	13	15	13	19
P*	0.053		0.001		0.001		0.392		<0.001		1.00		0.041		0.120	

DCIS, ductal carcinoma in situ
*Fisher's two sided exact

HIF-1α positivity was found in 29 of 34 specimens containing necrosis compared with 31 of 46 specimens without necrosis. The mean percentage of HIF-1α-positive cells was higher in specimens with necrosis (*P*=0.019), a trend also visible in the subgroups of DCIS (*P*=0.08) and invasive lesions (*P*=0.02).

DISCUSSION

The purpose of this study was to determine whether increased levels of HIF-1α could be detected during different stages of carcinogenesis in human breast tissue. HIF-1α appears to be involved in angiogenesis during prostate carcinogenesis [33], and recent data [34] show that increased levels of HIF-1α represent an unfavorable characteristic in early cervical cancer. In this study, we did not detect increased levels of HIF-1α in specimens from normal breast and areas with ductal hyperplasia, but we did detect increased levels in the majority of DCIS and invasive cancer specimens. Levels of HIF-1α increased as the degree of malignancy increased, confirming earlier

pilot data [1] suggesting that HIF-1α may be a biomarker of preinvasive human breast cancers. In addition, we observed that an increased level of HIF-1α was associated with high proliferation, strong VEGF expression, and ER positivity as well with angiogenesis but only in the subgroup of DCIS lesions. To our knowledge, this is the first report to implicate increased levels of HIF-1α overexpression in early human breast carcinogenesis.

A statistically significant association was observed between increased levels of HIF-1α and VEGF expression. Furthermore, a positive association was observed between increased levels of HIF-1α and intratumoral microvessel density in DCIS, supporting a role for HIF-1α in angiogenesis during breast carcinogenesis. Our findings are consistent with a previous report [4] in xenografts of animal tumors and transgenic models, which showed HIF-1α involvement in the angiogenic phenotype of cancer.

It is interesting that increased levels of HIF-1α overexpression were most pronounced in poorly differentiated lesions, which supports the previously proposed progression model for breast cancer [35,36]. In this model, well-differentiated cancers arise from well-differentiated precursor lesions and poorly differentiated cancers from poorly differentiated precursor lesions. Poorly differentiated lesions (in the preinvasive and invasive states) are clinically more aggressive. The observed increased levels of HIF-1α overexpression in poorly differentiated DCIS may indicate a higher likelihood that this lesion will acquire invasive properties and that the resulting poorly differentiated invasive lesions may have a poorer prognosis.

The association between increased levels of HIF-1α and ER status at first seemed surprising but was consistent with the following data: HIF-1α is known to stimulate the production of VEGF [7], and the VEGF gene has functional ER response elements [37]. Furthermore, estrogen stimulation increases phosphatidylinositol 3-hydroxykinase activity [38], which belongs to a signaling pathway that may play a role in HIF-1α activation [33]. The relationship of estrogen action, ER interactions, and HIF-1α-driven genes in breast cancer certainly merits further investigation.

Several mechanisms, which are not mutually exclusive, that induce elevated levels of HIF-1α may be active in breast carcinogenesis. First, neoplastic breast lesions may be hypoxic so that HIF-1α is induced by low oxygen tension, as demonstrated in cultured cells [39], animal models [16], and hypoxic myocardium [40]. Such hypoxic conditions occur in solid cancers, but it is not yet known whether this holds true in DCIS (microhypoxia). It is interesting to note, however, the presence of HIF-1α-positive cells around areas of necrosis. Cells with increased levels of HIF-1α were also found regularly around areas of necrosis in invasive cancers. In poorly differentiated DCIS and invasive lesions, necrosis occurred more frequently. The difference in levels of HIF-1α protein between well-differentiated and poorly differentiated lesions may, therefore, also be hypoxia related. Studies addressing the intratumoral oxygen level in human breast cancer are required to define this important epigenetic parameter, which may have functional effects on HIF-1-activated genes.

Second, activated oncogenes and loss-of-function mutations tumor suppressor genes, such as PTEN (phosphatase and tensin homolog deleted on chromosome 10), VHL (von Hippel-Lindau tumor suppressor gene), and p53, have been shown to modulate HIF-1α levels in certain tumor types [2]. For example, loss of p53 function

has been shown in vitro to augment HIF-1 and VEGF expression [21], and, under hypoxic conditions, HIF-1 is phosphorylated by extracellular regulated kinases [41,42] confirming the potential role of the PI(3)K and mitogen activated protein kinase pathways and upstream oncogenes. HER-2/*neu* activates both pathways, and mutations in this gene are among the most common genomic alteration in DCIS and early breast cancer. In our study, HIF-1α levels and HER-2/*neu* status were indeed positively associated. Increased levels of HIF-1α can also occur as an effect of growth factor activation of the PI(3)K/AKT (protein kinase B)/FRAP (FK506 binding protein [FKBP])-rapamycin-associated protein pathway [33,43], as has been shown in tumor types other than breast cancer.

Third, activated immune responses during inflammation by the proinflammatory cytokines interleukin 1β and tumor necrosis factor-α [44] may also modulate HIF-1 activity in tumors. Both cytokines are important in the growth and differentiation of human breast cancer [45,46].

Finally, this study suggests breast cancer angiogenesis may be driven in part by HIF-1. We detected a statistically significant positive association between increased levels of HIF-1α protein and VEGF expression in human breast tissue. HIF-1α activates angiogenesis by stimulating VEGF transcription [7], and VEGF is induced by both oncogenic transformation and hypoxia [21,47,48]. Increased expression of VEGF and its receptors Flt-1 and Flk-1 have been demonstrated in breast cancer [49,50]. Increased VEGF expression in primary breast cancer confers a poorer prognosis at clinical presentation. Increased angiogenesis has also been reported in DCIS [29]. In our study, increased levels of HIF-1α were positively associated with both VEGF and microvessel density. These data support an angiogenesis-inducing role for HIF-1α during breast carcinogenesis. HIF-1α immunostaining may serve as a surrogate biomarker of angiogenic potential of breast cancer and deserves further large-scale clinical investigations.

It was interesting to note occasional nuclei with increased levels of HIF-1α in usual ductal hyperplasia next to invasive cancers and areas of atypical ductal hyperplasia. Usual ductal hyperplasia (and even normal lobules) next to invasive cancer has been shown to harbor morphological [51] and genetic changes [52], and thus, should be considered to be a more advanced lesion than pure ductal hyperplasia. Atypical ductal hyperplasia should be placed between usual ductal hyperplasia and well-differentiated DCIS in the breast progression spectrum (36), and so may be the earliest pure preinvasive breast lesion with increased levels of HIF-1α.

Increased levels of HIF-1α protein were observed at the earliest pathologically detectable stages of breast cancer carcinogenesis and were increased in dedifferentiated malignant tissue. Thus, we urge that therapeutics that specifically targeting and inhibiting HIF-1 [33,53,54] be rationally tested to prevent malignant progression in early breast cancer.

ACKNOWLEDGMENTS

Supported by the AEGON International Scholarship in Oncology (Public Health Service [PHS] grant CA58236 [to R. Bos] from the National Cancer Institute [NCI], National Institutes of Health, Department of Health and Human Services); by the Avon Breast Cancer Crusade (H. Zhong, J.W. Simons); by grant DAMD 17-98-1-8475 from

the U.S. Department of Defense; by the CaPCure Foundation; and by PHS grant CA58236 from the NCI.

We thank Dr. A. M. DeMarzo (Department of Pathology, The Johns Hopkins University School of Medicine, Baltimore, MD) for providing the embedded HIF-1α TSU expressing cell line, Dr. H.V. Stel (a pathologist at the Gooi-Noord Hospital, Blaricum, The Netherlands) for some tumor material, Mrs. P. van der Groep for technical assistance with the immunostaining, and Mrs. K. Heaney for assisting in preparing the manuscript for submission.

REFERENCES

1. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, and Simons JW. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 1999;59:5830-5835.
2. Semenza GL. Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 1999;15:551-578.
3. Ratcliffe PJ, O'Rourke JF, Maxwell PH, and Pugh CW. Oxygen sensing, hypoxia-inducible factor-1 and the regulation of mammalian gene expression. J Exp Biol 1998;201:1153-1162.
4. Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshert E, and Keshet E. Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature 1998;394:485-490.
5. Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, and Giaccia AJ. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 1996;379:88-91.
6. Brizel DM, Scully SP, Harrelson JM, Layfield LJ, Bean JM, Prosnitz LR, and Dewhirst MW. Tumor oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. Cancer Res 1996;56:941-943.
7. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, and Semenza GL. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 1996;16:4604-4613.
8. Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, and Semenza GL. Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev 1998;12:149-162.
9. Plate KH, Breier G, Weich HA, and Risau W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. Nature 1992;359:845-848.
10. Ryan HE, Lo J, and Johnson RS. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. EMBO J 1998;17:3005-3015.
11. Huang LE, Arany Z, Livingston DM, and Bunn HF. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. J Biol Chem 1996;271:32253-32259.
12. Jiang BH, Semenza GL, Bauer C, and Marti HH. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. Am J Physiol 1996;271:C1172-C1180.
13. Sutter CH, Laughner E, and Semenza GL. Hypoxia-inducible factor 1alpha protein expression is controlled by oxygen-regulated ubiquitination that is disrupted by deletions and missense mutations [In Process Citation]. Proc Natl Acad Sci U S A 2000;97:4748-4753.
14. Salceda S and Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem 1997;272:22642-22647.
15. Reyes H, Reisz-Porszasz S, and Hankinson O. Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. Science 1992;256:1193-1195.
16. Martin C, Yu AY, Jiang BH, Davis L, Kimberly D, Hohimer AR, and Semenza GL. Cardiac hypertrophy in chronically anemic fetal sheep: Increased vascularization is associated with increased myocardial expression of vascular endothelial growth factor and hypoxia-inducible factor 1. Am J Obstet Gynecol 1998;178:527-534.
17. Nordsmark M, Overgaard M, and Overgaard J. Pretreatment oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. Radiother Oncol 1996;41:31-39.
18. Hockel M, Schlenger K, Knoop C, and Vaupel P. Oxygenation of carcinomas of the uterine cervix: evaluation by computerized O2 tension measurements. Cancer Res 1991;51:6098-6102.
19. Jiang BH, Agani F, Passaniti A, and Semenza GL. V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression. Cancer Res 1997;57:5328-5335.
20. Maxwell PH, Dachs GU, Gleadle JM, Nicholls LG, Harris AL, Stratford IJ, Hankinson O, Pugh CW, and Ratcliffe PJ. Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc Natl Acad Sci U S A 1997;94:8104-8109.
21. Ravi R, Mookerjee B, Bhujwalla ZM, Sutter CH, Artemov D, Zeng Q, Dillehay LE, Madan A, Semenza GL, and Bedi A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia- inducible factor 1alpha. Genes Dev 2000;14:34-44.
22. Zagzag D, Zhong H, Scalzitti JM, Laughner E, Simons JW, and Semenza GL. Expression of hypoxia-inducible factor 1alpha in brain tumors: Association with angiogenesis, invasion, and progression. Cancer 2000;88:2606-2618.
23. Semenza GL and Wang GL. A nuclear factor induced by hypoxia via de novo protein synthesis binds to

the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 1992;12:5447-5454.
24. Folkman J, Watson K, Ingber D, and Hanahan D. Induction of angiogenesis during the transition from hyperplasia to neoplasia. Nature 1989;339:58-61.
25. Guidi AJ, Schnitt SJ, Fischer L, Tognazzi K, Harris JR, Dvorak HF, and Brown LF. Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in patients with ductal carcinoma in situ of the breast. Cancer 1997;80:1945-1953.
26. Holland R, Peterse JL, Millis RR, Eusebi V, Faverly D, van de Vijver MJ, and Zafrani B. Ductal carcinoma in situ: a proposal for a new classification. Semin Diagn Pathol 1994;11:167-180.
27. Elston CW and Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991;19:403-410.
28. Guidi AJ, Fischer L, Harris JR, and Schnitt SJ. Microvessel density and distribution in ductal carcinoma in situ of the breast. J Natl Cancer Inst 1994;86:614-619.
29. Weidner N, Semple JP, Welch WR, and Folkman J. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. N Engl J Med 1991;324:1-8.
30. Weidner N, Folkman J, Pozza F, Bevilacqua P, Allred EN, Moore DH, Meli S, and Gasparini G. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. J Natl Cancer Inst 1992;84:1875-1887.
31. De Jong JS, van Diest PJ, and Baak JP. Hot spot microvessel density and the mitotic activity index are strong additional prognostic indicators in invasive breast cancer. Histopathology 2000;36:306-312.
32. Bosman FT, de Goeij AF, and Rousch M. Quality control in immunocytochemistry: experiences with the oestrogen receptor assay. J Clin Pathol 1992;45:120-124.
33. Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM, Simons JW, and Semenza GL. Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res 2000;60:1541-1545.
34. Birner P, Schindl M, Obermair A, Plank C, Breitenecker G, and Oberhuber G. Overexpression of hypoxia-inducible factor 1alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. Cancer Res 2000;60:4693-4696.
35. Buerger H, Otterbach F, Simon R, Schafer KL, Poremba C, Diallo R, Brinkschmidt C, Dockhorn-Dworniczak B, and Boecker W. Different genetic pathways in the evolution of invasive breast cancer are associated with distinct morphological subtypes. J Pathol 1999;189:521-526.
36. van Diest PJ. Ductal carcinoma in situ in breast carcinogenesis. J Pathol 1999;187:383-384.
37. Hyder SM, Nawaz Z, Chiappetta C, and Stancel GM. Identification of functional estrogen response elements in the gene coding for the potent angiogenic factor vascular endothelial growth factor. Cancer Res 2000;60:3183-3190.
38. Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, and Liao JK. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase [In Process Citation]. Nature 2000;407:538-541.
39. Zhong H, Agani F, Baccala AA, Laughner E, Rioseco-Camacho N, Isaacs WB, Simons JW, and Semenza GL. Increased expression of hypoxia inducible factor-1alpha in rat and human prostate cancer. Cancer Res 1998;58:5280-5284.
40. Lee SH, Wolf PL, Escudero R, Deutsch R, Jamieson SW, and Thistlethwaite PA. Early expression of angiogenesis factors in acute myocardial ischemia and infarction. N Engl J Med 2000;342:626-633.
41. Minet E, Arnould T, Michel G, Roland I, Mottet D, Raes M, Remacle J, and Michiels C. ERK activation upon hypoxia: involvement in HIF-1 activation. FEBS Lett 2000;468:53-58.
42. Richard DE, Berra E, Gothie E, Roux D, and Pouyssegur J. p42/p44 mitogen-activated protein kinases phosphorylate hypoxia- inducible factor 1alpha (HIF-1alpha) and enhance the transcriptional activity of HIF-1. J Biol Chem 1999;274:32631-32637.
43. Zundel W, Schindler C, Haas-Kogan D, Koong A, Kaper F, Chen E, Gottschalk AR, Ryan HE, Johnson RS, Jefferson AB, Stokoe D, and Giaccia AJ. Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev 2000;14:391-396.
44. Hellwig-Burgel T, Rutkowski K, Metzen E, Fandrey J, and Jelkmann W. Interleukin-1beta and tumor necrosis factor-alpha stimulate DNA binding of hypoxia-inducible factor-1. Blood 1999;94:1561-1567.
45. Binder C, Schulz M, Hiddemann W, and Oellerich M. Induction of inducible nitric oxide synthase is an essential part of tumor necrosis factor-alpha-induced apoptosis in MCF-7 and other epithelial tumor cells. Lab Invest 1999;79:1703-1712.
46. Speirs V, Kerin MJ, Newton CJ, Walton DS, Green AR, Desai SB, and Atkin SL. Evidence for transcriptional activation of ERalpha by IL-1beta in breast cancer cells. Int J Oncol 1999;15:1251-1254.
47. Mazure NM, Chen EY, Laderoute KR, and Giaccia AJ. Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras- transformed cells through a hypoxia inducible factor-1 transcriptional element. Blood 1997;90:3322-3331.
48. Shweiki D, Itin A, Soffer D, and Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 1992;359:843-845.
49. De Jong JS, van Diest PJ, van der Valk P, and Baak JP. Expression of growth factors, growth inhibiting factors, and their receptors in invasive breast cancer. I: An inventory in search of autocrine and paracrine loops. J Pathol 1998;184:44-52.
50. De Jong JS, van Diest PJ, van der Valk P, and Baak JP. Expression of growth factors, growth-inhibiting factors, and their receptors in invasive breast cancer. II: Correlations with proliferation and angiogenesis. J Pathol 1998;184:53-57.
51. Mommers EC, Poulin N, Meijer CJ, Baak JP, and van Diest PJ. Malignancy-associated changes in breast tissue detected by image cytometry. Anal Cell Pathol 2000;20:187-195.
52. Deng G, Lu Y, Zlotnikov G, Thor AD, and Smith HS. Loss of heterozygosity in normal tissue adjacent to breast carcinomas. Science 1996;274:2057-2059.
53. Kruger EA, Blagosklonny MV, Dixon SC, and Figg WD. UCN-01, a protein kinase C inhibitor, inhibits endothelial cell proliferation and angiogenic hypoxic response. Invasion Metastasis 1998;18:209-218.

54. Melillo G, Sausville EA, Cloud K, Lahusen T, Varesio L, and Senderowicz AM. Flavopiridol, a protein kinase inhibitor, down-regulates hypoxic induction of vascular endothelial growth factor expression in human monocytes. Cancer Res 1999;59:5433-5437.

Chapter 3

Expression of Hypoxia-Inducible Factor-1α and cell cycle proteins in invasive breast cancer are estrogen receptor related

Breast Cancer Res 2004;6:R450-459

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BACKGROUND. The transcription factor Hypoxia-Inducible Factor-1 (HIF-1) is a key regulator of the cellular response to hypoxia. Previous studies showed that levels of its subunit HIF-1α, the best surrogate for HIF-1 activity, are increased during breast carcinogenesis and can independently predict prognosis in breast cancer. During carcinogenesis, the cell cycle is progressively deregulated, and proliferation rate is a strong prognostic factor in breast cancer. Here, we undertook a detailed evaluation of the relationships between HIF-1α and cell cycle-associated proteins.

METHODS. In a representative group of 150 breast cancers, expression of HIF-1α, VEGF, the estrogen receptor (ER), HER-2/*neu*, Ki-67, cyclin A, cyclin D_1, p21, p53, and Bcl-2 was investigated by immunohistochemistry.

RESULTS. High levels ($\geq$5%) of HIF-1α were associated with increased proliferation as shown by positive correlations with Ki-67 (P<0.001) and the late S-G2 phase protein cyclin A (P<0.001), but not with the G_1-phase protein cyclin D_1. High HIF-1α levels were also strongly associated with p53-positivity (P<0.001) and loss of Bcl-2 expression (P=0.013). No association was found between p21 and HIF-1α (P=0.105) in the whole group of patients. However, the subgroup of ER-positive cancers was characterized by a strong positive association between HIF-1α and p21 (P=0.023) and HIF-1α lacked any relation with proliferation.

CONCLUSIONS. HIF-1α overexpression is associated with increased proliferation, which may explain the adverse prognostic impact of increased levels of HIF-1α in invasive breast cancer. In ER positive tumors, HIF-1α is associated to p21 but not to proliferation. This urges further functional analysis to unravel the role of HIF-1 in late cell cycle progression, and the link between HIF-1, p21 and ER.

HYPOXIA IS AN important cellular stressor that triggers a survival program by which the cells attempts to adapt to the new environment. This primarily involves adaptation of metabolism and/or stimulation of oxygen delivery. These cell-rescuing mechanisms can be conducted rapidly by a transcription factor which reacts to hypoxic conditions, the hypoxia-inducible factor-1 (HIF-1) [1]. HIF-1 stimulates processes such as angiogenesis, glycolysis and erythropoiesis [2], by activating genes that are responsible for these processes. The HIF-1 complex consists of two subunits, HIF-1α and HIF-1β. Protein levels of HIF-1α depend on the cellular oxygen concentration [3,4]. During normoxia the HIF-1α protein has a very short half-life due to its continuous Von Hippel-Lindau (VHL) protein mediated ubiquitination, which results in low protein levels in the cytoplasm. Hypoxia results in stabilization of the HIF-1α protein and translocation of the HIF-1 complex to the nucleus. In the nucleus HIF-1 binds to DNA of the consensus sequence 5'-RCGTG-3', the so called Hypoxia Response Elements in the promoters of target genes [5]. In this way HIF-1 allows the cell to adapt metabolism, increases O_2 delivery and stimulate cell survival [6]. Besides hypoxia, HIF-1 can be upregulated by loss of the tumor suppressor genes Phosphatase and tensin homologue deleted on chromosome ten (PTEN) [7] and loss of p53 [8], and overexpression of oncogenes, like HER-2/*neu* [9].

Cancer cells are able to survive and proliferate at extreme micro environmental circumstances and show changes in oncogenes and tumor suppressor genes. Hypoxia and HIF-1 have indeed been implicated in carcinogenesis and clinical behavior of tumors. Upregulation of HIF-1α was noted during breast carcinogenesis [10], especially in the poorly differentiated pathway. Hypoxia is related to poor response to therapy in various cancer types. In invasive breast cancer, high HIF-1α levels were associated with poor survival in lymph node negative patients [11]. As prognosis in breast cancer is closely related to proliferation rate [12], and poorly differentiated tumors usually exhibit high proliferation and HIF-1α overexpression, the prognostic value of HIF-1α may well be explained by a close association between HIF-1α and proliferation.

Proliferation is under control of many proteins involved in cell cycle regulation. We hypothesized that HIF-1, as a master regulator for surviving hypoxia, might interact with such cell cycle-related proteins. Therefore, we investigated if levels of HIF-1α were associated with aberrant expression of cell cycle proteins in human breast cancer. As a result, in this study we report that high levels of HIF-1α are associated with overexpression of p53 and markers of proliferation during the late S-G2 phase of the cell cycle. In the subgroup of ER-positive cancers only a positive association between HIF-1α and p21 was noticed. Probably, in ER positive cases, p21 causes cell cycle arrest as a response to increased HIF-1α levels.

PATIENTS AND METHODS

Patients

A representative and previously described group of 150 stage I/II breast cancer patients, diagnosed between 1985 and 1993 at the VU University Medical Center (Amsterdam, The Netherlands), was used [11]. By “representative” we mean that tumor size, distribution of histological subtypes and lymph node status were as expected in

a random group of stage I/II patients. Breast conserving therapy or modified radical mastectomy were the applied surgical procedures for the primary tumors, and axillary dissection including at least levels I and II was performed for all patients. All surgical specimens were directly fixed in neutral 4% buffered formaldehyde.

All invasive breast carcinomas were histologically classified according to WHO criteria as: ductal (n = 129), lobular (n = 11), mucinous (n = 4), tubular (n = 3), cribriform (n = 1), medullary (n = 1) or metaplastic (n = 1). Tumors were graded following the criteria of Elston [13] as grade I (n = 35), II (n = 49), or III (n = 66). The mean age at the time of diagnosis was 60 (range 30 to 86). The mean tumor diameter was 2.6 cm, ranging from 0.7 to 7.0 cm (according to the TNM system: 45 T_1, 94 T_2 and 11 T_3). Locally advanced breast cancers (TNM stage III) were excluded. The group included 81 (54%) lymph node-negative and 69 (46%) lymph node-positive patients. None of the patients received preoperative chemo-, hormonal or radiotherapy.

Immunohistochemistry

Paraffin embedded tumor tissue was derived from the archives of the Department of Pathology of the VU University Medical Center. Anonymous use of redundant tumor material for research purposes is part of the standard treatment agreement with patients in our hospital [14]. Immunohistochemistry was performed on 4 μm thick sections. After deparaffination and rehydration, sections were immersed in methanol containing 0.3% hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. For assessment of HIF-1α the Catalyzed Signal Amplification System (DAKO, Glostrup, Denmark) was used as described before [11]. All slides, except for HER-2/*neu*, were pretreated with a citrate buffer (10 mM, pH 6.0) for antigen retrieval either by heating the slides in a microwave oven or autoclave (for details see Table 1).

Table 1. Used mouse monoclonal antibodies, availability, dilution, incubation, antigen retrieval and detection method.

Antibody	Company	Dilution	Incubation time	Antigen-retrieval	Detection
Ki-67	DAKO	1:40	o/n, 4 °C	MW	ABC
Cyclin A	Novocastra	1:100	o/n, 4 °C	Autoclave	ABC
Cyclin D_1	Neomarkers	1:400	o/n, 4 °C	Autoclave	ABC
P21	Pharmingen	1:500	o/n, 4 °C	Autoclave	ABC#
P53	DAKO	1:50	o/n, 4 °C	MW	ABC
Bcl-2	DAKO	1:50	o/n, 4 °C	Autoclave	ABC#
ER	DAKO	1:50	o/n, 4 °C	Autoclave	ABC
VEGF	R&D systems	1:40	o/n, 4 °C	Autoclave	ABC
HIF-1α	Abcam	1:500	1h, 20°C	Waterbath	CSA kit
HER-2/*neu*	M.v/d Vijver[$]	1:10,000	o/n, 4 °C	none	ABC

o/n = overnight; MW = microwave in citrate buffer pH 6.0 for 10 minutes near to boiling; Autoclave = autoclave 20 minutes at 120 °C; Waterbath = 45 minutes at 95 °C; ABC = avidin-biotinyl complex; # biotinyl-tyramide enhancement; [$] M. v/d Vijver= Dr. M. van der Vijver, Dutch Cancer Institute, Amsterdam, The Netherlands

After cooling down and pre-incubation with normal serum of the species of the secondary antibody, the primary antibodies were incubated as described in Table 1. For recognition of VEGF a rabbit polyclonal antibody was used, which was visualized by a swine anti-rabbit antibody (DAKO). Subsequently, slides were incubated with biotinylated secondary antibodies for 30 minutes, followed by incubation with 1:200 streptavidin-biotinylated horseradish-peroxidase complex (DAKO) for 1 hour. For Bcl-2 and p21 staining, a biotinyl-tyramide enhancing step was introduced [15]. After a 30

minutes incubation of 1:1000 streptavidin-biotinylated horseradish-peroxidase complex (instead of 1:200), slides were incubated with 1:1000 diluted biotinyl-tyramide solution with 0.01% hydrogen peroxide in PBS for 10 minutes, followed by incubation with 1:200 streptavidin-biotinylated horseradish-peroxidase complex for 30 minutes. In all cases 3,3-diaminobenzidine was used as chromagen and hematoxylin as counterstaining. Positive controls consisted of a known immunoreactive carcinomas (VEGF, HIF-1α, ER, p53, cyclin D_1, p21, HER-2/*neu*) or tonsil tissue (Ki-67, Bcl-2, cyclin A). Negative controls were obtained by omission of the primary antibodies from the staining procedure.

TABLE 2. Association of HIF-1α expression with cell cycle-related proteins in 150 invasive human breast cancers.

	HIF-1α level		
	<5%	≥5%	*P* (χ^2)
Ki-67			
≤ 10 %	58	13	< 0.001
> 10 %	41	38	
Cyclin A			
≤ 10 %	67	19	< 0.001
> 10 %	31	32	
Cyclin D_1			
≤ 10 %	80	44	0.402
> 10 %	19	7	
p21			
≤ 10 %	89	41	0.105
> 10 %	10	10	
p53			
≤ 25 %	89	35	< 0.001
> 25 %	10	16	
Bcl-2			
Negative	4	5	
Moderate	35	26	0.025
Strong	60	19	

Quantification

For all cell cycle biomarkers, except Bcl-2, the percentage of positively stained nuclei of invasive tumor cells was scored blinded by one experienced observer (PJvD). ER status was determined by the Histoscore, regarding 100 or more as positive. HIF-1α staining was only considered positive when there was homogeneously dark nuclear staining; cytoplasmic staining was ignored. Cytoplasmic staining for Bcl-2 in invasive breast cancer epithelium was compared with adjacent normal breast tissue and scored as negative when absent, and as weak or strong when intensity was diminished respectively increased compared to adjacent normal tissue. Cytoplasmic VEGF expression was assessed as moderate or strong.

Statistical Methods

For statistical analysis, (SPSS for Windows Version 9.0.1., 1999, SPSS Inc., Chicago, IL, USA) the nonparametric Chi-square was used to evaluate correlations between HIF-1α and cell cycle-associated proteins. For the cell cycle-related proteins traditional cutoff values [16] (as shown in Table 2) and for HIF-1α 5% was used as cut off value as described previously [11]. Parametric tests and correlation tests using the continuous data were also done, but yielded similar results and have therefore not been described to keep description of the statistical results as succinct as possible. Two-sided *P* values below 0.050 were regarded as significant.

RESULTS

Whole group

Analyzing the whole group of patients (n = 150), there were (table 2) significant and positive associations between HIF-1α and Ki-67 ($P < 0.001$), cyclin A ($P < 0.001$), and p53 ($P < 0.001$). A positive trend between high levels of HIF-1α and expression of p21 ($P = 0.105$) was noticed, and there was a significant inverse association between Bcl-2 ($P = 0.025$) and HIF-1α expression. No association between HIF-1α and cyclin D_1 was found. In addition, both ER positivity and overexpression of p21 were positively associated with overexpression of cyclin D_1 ($P < 0.001$) in the whole group of patients.

TABLE 3. Association of HIF-1α with cell cycle-related proteins in ER positive (n=52) and negative (n=98) breast cancers.

		ER-positive			ER-negative		
		HIF-1α			HIF-1α		
		< 5%	≥ 5%	P (χ^2)	< 5%	≥ 5%	P (χ^2)
Ki-67	≤ 10 %	24	7	.624	34	6	< .001
	> 10 %	15	7		26	32	
Cyclin A	≤ 10 %	28	9	.756	39	10	< .001
	> 10 %	10	4		21	28	
Cyclin D_1	≤ 10 %	25	9	.736	55	35	.938
	> 10 %	14	4		5	3	
p21	≤ 10 %	33	7	.023	56	34	.497
	> 10 %	6	6		4	4	
p53	≤ 25 %	36	11	.415	53	24	.003
	> 25 %	3	2		7	14	
Bcl-2	Negative	0	0	.609	4	5	.048
	Moderate	12	5		23	21	
	Strong	27	8		33	11	
VEGF	Weak	8	2	.685	21	4	.007
	Strong	31	11		39	34	
HER-2/*neu*	Negative	36	9	.035	55	26	.003
	Positive	3	4		5	12	

ER, estrogen receptor

ER subgroups

Interestingly, subgroup analysis of ER positive cancers (n = 52) revealed different associations (table 3). Only p21-positivity was significantly associated with high levels of HIF-1α ($P = 0.023$). No associations for HIF-1α were found with Ki-67, cyclins A and D_1, p53, and Bcl-2. In this subgroup, p21 was positively associated with cyclin D_1 ($P < 0.001$) but not with p53. On the other hand, the subgroup of ER-negative cancers (n = 98) showed strongly significant positive associations between HIF-1α on the one hand and Ki-67 ($P < 0.001$), cyclin A ($P < 0.001$), and p53 ($P = 0.003$) on the other. Again, loss of Bcl-2 ($P = 0.048$) was significantly negatively associated with high levels of HIF-1α and no association between HIF-1α, p21 and cyclin D_1 was found. The striking difference in biomarker expression between the ER positive and negative subgroups made us further test if this was associated with proliferation or with HIF-1α. In the ER-positive subgroup HER-2/*neu*-positivity was associated with high levels of HIF-1α ($P = 0.035$), but no association was found between HIF-1α, p53 and VEGF. Further, in this subgroup we observed low ER expression in HIF-1α posi-

tive regions. In the ER-negative subgroup strong VEGF expression (P = 0.007), p53 accumulation (P = 0.003), and HER-2/*neu*-positivity (P = 0.003) were all positively associated with high levels of HIF-1α.

p53 subgroups

To exclude that the data which were found in the ER subgroups are dependent on p53 status, we also performed subgroup analysis based upon p53 status (table 4). In short, the subgroup assumed to be “wild type” p53 was characterized by different associations than the subgroup of ER positive cases.

TABLE 4. Association of HIF-1α with cell cycle-related proteins in WT-p53 (n=124) and M-p53 (n=26) breast cancers.

		“Wild type” p53			“Mutated” p53		
		HIF-1α			HIF-1α		
		< 5%	≥ 5%	P (χ^2)	< 5%	≥ 5%	P (χ^2)*
Ki-67	≤ 10 %	55	12	0.006	3	1	0.264
	> 10 %	34	23		7	15	
Cyclin A	≤ 10 %	62	15	0.004	5	4	0.192
	> 10 %	26	20		5	12	
Cyclin D_1	≤ 10 %	71	31	0.248	9	13	1.00
	> 10 %	18	4		1	3	
p21	≤ 10 %	80	28	0.139	9	13	1.00
	> 10 %	9	7		1	3	
Bcl-2	Negative	3	3	0.273	1	2	0.132
	Moderate	31	15		4	11	
	Strong	55	17		5	2	
VEGF	Weak	28	5	0.051	1	1	1.00
	Strong	61	30		9	15	
HER-2/*neu*	Negative	82	27	0.021	9	8	0.037
	Positive	7	8		1	8	

*= Fisher Exact Test when appropriate, WT-p53, probably wild type p53, M-p53, probably mutated p53

Lymph node status subgroups

Because in our former study we found an independent prognostic value for HIF-1α only in lymph node (LN)-negative breast cancers, we analyzed the subgroups of lymph node negative (n = 81) and positive cases (n = 69) separately (table 5). Positive significant associations were found in the subgroup of LN negative cancers between HIF-1α and Ki-67 (P < 0.001), cyclin A (P < 0.001), p21 (P = 0.049), and p53 (P = 0.001). Also in this subgroup Bcl-2 (P = 0.008) was significantly inversely associated with HIF-1α. No association was found for cyclin D_1. In the subgroup of LN positive cancers, Ki-67 (P = 0.035) and p53 (P = 0.042) revealed a positive association with HIF-1α. No relation with HIF-1α was found for cyclins A and D_1, p21 and Bcl-2.

Ductal type subgroup

To exclude confounding mechanisms based on the mixed group of different types of breast cancer, we also performed the same above described statistical analysis on the subgroup of ductal type of invasive breast cancers (n=129) as shown in table 6 and 7. In short, the same associations were found as described for the

group of 150 patients.

TABLE 5. Association of HIF-1α with cell cycle-related proteins in LN negative (n=81) and positive (n=69) breast cancers.

		LN-negative			LN-positive		
		HIF-1α			HIF-1α		
		< 5%	≥ 5%	P (χ^2)	< 5%	≥ 5%	P (χ^2)
Ki-67	≤ 10 %	36	9	< .001	22	4	.035
	> 10 %	15	21		26	17	
Cyclin A	≤ 10 %	40	10	< .001	27	9	.305
	> 10 %	10	20		21	12	
Cyclin D_1	≤ 10 %	40	25	.593	40	19	.438
	> 10 %	11	5		8	2	
p21	≤ 10 %	47	23	.049	42	18	.839
	> 10 %	4	7		6	3	
p53	≤ 25 %	47	21	.009	42	14	.042
	> 25 %	4	9		6	7	
Bcl-2	Negative	0	3	.008	4	2	.459
	Moderate	17	15		18	11	
	Strong	34	11		26	8	

LN, lymph node

TABLE 6. Association of HIF-1α expression with cell cycle-related proteins in all ductal type (129 out of 150) invasive human breast cancers.

	HIF-1α level		
	< 5%	≥ 5%	P (χ^2)
Ki-67			
≤ 10 %	44	11	0.001
> 10 %	39	35	
Cyclin A			
≤ 10 %	52	16	0.002
> 10 %	30	30	
Cyclin D_1			
≤ 10 %	67	39	0.564
> 10 %	16	7	
p21			
≤ 10 %	75	36	0.057
> 10 %	8	10	
p53			
≤ 25 %	68	25	0.001
> 25 %	15	21	
Bcl-2			
Negative	4	5	
Moderate	29	23	0.042
Strong	50	17	

DISCUSSION

In this study positive associations between HIF-1α and the cell cycle-related proteins cyclin A, Ki-67 and p53 in invasive human breast cancers were found. These associations were most evident in the lymph node-negative cases and, therefore, may attribute to the poor prognosis of HIF-1α positive cancers described before [11]. Further, the ER-subgroups showed differential expression of biomarkers, suggesting an interaction between HIF-1 and ER.

In general, expression of cyclin A and Ki-67 indicate that cells are in the S or G_2 phase. Cyclin A expression is stimulated by the protein-tyrosine phosphatase cdc25A [17] and is associated with undifferentiated and ER-negative breast tumors harboring poor prognosis [18,19]. It is common to use protein levels of Ki-67 as a proliferation marker although its function is unknown; it is most present in the S-phase but also in the $G_{1\text{-}2}$ [20]. Like cyclin A, Ki-67 overexpression represents a high proliferation rate and thus poor prognosis. Our data show that high levels of HIF-1α are associated with high levels of cyclin A and Ki-67 as markers of proliferation.

TABLE 7. Association of HIF-1α with cell cycle-related proteins in only ductal carcinoma, subdivided into ER positive (n=46) and negative (n=83) breast cancers.

		ER-positive			ER-negative		
		HIF-1α			HIF-1α		
		< 5%	≥ 5%	*P* (χ^2)*	< 5%	≥ 5%	*P* (χ^2)*
Ki-67	≤ 10 %	20	5	0.730	24	6	0.002
	> 10 %	15	6		24	29	
Cyclin A	≤ 10 %	24	7	0.717	28	9	0.003
	> 10 %	10	4		20	26	
Cyclin D_1	≤ 10 %	22	7	0.100	45	32	0.693
	> 10 %	13	4		3	3	
p21	≤ 10 %	29	5	0.022	46	31	0.235
	> 10 %	6	6		2	4	
p53	≤ 25 %	29	7	0.175	39	18	0.008
	> 25 %	6	4		9	17	
Bcl-2	Negative	0	0	0.477	4	5	0.141
	Moderate	11	5		18	18	
	Strong	24	6		26	11	
VEGF	Weak	8	2	1.00	18	3	0.003
	Strong	27	9		30	32	
HER-2/*neu*	Negative	33	8	0.080	43	23	0.008
	Positive	2	3		5	12	

*= Fisher Exact Test when appropriate, ER, estrogen receptor

The association of HIF-1 with proliferation was noticed before [10], but is still not fully understood [21,22]. The main question is whether HIF-1 is acting on or a reaction to tumor proliferation. The latter mechanism assumes that unorganized rapidly growing tumors will outgrowth their own vasculature leading to a lack of oxygen supply, necessitating adaptation by switching to anaerobic metabolism and induction of angiogenesis. As HIF-1 plays a crucial role in these latter processes, it may be postulated that rapidly proliferating tumors need HIF-1. In this light the association between HIF-1 and proliferation is more or less epigenetic or due to tumor necrosis. According to the former mechanism, primary HIF-1 overexpression might also lead to tumor proliferation. One argument for this hypothesis is based on the observation that HIF-1α expression is not restricted to necrotic tumor areas. Another argument might be the influence of oncogenes (e.g. HER-2/*neu*, Bcl-6), tumor suppressor genes (e.g. pVHL, PTEN), or growth factors (e.g. IGF-2) on the protein levels of HIF-1α [23,24,9,25,26,7]. These tumorigenic mechanisms also stimulate proliferation. Also, the recent demonstration that pulmonary artery fibroblasts, when exposed to hypoxia, stimulate the proliferation of adjacent pulmonary artery smooth muscle cells via hypoxia-regulated genes indicates a stimulating role for HIF-1 in the cell cycle machinery [27].

In contrast, recent work from Goda et al showed in two different primary differentiated cell types that HIF-1 is necessary for induction of growth arrest during hypoxia [28]. HIF-1 alters the cell cycle during hypoxia by increasing cyclin-dependent kinase inhibitors p21 and p27. Also hypophosphorylation of retinoblastoma proteins is HIF-1 dependent. In addition, Goda also showed that cells lacking the HIF-1α gene had an increased progression into S-phase [28]. These *in vitro* data are logic in physiologic circumstances but do not comply with our observations in human cancers. Thus, if these mechanisms also occur in human breast cancer is difficult to say. More likely, it might be postulated that cancer cells have lost control over the cell cycle and its potential interplay with HIF-1α.

Subgroup analyses based on ER status revealed that only in ER-positive cases, positivity for p21 was associated with high levels of HIF-1α, without correlation between HIF-1 and proliferation. This points to an intact feedback loop in ER expressing cells where p21 might cause cell cycle arrest as a response to increased HIF-1α levels, which may be cyclin D_1 regulated [29]. In contrast, in ER-negative cases, a positive association between HIF-1 on the one hand and Ki-67, cyclin A, p53 and loss of Bcl-2 on the other was noted. Apparently, the p21 feedback loop is not functional in ER negative cells. In addition, in the ER-positive cases no association for HIF-α with VEGF and p53 could be demonstrated, which was opposite to the ER-negative subgroup. These findings suggests that the different associations in both ER subgroups can not only be attributed to a different proliferation rate. We excluded the option that the p53 status might be the underlying cause for these differences, as shown in table 4. Therefore, it is tempting to suggest that these differences might be caused by an interaction between HIF-1 and ER. Little is known about the interaction between HIF-1 and ER, but an almost significant positive association was found in endometrial cancer [30] but not in breast cancer [11,31]. In prostate cancer, the presence of the androgen receptor seems necessary to potentate the angiogenic effects of HIF-1α, although this effect is mediated by the EGF/phosphatidylinositol 3'-kinase/protein kinase B pathway [32]. In two studies, it was shown that hypoxia down regulates ER in breast cancer cell lines [33,34]. Therefore, more studies are warranted to investigate the interaction between ER and HIF-1.

Most knowledge about the interaction between HIF-1 and the cell cycle has been gathered around p53. Some of this interplay between p53 and HIF-1 has been defined by An et al. who demonstrated that during hypoxia p53 could not stabilize without the presence of HIF-1α [35]. Even a direct association between p53 and HIF-1α was shown by co-immunoprecipitation in hypoxic cells. Subsequently, it was shown that p53 depends on HIF-1α when it initiates apoptosis during hypoxia [21]. On the other hand Ravi et al. showed that the level of HIF-1α increased when no p53 was present in tumor cells who responded to hypoxia [8]. Therefore, HIF-1 and p53 can be seen as competitors because both are upregulated by hypoxia, p53-inducing apoptosis while HIF-1 rather maintains homeostasis, although HIF-1 may also induce apoptosis in concert with p53 [36] and through NIP3 [37]. Other factors influencing the balance between p53 and HIF-1 are competition for the cofactor p300 [38] and MDM-2 degradation of HIF-1α via p53 [8]. Thus, loss of wild-type p53 might be associated with increased tumor growth during hypoxia because of diminished apoptosis and augmented HIF-1 induced transcriptional activation of VEGF. Indeed, the positive association between VEGF and accumulation of p53, which is associated with p53 mutation, has been noticed in breast cancer [39]. In the present study we found a positive association between accumulation of p53 with HIF-1α and p53 with VEGF (the positive association of HIF-1α and VEGF has been described elsewhere [11]). The p53/HIF-1 data are in concordance of Zhong et al who noticed such association in a mixed group of colon and breast cancer patients [22]. In contrast, in lymph node-positive breast cancer and epithelial ovarian cancer no relation between p53 and HIF-1α was found [40,33,31,34]. Interestingly, we noticed that the classical association of HIF with proliferation and VEGF is true in wildtype p53, but not in the “mutated” subgroup (assuming that more than 25% nuclear p53 accumulation points to a p53 mutation).

Thus, these data imply that HIF needs wildtype p53 to exert its downstream effects. Combined high expression of p21 and cyclin D_1 was positively associated with high differentiation and low proliferation in various carcinomas including breast cancer [29,41]. Interestingly, in this study, increased levels of cyclin D_1 corresponded to high p21 (as before [29]), but not HIF-1α. Many investigators have searched for an explanation why cyclin D_1 becomes upregulated in breast cancer. Most validated is the assumption that an amplification or translocation of the cyclin D_1 gene is responsible, but also other mechanisms seem to be involved because of the low incidence of cyclin D_1 amplification. It was shown that cyclin D_1 exerts the effects of ER [42] confirming the importance of cyclin D_1 in breast cancer. The results reported here may provide circumstantial evidence that upregulation of cyclin D_1 might indirectly be caused either by hypoxia or oncogenes who can stimulate HIF-1 and thereby p21. In fact, some specific pVHL-deficient renal cell carcinomas cell lines showed such an association, although a feasible mechanism was not described [43].

BCL-2 is known as an inhibitor of apoptosis. In this study we found an inverse association between HIF-1α and BCL-2. This is in contradiction to an earlier study on melanoma cell lines that showed that BCL-2 augment the angiogenic potential of HIF-1 via increased VEGF transcription and prolonged VEGF mRNA stabilization [44]. Another paper suggested, however, that VEGF itself stimulated BCL-2 expression in breast cancer [45]. Meanwhile, loss of BCL-2 fits to the model in which upregulation of HIF-α is associated with breast cancer aggressiveness, because loss of BCL-2 is associated with tumor aggressiveness [46]. We described earlier that the rate of apoptosis and the levels of HIF-1α are both increased in aggressive breast cancers [47]. This could be an epigenetic phenomena, but other studies indeed point to a direct apoptotic effect of HIF-1 when the cell losses control of homeostasis despite HIF-1 activation [48].

Conclusion

HIF-1α overexpression is associated with increased proliferation, which may explain the adverse prognostic impact of increased levels of HIF-1α in invasive breast. In ER positive tumors HIF-1α is associated to p21, but not proliferation. This urges further functional analysis to unravel the role of HIF-1 in late cell cycle progression, and the link between HIF-1, p21 and ER.

ACKNOWLEDGEMENTS

This work was supported by unrestricted research support from the AEGON International Scholarship in Oncology [RB, PvdG,AS, AEG]. We thank Wim Vos for help with immunostaining.

REFERENCES

1. Semenza GL and Wang GL. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 1992;12:5447-5454.

2. Jiang BH, Rue E, Wang GL, Roe R, and Semenza GL. Dimerization, DNA binding, and transactivation properties of hypoxia- inducible factor 1. J Biol Chem 1996;271:17771-17778.
3. Huang LE, Arany Z, Livingston DM, and Bunn HF. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. J Biol Chem 1996;271:32253-32259.
4. Jiang BH, Semenza GL, Bauer C, and Marti HH. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. Am J Physiol 1996;271:C1172-C1180.
5. Semenza GL. Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 1999;15:551-578.
6. Ratcliffe PJ, O'Rourke JF, Maxwell PH, and Pugh CW. Oxygen sensing, hypoxia-inducible factor-1 and the regulation of mammalian gene expression. J Exp Biol 1998;201:1153-1162.
7. Zundel W, Schindler C, Haas-Kogan D, Koong A, Kaper F, Chen E, Gottschalk AR, Ryan HE, Johnson RS, Jefferson AB, Stokoe D, and Giaccia AJ. Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev 2000;14:391-396.
8. Ravi R, Mookerjee B, Bhujwalla ZM, Sutter CH, Artemov D, Zeng Q, Dillehay LE, Madan A, Semenza GL, and Bedi A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia- inducible factor 1alpha. Genes Dev 2000;14:34-44.
9. Laughner E, Taghavi P, Chiles K, Mahon PC, and 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;21:3995-4004.
10. Bos R, Zhong H, Hanrahan CF, Mommers EC, Semenza GL, Pinedo HM, Abeloff MD, Simons JW, van Diest PJ, and van der Wall E. Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. J Natl Cancer Inst 2001;93:309-314.
11. Bos R, van der Groep P, Greijer AE, Shvarts A, Meijer S, Pinedo HM, Semenza GL, van Diest PJ, and van der Wall E. Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. Cancer 2003;97:1573-1581.
12. van Diest PJ and Baak JP. The morphometric prognostic index is the strongest prognosticator in premenopausal lymph node-negative and lymph node-positive breast cancer patients. Hum Pathol 1991;22:326-330.
13. Elston CW and Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991;19:403-410.
14. van Diest PJ. No consent should be needed for using leftover body material for scientific purposes. BMJ 2002;325:648-651.
15. Bobrow MN, Litt GJ, Shaughnessy KJ, Mayer PC, and Conlon J. The use of catalyzed reporter deposition as a means of signal amplification in a variety of formats. J Immunol Methods 1992;150:145-149.
16. Mommers EC, Leonhart AM, Falix F, Michalides R, Meijer CJ, Baak JP, and Diest PJ. Similarity in expression of cell cycle proteins between in situ and invasive ductal breast lesions of same differentiation grade. J Pathol 2001;194:327-333.
17. Blomberg I and Hoffmann I. Ectopic expression of Cdc25A accelerates the G(1)/S transition and leads to premature activation of cycl. Mol Cell Biol 1999;19:6183-6194.
18. Bukholm IR, Bukholm G, and Nesland JM. Over-expression of cyclin A is highly associated with early relapse and reduced survival in patients with primary breast carcinomas. Int J Cancer 2001;93:283-287.
19. Michalides R, van Tinteren H, Balkenende A, Vermorken JB, Benraadt J, Huldij J, and van Diest P. Cyclin A is a prognostic indicator in early stage breast cancer with and without tamoxifen treatment. Br J Cancer 2002;86:402-408.
20. van Diest PJ, Brugal G, and Baak JP. Proliferation markers in tumours: interpretation and clinical value. J Clin Pathol 1998;51:716-724.
21. Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshert E, and Keshet E. Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature 1998;394:485-490.
22. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, and Simons JW. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 1999;59:5830-5835.
23. Bos R, van Diest PJ, van der GP, Greijer AE, Hermsen MA, Heijnen I, Meijer GA, Baak JP, Pinedo HM,

van der WE, and Shvarts A. Protein expression of B-cell lymphoma gene 6 (BCL-6) in invasive breast cancer is associated with cyclin D1 and hypoxia-inducible factor-1alpha (HIF-1alpha). Oncogene 2003;22:8948-8951.
24. Feldser D, Agani F, Iyer NV, Pak B, Ferreira G, and Semenza GL. Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. Cancer Res 1999;59:3915-3918.
25. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, and Ratcliffe PJ. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 1999;399:271-275.
26. Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM, Simons JW, and Semenza GL. Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res 2000;60:1541-1545.
27. Rose F, Grimminger F, Appel J, Heller M, Pies V, Weissmann N, Fink L, Schmidt S, Krick S, Camenisch G, Gassmann M, Seeger W, and Hanze J. Hypoxic pulmonary artery fibroblasts trigger proliferation of vascular smooth muscle cells: role of hypoxia-inducible transcription factors. FASEB J 2002;16:1660-1661.
28. Goda N, Ryan HE, Khadivi B, McNulty W, Rickert RC, and Johnson RS. Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia. Mol Cell Biol 2003;23:359-369.
29. De Jong JS, van Diest PJ, Michalides RJ, and Baak JP. Concerted overexpression of the genes encoding p21 and cyclin D1 is associated with growth inhibition and differentiation in various carcinomas. Mol Pathol 1999;52:78-83.
30. Sivridis E, Giatromanolaki A, Gatter KC, Harris AL, and Koukourakis MI. Association of hypoxia-inducible factors 1alpha and 2alpha with activated angiogenic pathways and prognosis in patients with endometrial carcinoma. Cancer 2002;95:1055-1063.
31. Schindl M, Schoppmann SF, Samonigg H, Hausmaninger H, Kwasny W, Gnant M, Jakesz R, Kubista E, Birner P, and Oberhuber G. Overexpression of Hypoxia-inducible Factor 1alpha Is Associated with an Unfavorable Prognosis in Lymph Node-positive Breast Cancer. Clin Cancer Res 2002;8:1831-1837.
32. Mabjeesh NJ, Willard MT, Frederickson CE, Zhong H, and Simons JW. Androgens stimulate hypoxia-inducible factor 1 activation via autocrine loop of tyrosine kinase receptor/phosphatidylinositol 3'-kinase/protein kinase B in prostate cancer cells. Clin Cancer Res 2003;9:2416-2425.
33. Helczynska K, Kronblad A, Jogi A, Nilsson E, Beckman S, Landberg G, and Pahlman S. Hypoxia promotes a dedifferentiated phenotype in ductal breast carcinoma in situ. Cancer Res 2003;63:1441-1444.
34. Stoner M, Saville B, Wormke M, Dean D, Burghardt R, and Safe S. Hypoxia induces proteasome-dependent degradation of estrogen receptor alpha in ZR-75 breast cancer cells. Mol Endocrinol 2002;16:2231-2242.
35. An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV, and Neckers LM. Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. Nature 1998;392:405-408.
36. Piret JP, Mottet D, Raes M, and Michiels C. Is HIF-1alpha a pro- or an anti-apoptotic protein? Biochem Pharmacol 2002;64:889-892.
37. Bruick RK. Expression of the gene encoding the proapoptotic Nip3 protein is induced by hypoxia. Proc Natl Acad Sci U S A 2000;97:9082-9087.
38. Blagosklonny MV, An WG, Romanova LY, Trepel J, Fojo T, and Neckers L. p53 inhibits hypoxia-inducible factor-stimulated transcription. J Biol Chem 1998;273:11995-11998.
39. Linderholm B, Lindh B, Tavelin B, Grankvist K, and Henriksson R. p53 and vascular-endothelial-growth-factor (VEGF) expression predicts outcome in 833 patients with primary breast carcinoma. Int J Cancer 2000;89:51-62.
40. Birner P, Schindl M, Obermair A, Breitenecker G, and Oberhuber G. Expression of hypoxia-inducible factor 1alpha in epithelial ovarian tumors: its impact on prognosis and on response to chemotherapy. Clin Cancer Res 2001;7:1661-1668.
41. van Diest PJ, Michalides RJ, Jannink L, van d, V, Peterse HL, De Jong JS, Meijer CJ, and Baak JP. Cyclin D1 expression in invasive breast cancer. Correlations and prognostic value. Am J Pathol 1997;150:705-711.
42. Zwijsen RM, Wientjens E, Klompmaker R, van der SJ, Bernards R, and Michalides RJ. CDK-independent activation of estrogen receptor by cyclin D1. Cell 1997;88:405-415.
43. Bindra RS, Vasselli JR, Stearman R, Linehan WM, and Klausner RD. VHL-mediated hypoxia regulation of cyclin D1 in renal carcinoma cells. Cancer Res 2002;62:3014-3019.

44. Iervolino A, Trisciuoglio D, Ribatti D, Candiloro A, Biroccio A, Zupi G, and Del Bufalo D. Bcl-2 overexpression in human melanoma cells increases angiogenesis through VEGF mRNA stabilization and HIF-1-mediated transcriptional activity. FASEB J 2002;16:1453-1455.
45. Pidgeon GP, Barr MP, Harmey JH, Foley DA, and Bouchier-Hayes DJ. Vascular endothelial growth factor (VEGF) upregulates BCL-2 and inhibits apoptosis in human and murine mammary adenocarcinoma cells. Br J Cancer 2001;85:273-278.
46. Villar E, Redondo M, Rodrigo I, Garcia J, Avila E, and Matilla A. bcl-2 Expression and apoptosis in primary and metastatic breast carcinomas. Tumour Biol 2001;22:137-145.
47. Bos R, van Diest PJ, and van der Wall E. RESPONSE: Re: Levels of Hypoxia-Inducible Factor-1 During Breast Carcinogenesis. J Natl Cancer Inst 2001;93:1177-1177.
48. Sowter HM, Ratcliffe PJ, Watson P, Greenberg AH, and Harris AL. HIF-1-dependent regulation of hypoxic induction of the cell death factors BNIP3 and NIX in human tumors. Cancer Res 2001;61:6669-6673.

Chapter 4

Protein expression of B-cell lymphoma gene 6 (BCL-6) in invasive breast cancer is associated with cyclin D1 and hypoxia-inducible factor-1α (HIF-1α)

Oncogene 2003;22:8948-8951

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BACKGROUND. B-cell lymphoma gene (BCL-6) upregulation contributes to immortalization of mouse embryo fibroblast and primary B cells via upregulation of cyclin D_1. As cyclin D_1 overexpression is a common phenomenon in different cancers, BCL-6 protein overexpression may not be restricted to lymphomas.

METHODS & RESULTS. In this study, expression of BCL-6 was investigated by immunohistochemistry on paraffin-embedded specimens from 150 breast cancer patients and 10 specimens of normal breast tissue. The results showed BCL-6 overexpression (≥10% of cells) in 24/150 (16%) breast cancer patients, whereas in normal breast low expression (<1%) of BCL-6 was observed. In linear regression analysis BCL-6 expression was associated with cyclin D_1 (r=0.197, P=0.016). Further, in χ^2 analyses, BCL-6-positivity was associated with overexpression of p53 (P=0.016), and hypoxia-inducible factor-1α (P<0.001). Involvement of BCL-6 in breast carcinogenesis is further underscored by comparative genomic hybridization analysis that showed gains at the BCL-6 locus (3q27) in 14/86 (16%) breast cancer tissues. The cases with amplification in BCL-6 showed an increased (25%) incidence of BCL-6 protein overexpression.

CONCLUSIONS. Thus, this study is the first to show that BCL-6 oncogene activation plays a role in cancers other than lymphomas.

THE PROTO-ONCOGENE B-cell lymphoma gene 6 (BCL-6) is located on 3q27, which is the third most common translocated region in non-Hodgkin lymphoma (NHL). The BCL-6 gene encodes a protein that harbors transcriptional repressor activity [1]. Genes carrying its recognition site in the promotor region include many lymphocyte activation genes such as MIP-1α and IP-10 and Blimp-1, and cyclin D_2 [2]. As yet, not all target genes of BCL-6 have been identified and its real impact and function is still not clarified. BCL-6 is believed to be involved in germinal center B-cell function and the immune response [3]. Deregulation of BCL-6 seemed to be uniquely associated with lymphomagenesis, especially for non-Hodgkin B-cell lymphomas such as the diffuse large cell, follicular and marginal zone subtypes [4], with overexpression levels up to 90% [5]. Recently, we reported the involvement of BCL-6 in mouse embryonic fibroblast senescence via upregulation of cyclin D_1 [6], indicating a more general role for BCL-6 in oncogenesis. Cyclin D_1 is frequently expressed in invasive breast cancer [7], partly explained by amplification and estrogen receptor α (ERα) activation, but the mechanism is not clear in all cases. BCL-6 could be involved here.

Therefore, we investigated if BCL-6 is expressed in invasive breast cancer and whether this is associated with expression of cyclin D_1 and other biomarkers. We analyzed protein levels of BCL-6 by immunohistochemistry in 150 breast cancers previously characterized for expression of the hypoxia-inducible factor-1α (HIF-1α), cyclin D_1 and other common clinicopathologic features [8].

PATIENTS & METHODS

Patients

As described previously [8], a representative group of 150 breast cancer patients, diagnosed between 1985 and 1993 at the VU University Medical Center, Amsterdam, The Netherlands, was randomly selected. All patients underwent either breast conserving therapy or modified radical mastectomy. For all patients, axillary dissection including at least levels I and II was performed. Histological classification was performed using the WHO criteria. Tumors were graded following the criteria of Elston and Ellis [9]. The mitotic activity index (MAI) was assessed according to an established reproducible protocol [10]. None of the patients received preoperative chemo-, hormonal or radiotherapy.

For comparative genomic hybridization, a separate group of 86 patients was used and has in part (n = 53) been described before [11], and was diagnosed between 1993 and 1998 at the Department of Pathology of VUMC (n = 43) and the Medical Centre Alkmaar (Alkmaar, The Netherlands) (n = 43). These tumors had a mean size of 1.9 cm (range 0.5–6.5), and mean age at the time of diagnosis was 53, ranging from 28 to 84.

Immunohistochemistry

After deparaffination and rehydration, antigen retrieval for BCL-6 was performed in an autoclave (120°C, 20 min) with 4 μm thick slides placed in a citrate buffer (pH 6). A mouse monoclonal antibody against BCL-6 (1 : 25, clone PG-B6P, DAKO, Glostrup, Denmark) was incubated 60 min at room temperature. The primary antibody was detected using a biotinylated rabbit anti-mouse antibody (DAKO). This sig-

nal was amplified by avidin–biotin complex formation and developed with diaminobenzidine. Appropriate negative (obtained by omission of the primary antibody) and positive controls (normal tonsil with germinal centers) were used throughout. The percentage of tumor cells harboring nuclear BCL-6 expression was estimated in each case by one pathologist (PvD), who was blinded to any outcome. Staining and scoring protocols for the other biomarkers have been described elsewhere [8].

Comparative genomic hybridization

Comparative genomic hybridization (CGH) was performed as described before [11] on DNA extracted from frozen tumor tissue with the QiAmp Tissue Kit (Qiagen GmbH, Hilden, Germany). Results of tumor to normal fluorescence ratios with 95% confidence intervals were plotted in relative copy number karyotypes

Statistical analysis

Nonparametric χ^2 and Mann–Whitney test (SPSS for Windows Version 9.0.1, 1999, SPSS Inc., Chicago, IL, USA) were used to evaluate correlations. Traditional cutoff values for the other biomarkers were used. For BCL-6, a cut off value of 10% for high BCL-6 protein expression was chosen based on Ree et al. [12].

Results and Discussion

BCL-6 immunohistochemistry

In normal breast tissue (n = 10), weak nuclear staining (<1% of cells) of BCL-6 was noted in glandular epithelial cells. In 53/150 (35%) cases of invasive breast cancer, BCL-6 nuclear expression (≥1%) was noted (mean 14%, range 1–75%). BCL-6 overexpression (≥10%) was found in 24/150 (16%) breast cancers. Nuclear expression of BCL-6 was sometimes seen in adjacent morphologically normal breast, ductal hyperplasia of usual type and ductal carcinoma in situ (DCIS). A few cases showed nuclear expression of BCL-6 in DCIS but not in the invasive tumor. The staining pattern of BCL-6 varied between scattered, diffuse (Figure 1A) and in two cases around necrotic tumor areas (Figure 1B).

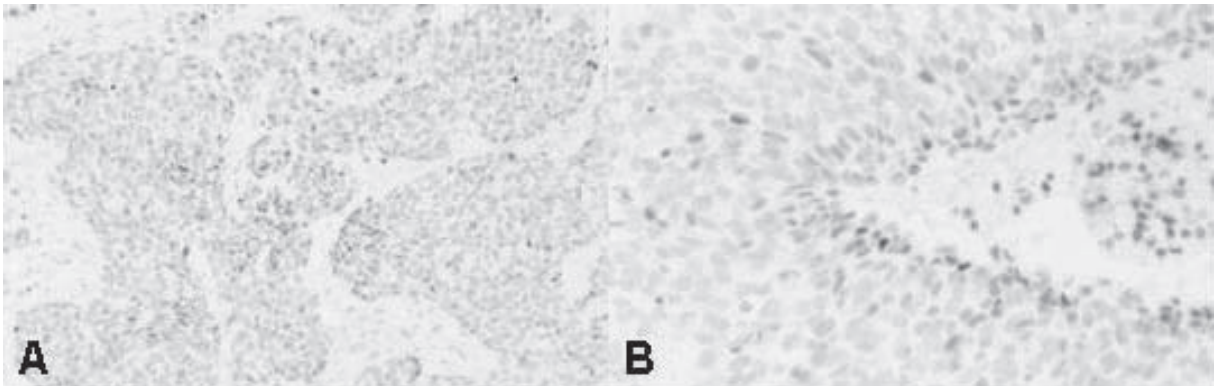

Figure 1 Invasive ductal breast cancer showing diffuse (A) and perinecrotic (B) nuclear BCL-6 immunohistochemical staining (microscope magnification ×20). (for **color plate** see **p.126**)

Thus, the dogma that BCL-6 overexpression is a unique feature of lymphomas

should be discarded now that BCL-6 protein expression has been noticed in invasive breast cancer. Increased BCL-6 nuclear expression was seen in 35% of invasive breast cancers and BCL-6 protein overexpression was found in 16% of cases. Also, the recognized breast cancer precursor lesions ductal hyperplasia and DCIS showed increased expression sometimes. The expression was largely nuclear where the protein is functionally active, but in two tissues a strong diffuse cytoplasmic staining was seen, which could point to truncated forms or splice variants, sequestered in the cytoplasm.

CGH: 3q27 gains and protein levels of BCL-6

For determining a genetic basis of BCL-6 overexpression, we investigated 3q27 gains in 86 breast cancers by comparative genomic hybridization (CGH). in a separate patient group, in part (n = 53) described before [11]. In 14/86 (16%) patients, a gain of 3q27, the BCL-6 locus was found. Paraffin blocks were available for 12 of these 14 patients. BCL-6 immunohistochemistry on these cases revealed that 3/12 (25%) had overexpression (≥10%) of BCL-6, another three cases showed 5% of BCL-6 expression, two cases showed diffuse strong cytoplasmic expression, while in four of 12 (33%) cases no BCL-6 protein could be detected (Table 1). In our reference group of 150 breast cancers, 97 cases were completely BCL-6 negative (P=0.03, χ^2 test) (Table 1). Four of the 14 cases with a gain of 3q27 (28%) had an amplification of 11q13 (locus of cyclin D_1), while gain at 11q13 was seen in 24/86 (28%) of the reference group (P=0.950).

TABLE 1 Comparison in BCL-6 protein expression levels between the group with CGH detected gains in the BCL-6 locus and the original group of 150 breast cancer patients

	BCL-6 protein expression			
	≥ 10%	≥ 5%	no expression	Total
Selected group with gains in BCL-6 locus	3 (25%)	3 (25%)	4 (33%)	12 (100%)
Original group of 150 patients	24 (16%)	16 (11%)	97 (64%)	150 (100%)

Genetic alterations are the main reason for the tumor biological activity of BCL-6 in lymphomas. The data from this study imply that BCL-6 gene amplification could be responsible for protein overexpression in these cases, since the CGH data of 86 breast cancers revealed a gain at the locus of BCL-6 (3q27) in 16% of cases, of which 25% had BCL-6 overexpression at the protein level. However, 33% of the 3q27-positive cases showed no BCL-6 protein expression at all, which might be explained by promoter methylation, or post-transcriptional regulation of the gene. On the other hand, the gain may be due to amplification of another gene located at 3q27.

Immunohistochemistry: association of BCL-6 with other markers

As shown in Table 2, BCL-6 nuclear overexpression was significantly associated with overexpression of HIF-1α (P<0.001). HIF-1 is a transcription factor consisting of two protein subunits, HIF-1α and HIF-1β. While HIF-1β is constitutively present, HIF-1α is under normoxia continuously degraded by the proteasome via ubiquitination by

the von Hippel–Lindau tumor suppressor E3 ligase complex [13].

TABLE 2. Associations of BCL-6 protein expression with clinicopathologic variables in 150 invasive breast cancers.

	BCL-6 expression		
	< 10%	≥ 10%	*P* (χ^2)*
Histologic grade			
Well	28	7	0.621
Moderate	43	6	
Poor	55	11	
Mitotic Activity Index			
≤ 10	72	15	0.626
>10	54	9	
Lymph node status			
Negative	65	16	0.174
Positive	61	8	
Tumor size			
T_1 (0-2 cm)	36	9	0.268
T_2 (2-5 cm)	79	15	
T_3 (>5 cm)	11	0	
Age			
< 55 year	42	6	0.422
≥ 55 year	84	18	
HER-2/neu			
Negative	108	18	0.189
Positive	18	6	
Estrogen Receptor			
Negative	84	14	0.432
Positive	42	10	
Progesteron Receptor			
Negative	75	17	0.297
Positive	51	7	
Ki-67			
≤ 10 %	61	10	0.544
> 10 %	65	14	
Cyclin D_1			
≤ 10 %	107	17	0.095
> 10 %	19	7	
p21			
≤ 10 %	110	20	0.600
> 10 %	16	4	
p53			
≤ 5 %	98	13	0.016
> 5 %	28	11	
HIF-1α			
≤ 5 %	91	8	<0.001
> 5 %	35	16	

During hypoxia, ubiquitination of HIF-1α is blocked and HIF-1α becomes overexpressed, leading to the formation of the active HIF-1 complex in the nucleus. As a survival response, HIF-1 activates several target genes involved in angiogenesis, and glucose/iron metabolism such as vascular endothelial growth factor, glucose transporter1, erythropoietin, transferrin and insulin growth factor-2 [14]. Recently, several other mechanisms of activating HIF-1 have been defined such as the tumor suppressor genes p53, pVHL and PTEN, oncogenes as HER-2/*neu*, and growth factors such as insulin-like growth factor-1 [14]. Hereby, a more direct role of HIF-1 in oncogenesis is postulated. This potential role of HIF-1 in cancer is elucidated by the presence of HIF-1α in different types of solid tumors [15], increased levels of HIF-1α during breast carcinogenesis [16] and high levels predicting poor prognosis in lymph node-negative breast cancer [8]. In this study, BCL-6 and HIF-1α overexpression were found to be positively correlated. As yet, the question arises whether BCL-6 is the next oncogene with HIF-1 activating capacity.

BCL-6 bypasses the senescence response in mouse embryonic fibroblasts and primary B cells by ignoring p53 and causing sequestration of p21 by cyclin D_1 [6]. As HIF-1α is strongly associated with p21 expression [17], this might explain the relation between HIF-1 and BCL-6 reported in the present study. Growth inhibitory signals from hypoxia via the HIF-1 pathway would be counteracted by the presence of BCL-6 and BCL-6-regulated genes. The presence of high levels of p21 would thereby reflect the initial response of the cells to stress from hypoxia.

The current finding that HIF-1α is associated with BCL-6 raises the question as to how HIF-1, as a transcription factor, and BCL-6, as a transcription repressor, are related to each other. This is not unique for breast cancer, as we have also observed

concerted overexpression of HIF-1α and BCL-6 in diffuse largecell B-cell lymphomas (unpublished results). No hypoxia response element has been reported in the transcriptional region of BCL-6. One possible explanation could be the repression of VHL by BCL-6. VHL degrades the HIF-1α protein by making it eligible for ubiquitination. Loss of VHL has already been associated with high levels of cyclin D_1 [18]. Downregulation of VHL by BCL-6 would lead to higher levels of HIF-1 α. However, to date we have no direct data to support this hypothesis. On the other hand, we also occasionally noticed BCL-6 expression in perinecrotic areas as for HIF-1α staining, implying that BCL-6 may be induced by hypoxia. Further research is definitely required to test these assumptions and hypotheses.

BCL-6 nuclear overexpression was also significantly associated with p53 overexpression (*P*=0.016). This finding might be due to the general genetic instability caused by p53 mutations, which might give rise to genetic alterations in the BCL-6 gene. A trend was found with cyclin D_1 (*P*=0.095), whereas other markers (Table 2) did not show a relationship with BCL-6. Linear regression analysis showed that HIF-1α (*r*=0.225, *P*=0.006) and cyclin D_1 (*r*=0.197, *P*= 0.016), but not p53, p21, MAI or tumor size, were associated with BCL-6 expression (Table 3).

TABLE 3 Relationship between BCL-6 protein expression and different biomarkers in breast cancer using Pearson correlation test

Biomarker	*r**	*P*
Mitotic activity index	0.016	0.850
Tumor size (cm)	-1.08	0.189
HIF-1α	0.225	0.006
P53	0.007	0.930
P21	0.093	0.258
Cyclin D_1	0.197	0.016

*linear regression analysis with two-sided *P* values below 0.05 were regarded as significant.

In addition, a strong positive association between overexpression of both p21 and cyclin D_1 (P<0.001) was found in χ^2 analysis (data not shown), confirming our previous results [19].

In concordance with the potential of BCL-6 to immortalize mouse embryonic fibroblasts by activating cyclin D_1 [6], this study shows that BCL-6 and cyclin D_1 overexpression are associated in breast cancer. This .finding might be important since many investigators have searched for the cause of cyclin D_1 upregulation in breast cancer, which is a common phenomenon which is just partly explained by a genetic alteration (amplification, translocation) involving the cyclin D_1 gene at locus 11q13 [7]. BCL-6 therefore seems to qualify as a next upregulator of cyclin D_1. We found no correlation between 3q27 and 11q13 gains in the present study, suggesting that there is no direct causal relationship between these genetic events in breast cancer.

In conclusion, the BCL-6 locus is amplified and the BCL-6 protein is overexpressed in 16% of invasive breast cancers and is related to cyclin D_1, p53 and HIF-1α overexpression. This is the first example of overexpression of BCL-6 in other malignancies than lymphomas. Further research is necessary to investigate the potential carcinogenetic role of BCL-6 in breast cancer and other solid malignancies, and the potential therapeutic implications of these new findings.

ACKNOWLEDGEMENTS

We are grateful to Dr Mehdi Jiwa from the Medical Center Alkmaar for providing tissue material of patients for CGH and BCL-6 staining.

This work was supported by generous research support from the AEGON International Scholarship in Oncology (RB, PvdG, AEG, IH, AS), and in part by the KWF Grant 03-2825 (MAJAH).

REFERENCES

1. Chang CC, Ye BH, Chaganti RS, and Dalla-Favera R. BCL-6, a POZ/zinc-finger protein, is a sequence-specific transcriptional repressor. Proc Natl Acad Sci U S A 1996;93:6947-6952.
2. Shaffer AL, Yu X, He Y, Boldrick J, Chan EP, and Staudt LM. BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control. Immunity 2000;13:199-212.
3. Ye BH, Cattoretti G, Shen Q, Zhang J, Hawe N, de Waard R, Leung C, Nouri-Shirazi M, Orazi A, Chaganti RS, Rothman P, Stall AM, Pandolfi PP, and Dalla-Favera R. The BCL-6 proto-oncogene controls germinal-centre formation and Th2- type inflammation. Nat Genet 1997;16:161-170.
4. Ye BH. BCL-6 in the pathogenesis of non-Hodgkin's lymphoma. Cancer Invest 2000;18:356-365.
5. Dunphy CH, Polski JM, Lance EH, and Gardner LJ. Paraffin immunoreactivity of CD10, CDw75, and Bcl-6 in follicle center cell lymphoma. Leuk Lymphoma 2001;41:585-592.
6. Shvarts A, Brummelkamp TR, Scheeren F, Koh E, Daley GQ, Spits H, and Bernards R. A senescence rescue screen identifies BCL6 as an inhibitor of anti- proliferative p19(ARF)-p53 signaling. Genes Dev 2002;16:681-686.
7. van Diest PJ, Michalides RJ, Jannink L, van d, V, Peterse HL, De Jong JS, Meijer CJ, and Baak JP. Cyclin D1 expression in invasive breast cancer. Correlations and prognostic value. Am J Pathol 1997;150:705-711.
8. Bos R, van der Groep P, Greijer AE, Shvarts A, Meijer S, Pinedo HM, Semenza GL, van Diest PJ, and van der Wall E. Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. Cancer 2003;97:1573-1581.
9. Elston CW and Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991;19:403-410.
10. van Diest PJ, Baak JP, Matze-Cok P, Wisse-Brekelmans EC, van Galen CM, Kurver PH, Bellot SM, Fijnheer J, van Gorp LH, Kwee WS, and . Reproducibility of mitosis counting in 2,469 breast cancer specimens: results from the Multicenter Morphometric Mammary Carcinoma Project. Hum Pathol 1992;23:603-607.
11. Hermsen MA, Baak JP, Meijer GA, Weiss JM, Walboomers JW, Snijders PJ, and van Diest PJ. Genetic analysis of 53 lymph node-negative breast carcinomas by CGH and relation to clinical, pathological, morphometric, and DNA cytometric prognostic factors. J Pathol 1998;186:356-362.
12. Ree HJ, Ohsima K, Aozasa K, Takeuchi K, Kim CW, Yang WI, Huh JY, Lee SS, Ko YH, Kwon MS, Cho EY, Choi YL, Rhee JC, Kikuchi M, and Mori S. Detection of germinal center B-cell lymphoma in archival specimens: critical evaluation of Bcl-6 protein expression in diffuse large B-cell lymphoma of the tonsil. Hum Pathol 2003;34:610-616.
13. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, and Ratcliffe PJ. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science 2001;292:468-472.
14. Semenza GL. HIF-1 and tumor progression: pathophysiology and therapeutics. Trends Mol Med 2002;8:S62-S67.
15. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, and Simons JW. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 1999;59:5830-5835.
16. Bos R, Zhong H, Hanrahan CF, Mommers EC, Semenza GL, Pinedo HM, Abeloff MD, Simons JW, van Diest PJ, and van der Wall E. Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. J Natl Cancer Inst 2001;93:309-314.
17. Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshert E, and Keshet E. Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature 1998;394:485-490.
18. Bindra RS, Vasselli JR, Stearman R, Linehan WM, and Klausner RD. VHL-mediated hypoxia regulation of cyclin D1 in renal carcinoma cells. Cancer Res 2002;62:3014-3019.
19. De Jong JS, van Diest PJ, Michalides RJ, and Baak JP. Concerted overexpression of the genes encoding p21 and cyclin D1 is associated with growth inhibition and differentiation in various carcinomas. Mol Pathol 1999;52:78-83.

Chapter 5

Hypoxia-Inducible Factor-1α is associated with angiogenesis, and expression of bFGF, PDGF-BB, and EGFR in invasive breast cancer

Histopathology 2004; in press

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BACKGROUND. . Hypoxia-inducible factor-1 (HIF-1) is the key transcription factor regulating the cellular response to hypoxia including angiogenesis. Growth factors play an important role in tumor growth and angiogenesis and some have been shown to be induced by HIF-1 *in vitro*. This study investigated if angiogenesis or growth factors or their receptors are associated with HIF-1α in invasive breast cancer.

METHODS/RESULTS. High levels of HIF-1α, detected by immunohistochemistry in 45 breast cancers, were positively associated with increased microvessel density (as a degree for angiogenesis) (P=0.023). Further, high levels of HIF-1α were associated with epithelial expression of epidermal growth factor (EGFR) (P=0.011), platelet-derived growth factor (PDGF-BB) (P<0.001), and basic fibroblast growth factor (bFGF) (P=0.045). A positive, yet insignificant, trend for HIF-1α with epithelial expression of transforming growth factor (TGF) alpha (P=0.081) and vascular endothelial growth factor (P=0.109) was noticed as well as an inverse association with stromal expression of TGFβ-R1 (P=0.070).

CONCLUSIONS. In invasive breast cancer, HIF-1α is associated with angiogenesis, and expression of growth factors bFGF and PDGF-BB, and the receptor EGFR. Thus, agents targeting HIF-1 may combine different pathways of inhibiting breast cancer growth, including angiogenesis and growth factors.

IT HAS BEEN WELL recognized that angiogenesis plays an important role in the development, progression and dissemination of human breast cancer [1,2]. In recent years, many growth factors, growth factor receptors and growth inhibiting factors have been shown to be expressed in invasive breast cancer. In a former study [3], we showed that the expression of such factors was correlated with angiogenesis in invasive breast cancer. Therefore, growth factors and their receptors are potential targets for anti-angiogenesis therapy. Here we will rehearse shortly the characteristics of the growth factors investigated in this study.

Probably the most well studied growth factor in breast cancer is vascular endothelial growth factor A (VEGF-A). Several other members of the VEGF family have been cloned (VEGF-B, -C, and –D), but little is known about their function and regulation [4,5]. VEGF-A, a heparin-binding homodimeric glycoprotein, was originally identified as a factor that induces vascular permeability and endothelial growth. However, VEGF-A is also a potent initiator of angiogenesis and has been intensively studied as a therapeutic target [6]. Increased levels of VEGF-A indicate poor prognosis in breast cancer [7]. VEGF-A binds to its receptors VEGF R1 (also known as Flt-1) and VEGF R2 (also known as Flk-1 or KDR), which are characterized by a high affinity. In addition, VEGF-A can also bind to neuropilin-1 and neuropilin 2, but there is no evidence for intracellular signaling after binding of these proteins with VEGF-A [8,9,10].
Structurally related to VEGF is platelet-derived growth factor (PDGF). PDGF is a dimer of two polypeptide chains (A and B). Several combinations of subchains (AA, AB, BB) are possible, but in this study we focus on PDGF-AA and its PDGF-α receptor (PDGFαR), and PDGF-BB and its PDGF-β receptor (PDGFβR), because of their presence in breast cancer. The physiologic role of PDGF is to control connective tissue growth [11]. In cancer PDGF and its receptors stimulate microvascular sprouting by attracting pericytes and smooth muscle cells during angiogenesis [12]. The growth inhibiting factors, transforming growth factor β-1 (TGFβ1) and TGFβ2 are homodimers that bind their receptor couple TGFβR-I and TGFβR-II. In general, the TGFβs contribute to the maturation phase of angiogenesis. At low doses, they stimulate angiogenic growth, but at high doses they block endothelial proliferation and stimulate smooth muscle cell differentiation and recruitment (angiogenic maturation).

Basic fibroblast growth factor (bFGF, also known as FGF-2) is a member of the large fibroblast growth factor family, which share their growth and differentiation potential for cells of mesodermal and neuroectodermal origin. Due to its strong angiogenic potential, bFGF is a well studied family member. bFGF is involved in different ways in the process of angiogenesis. One way is by stimulating the synthesis of plasminogen activator and other proteases which are involved in angiogenesis [13,14]. Further, bFGF stimulates migration and DNA synthesis of endothelial cells in vitro [15].

Transforming growth factor alpha (TGFα) is released by proteolysis of membrane bound precursors, and binds to epidermal growth factor receptor (EGFR), which has sequence similarity to the HER-2/*neu* oncogene. EGFR can also be bound by epidermal growth factor (EGF). TGFα has a critical role in the normal and neoplastic development of the mammary gland [16]. In recent years, multiple pharmacological agents counteracting EGFR have been developed and tested in clinical studies [17]. Growing interest in the role of hypoxia during development of cancer and

angiogenesis showed some striking relations to some of the above mentioned growth factors/receptors. This link was found by Semenza et al, who showed that the erythropoietin gene has a hypoxia response element (HRE) in its promoter region, which can be stimulated by the transcription factor Hypoxia-Inducible Factor-1 (HIF-1) [18,19]. In fact, HIF-1 regulates many more processes to survive cellular hypoxia [20]. HIF-1 is a basic helix-loop-helix transcription factor, which consists of the two protein subunits HIF-1α and HIF-1β [21]. HIF-1β is a constitutively expressed nuclear protein, while the HIF-1α protein is subject to ongoing proteolysis. Hypoxia will lessen prolyl hydroxylation and von Hippel-Lindau E3 ubiquitination, and thus block proteolysis of HIF-1α in an oxygen dependent manner [22,23]. Therefore, levels of HIF-1α are closely correlated to the oxygen status of the cell. Besides this physiological induction, HIF-1 can also be activated by oncogenes and tumor suppressor genes such as HER-2/*neu*, PTEN, p53 and pVHL as reviewed elsewhere [24]. Following overexpression of HIF-1α, the HIF-1 complex will be formed and activate many target genes including several growth factors and receptors, such as VEGF [25], TGFβ3 [26], IGF-II [27] and VEGF R1 [28]

In breast cancer, we have shown that HIF-1α is overexpressed in preinvasive and invasive cancer, especially in the poorly differentiated types [29]. Further, we found that high levels of HIF-1α are associated with HER-2/*neu* overexpression and are independently correlated to poor prognosis in lymph node-negative breast cancer [30]. The aim of this study was to investigate if levels of HIF-1α are related to angiogenesis and several growth (inhibiting) factors and their receptors in invasive breast cancer.

MATERIAL AND METHODS

TABLE 1 Tumor characteristics of 45 cases of invasive breast cancer

Characteristic	Number	%
Tumor Grade		
I	14	31
II	19	42
III	12	27
Tumor size		
$\leq$ 2 cm	16	36
2-5 cm	27	60
$\geq$ 5 cm	2	4
Tumor type		
Ductal	39	87
Lobular	5	11
Medullary	1	2
LN status		
negative	25	56
positive	20	44
ER status		
negative	25	56
positive	20	44

LN, lymph node; ER, estrogen receptor

At the department of Pathology (VU University medical center, Amsterdam) fresh operation specimens from 45 invasive breast cancer patients were cut in slices of approximately 0.5 cm. Of each tumor one piece was snap-frozen in liquid nitrogen and stored at -80°C. The other tissue pieces were fixed in neutral 4% buffered formaldehyde and embedded in paraffin. Haematoxylin and eosin-stained sections were used for histological typing (according to the WHO) and grading [31]. Tumor characteristics are shown in table 1. Anonymous use of redundant tumor material for research purposes is part of the standard treatment agreement with patients in our hospital [32]. Paraffin-embedded 4 µm sections of two to four tissue blocks were cut for CD31 immunohistochemistry for selection of the most vascularized tumor area and microvessel counting. From the tumor block with the highest microvessel density an adjacent section was used for HIF-1α immunohistochemistry.

Immunohistochemistry for the growth (inhibiting) factors and their receptors was previously done as described earlier in detail [33] on 4

µm cryostat sections. Staining in epithelium, stroma and blood vessels were as before scored by three observers as negative (<10% cells stained) or positive (≥10% cells stained). CD31 immunohistochemistry was done as described earlier on paraffin sections [34]. After staining multiple tumor blocks from each patient for CD31, an adjacent paraffin-embedded tumor tissue sections of the "hot spot tissue block" was used for HIF-1α immunohistochemistry. Both staining procedures are based upon an avidin-biotinyl peroxidase technique. After deparaffination and rehydration, the Catalyzed Signal Amplification System (DAKO, Glostrup, Denmark) was used for detecting HIF-1α as described before [29]. In short, target retrieval solution (DAKO) was used for antigen retrieval with all slides placed in a water bath for 45 minutes at 97°C. A cooling off period of 20 minutes preceded the incubation of the anti-HIF-1α mouse monoclonal H1α67 [35] at a dilution of 1:500 (Abcam, Cambridge, United Kingdom).

Microvessel counting

Two to four paraffin-embedded tissue blocks were used for selection of the most vascularized tumor area (hot spot) for microvessel counting. Microvessels were counted by a strictly reproducible counting protocol in each available section by two observers, in the area subjectively having the highest microvessel density (MVD) at a 400x magnification (objective 40x, field diameter 445 µm) in four fields of vision [36,22,23,37,38]. The highest MVD was used for correlation with HIF-1α. All microvessel counts in the hot spots of a tumor were converted to mm^2 to obtain the hotspot MVD. The cut-off value for the hot-spot MVD for discriminating between high and low vascularized tumors was 110 microvessels per mm^2 (median).

Data analysis

For statistical analysis, (SPSS for Windows Version 9.0.1., 1999, SPSS Inc., Chicago, IL, USA) the nonparametric Chi-square or Fisher's exact test was used to evaluate correlations between HIF-1α and MVD, individual growth factors, growth factor receptors, growth inhibiting factors. For the MVD the median was used as cut-off value, for the growth factors/receptors 10% positivity of cells in either epithelium, stroma or vasculature was used as cutoff (as shown in Table 3). For HIF-1α 5% was used as cut off value as described previously [30]. Two-sided *P* values below 0.050 were regarded as significant.

RESULTS

HIF-1α overexpression was found in 25 (56%) out of 45 patients. The mean expression of HIF-1α was 13% (minimum 0%, maximum 80%) and the median was 5%. As noticed before [29,30], HIF-1α expression was predominantly perinecrotic, but occasionally also seen in tumor fields without necrosis.

As shown in table 2, high levels of HIF-1α were positively associated with increased microvessel density (*P*=0.023), epithelial expression (≥10%) of EGFR (*P*=0.011), PDGF-BB (*P*<0.001), and bFGF (*P*=0.045). A positive but insignificant trend for HIF-1α with epithelial expression of TGFα (*P*=0.081) and VEGF (*P*=0.109) was found, and a trend for an inverse association between stromal expression of

TGFβR-II (*P*=0.070) and HIF-1α. All other factors showed no associations with HIF-1α.

TABLE 2 Significant and almost significant associations of HIF-1α expression with several growth factors and microvessel density (MVD) in 45 invasive human breast cancers.

	Level of HIF-1α		
	< 5%	≥ 5%	*P* (χ^2)
MVD			
Low	14	9	0.023
High	6	16	
*EGFR**			
≤ 10 %	14	8	0.011
> 10 %	6	17	
*PDGF-BB**			
≤ 10 %	15	6	<0.001
> 10 %	5	19	
*bFGF**			
≤ 10 %	14	10	0.045
> 10 %	6	15	
*TGFα**			
≤ 10 %	14	11	0.081
> 10 %	6	14	
*TGFβ-RII^*			
≤ 10 %	10	19	0.070
> 10 %	10	6	
*VEGF**			
≤ 10 %	12	9	0.109
> 10 %	8	16	

* epithelial expression, ^ stromal expression

DISCUSSION

This translational study was undertaken to investigate if the *in vitro* associations between HIF-1 and angiogenesis, growth factors and their receptors can be reproduced in clinical human breast cancer.

To our knowledge, this is the first study reporting a positive association between HIF-1α and a high microvessel density as a measure of angiogenesis in human breast cancer. These data are in line with other tumors like oligodendrogliomas [39], endometrial cancer [40], gastrointestinal stromal tumors of the stomach [41], and colorectal carcinomas [42], where also a positive association between HIF-1α and angiogenesis was found. Since both high levels of HIF-1α and a high microvessel density are associated with a poor prognosis in breast cancer [43], and because both are related to each other, there may be a rationale for anti-HIF-1 therapy in breast cancer. Therefore, 2-methoxyestradiol (2ME2) a novel anti-tumor and anti-angiogenic agent, is of special interest, because its anti-angiogenic mechanism is based on inhibition of the HIF-1 pathway [44]. However, other anti-HIF blocking agents (as comprehensively reviewed elsewhere [45]), such as 17-AAG (HSP90 inhibition), or YC-1 (unknown mechanism) may also block HIF-1 and angiogenesis, and the results of the ongoing clinical trials should be awaited to determine the clinically most effective anti-HIF-1 drug.

In a tumor model it was suggested that HIF-1α causes the angiogenic switch via VEGF, independent from bFGF [46]. This mechanism is widely accepted and confirmed in this study, but in this study we also found a positive association between HIF-1α on the one hand and EGFR, PDGF-BB, and bFGF on the other. Thus, our *in vivo* data also favor links to other growth factors than VEGF-A. Our data are further underlined by our former study, where primarily associations between angiogenesis and bFGF, TGFα and EGFR expression in the tumor cells were found.; TGFβ2 in the stromal cells; and TGFα and TGFβ2 expression in the endothelium [47]. This may make a case for combining anti-VEGF treatment with drugs inhibiting other growth factors in breast cancer patients.

As mentioned above, we found a positive association between HIF-1α and

EGFR, PDGF-BB, and bFGF. To date, two studies investigated the interaction between HIF and EGFR, and no HRE has been defined at the EGFR locus. The association between HIF-1α and EGFR might perhaps be explained by the sequence similarity of EGFR to the HER-2/*neu* oncogene. HER-2/*neu* is known to induce HIF-1 activity [48] and both the EGFR and HER-2/*neu* proteins are receptors with tyrosine kinase activity. EGFR might be stimulated in an autocrine or paracrine fashion through TGF-α, which showed a nearly statistically significantly association with HIF-1α. Otherwise, EGFR might be stimulated by Epidermal Growth Factor which has been shown to activate the PI3K/PTEN/AKT/FRAP pathway and thereby increase levels of HIF-1α in prostate cancer *in vitro* [49]. In contrast, a central role for HIF-1 in oncogenesis was found by its activation of the TGF-α/EGFR pathway in a recent *in vitro* study in clear cell renal cell carcinomas [50]. Thus, further research to elucidate these contradictive results seems necessary.

The association between HIF-1α and PDGF-BB confirms the data of Kourembanas et al, who showed that hypoxia induced mRNA expression of PDGF *in vitro* [51]. To our knowledge, no functional HRE has been identified in the PDGF promoter, and the exact mechanism for upregulation of PDGF by hypoxia has still to be clarified. A putative HRE in the PDGF-B promoter was not eligible for hypoxic induction. Instead, it functioned as a hypoxia independent enhancer element in hepatocellular carcinoma cells [52]. However, it was recently suggested that the PDGF/PDGF-beta receptor/Akt pathway induces downstream HIF-1α gene transcription. This was based on the observation that hypoxia induced time-dependent increases in expression of PDGF and permitted induction of survival genes in neuronal cells [53]. Thus, PDGF may activate HIF-1 instead of the opposite.

The association between HIF-1α and bFGF was noted before in non-small cell lung cancer [54], although no feasible explanation was given. Two in vitro studies addressed the interaction between HIF-1 and bFGF. One study showed that endothelial cells were during hypoxia more responsive to bFGF due to an increase in the enzymes GlnNAcT-I and HS2ST, which synthesizes heparan sulfate, which in turn act as a co-receptor for bFGF and were found to be upregulated by HIF-1 [55]. Another study showed a more direct interaction between growth factors and HIF-1 in serum-starved cells [27]. Here, besides insulin, IGF-1, IGF-2, and EGF also bFGF could induce HIF-1α expression [27]. For IGF-2 Feldser et al. further showed that there is a reciprocal regulation with HIF-1α, because IGF-2 was transcriptionally activated by HIF-1, although in the absence of a HRE. Such autocrine stimulation could also exist for bFGF and HIF-1α, because growth factor signaling is generally based upon this concept. Further research, investigating this hypothesis, seems justified for a better understanding of potential effects induced by anti-HIF-1 therapy.

In conclusion, HIF-1α is associated with angiogenesis in human breast cancer and expression of growth factors PDGF-BB and bFGF, and growth factor receptor EGFR. Thus, development of drugs targeting HIF-1 may open up news ways of inhibiting breast cancer growth.

ACKNOWLEDGEMENT

This work was supported by unrestricted research support from the AEGON Interna-

tional Scholarship in Oncology [R.B., & P.vdG.].

REFERENCES

1. Folkman J, Merler E, Abernathy C, and Williams G. Isolation of a tumor factor responsible or angiogenesis. J Exp Med 1971;133:275-288.
2. Folkman J, Watson K, Ingber D, and Hanahan D. Induction of angiogenesis during the transition from hyperplasia to neoplasia. Nature 1989;339:58-61.
3. de Jong JS, van Diest PJ, van d, V, and Baak JP. Expression of growth factors, growth-inhibiting factors, and their receptors in invasive breast cancer. II: Correlations with proliferation and angiogenesis. J Pathol 1998;184:53-57.
4. Carmeliet P and Collen D. Role of vascular endothelial growth factor and vascular endothelial growth factor receptors in vascular development. Curr Top Microbiol Immunol 1999;237:133-158.
5. Eriksson U and Alitalo K. Structure, expression and receptor-binding properties of novel vascular endothelial growth factors. Curr Top Microbiol Immunol 1999;237:41-57.
6. Dachs GU, Patterson AV, Firth JD, Ratcliffe PJ, Townsend KM, Stratford IJ, and Harris AL. Targeting gene expression to hypoxic tumor cells. Nat Med 1997;3:515-520.
7. Gasparini G, Toi M, Gion M, Verderio P, Dittadi R, Hanatani M, Matsubara I, Vinante O, Bonoldi E, Boracchi P, Gatti C, Suzuki H, and Tominaga T. Prognostic significance of vascular endothelial growth factor protein in node-negative breast carcinoma. J Natl Cancer Inst 1997;89:139-147.
8. Neufeld G, Cohen T, Gengrinovitch S, and Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J 1999;13:9-22.
9. Neufeld G, Cohen T, Shraga N, Lange T, Kessler O, and Herzog Y. The neuropilins: multifunctional semaphorin and VEGF receptors that modulate axon guidance and angiogenesis. Trends Cardiovasc Med 2002;12:13-19.
10. Soker S, Takashima S, Miao HQ, Neufeld G, and Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell 1998;92:735-745.
11. Westermark B, Claesson-Welsh L, and Heldin CH. Structural and functional aspects of the receptors for platelet-derived growth factor. Prog Growth Factor Res 1989;1:253-266.
12. Carmeliet P and Jain RK. Angiogenesis in cancer and other diseases. Nature 2000;407:249-257.
13. Mignatti P, Tsuboi R, Robbins E, and Rifkin DB. In vitro angiogenesis on the human amniotic membrane: requirement for basic fibroblast growth factor-induced proteinases. J Cell Biol 1989;108:671-682.
14. Moscatelli D and Rifkin DB. Membrane and matrix localization of proteinases: a common theme in tumor cell invasion and angiogenesis. Biochim Biophys Acta 1988;948:67-85.
15. Moscatelli D, Presta M, and Rifkin DB. Purification of a factor from human placenta that stimulates capillary endothelial cell protease production, DNA synthesis, and migration. Proc Natl Acad Sci U S A 1986;83:2091-2095.
16. Humphreys RC and Hennighausen L. Transforming growth factor alpha and mouse models of human breast cancer. Oncogene 2000;19:1085-1091.
17. Giaccone G. Clinical impact of novel treatment strategies. Oncogene 2002;21:6970-6981.
18. Semenza GL and Wang GL. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 1992;12:5447-5454.
19. Wang GL, Jiang BH, Rue EA, and Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A 1995;92:5510-5514.
20. Semenza GL. HIF-1 and human disease: one highly involved factor. Genes Dev 2000;14:1983-1991.
21. Wang GL, Jiang BH, Rue EA, and Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A 1995;92:5510-5514.
22. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, and Kaelin WG, Jr. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 2001;292:464-468.
23. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, and Ratcliffe PJ. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science 2001;292:468-472.
24. Semenza GL. Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. Crit Rev Biochem Mol Biol 2000;35:71-103.

25. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, and Semenza GL. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 1996;16:4604-4613.
26. Caniggia I, Mostachfi H, Winter J, Gassmann M, Lye SJ, Kuliszewski M, and Post M. Hypoxia-inducible factor-1 mediates the biological effects of oxygen on human trophoblast differentiation through TGFbeta(3). J Clin Invest 2000;105:577-587.
27. Feldser D, Agani F, Iyer NV, Pak B, Ferreira G, and Semenza GL. Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. Cancer Res 1999;59:3915-3918.
28. Gerber HP, Condorelli F, Park J, and Ferrara N. Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. J Biol Chem 1997;272:23659-23667.
29. Bos R, Zhong H, Hanrahan CF, Mommers EC, Semenza GL, Pinedo HM, Abeloff MD, Simons JW, van Diest PJ, and van der Wall E. Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. J Natl Cancer Inst 2001;93:309-314.
30. Bos R, van der Groep P, Greijer AE, Shvarts A, Meijer S, Pinedo HM, Semenza GL, van Diest PJ, and van der Wall E. Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. Cancer 2003;97:1573-1581.
31. Elston CW and Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991;19:403-410.
32. van Diest PJ. No consent should be needed for using leftover body material for scientific purposes. BMJ 2002;325:648-651.
33. de Jong JS, van Diest PJ, van d, V, and Baak JP. Expression of growth factors, growth inhibiting factors, and their receptors in invasive breast cancer. I: An inventory in search of autocrine and paracrine loops. J Pathol 1998;184:44-52.
34. de Jong JS, van Diest PJ, van d, V, and Baak JP. Expression of growth factors, growth-inhibiting factors, and their receptors in invasive breast cancer. II: Correlations with proliferation and angiogenesis. J Pathol 1998;184:53-57.
35. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, and Simons JW. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 1999;59:5830-5835.
36. de Jong JS, van Diest PJ, and Baak JP. Heterogeneity and reproducibility of microvessel counts in breast cancer. Lab Invest 1995;73:922-926.
37. Weidner N, Semple JP, Welch WR, and Folkman J. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. N Engl J Med 1991;324:1-8.
38. Weidner N, Folkman J, Pozza F, Bevilacqua P, Allred EN, Moore DH, Meli S, and Gasparini G. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. J Natl Cancer Inst 1992;84:1875-1887.
39. Birner P, Gatterbauer B, Oberhuber G, Schindl M, Rossler K, Prodinger A, Budka H, and Hainfellner JA. Expression of hypoxia-inducible factor-1 alpha in oligodendrogliomas: its impact on prognosis and on neoangiogenesis. Cancer 2001;92:165-171.
40. Sivridis E, Giatromanolaki A, Gatter KC, Harris AL, and Koukourakis MI. Association of hypoxia-inducible factors 1alpha and 2alpha with activated angiogenic pathways and prognosis in patients with endometrial carcinoma. Cancer 2002;95:1055-1063.
41. Takahashi R, Tanaka S, Hiyama T, Ito M, Kitadai Y, Sumii M, Haruma K, and Chayama K. Hypoxia-inducible factor-1alpha expression and angiogenesis in gastrointestinal stromal tumor of the stomach. Oncol Rep 2003;10:797-802.
42. Kuwai T, Kitadai Y, Tanaka S, Onogawa S, Matsutani N, Kaio E, Ito M, and Chayama K. Expression of hypoxia-inducible factor-1alpha is associated with tumor vascularization in human colorectal carcinoma. Int J Cancer 2003;105:176-181.
43. de Jong JS, van Diest PJ, and JP AB. Hot spot microvessel density and the mitotic activity index are strong additional prognostic indicators in invasive breast cancer. Histopathology 2000;36:306-312.
44. Mabjeesh NJ, Escuin D, LaVallee TM, Pribluda VS, Swartz GM, Johnson MS, Willard MT, Zhong H, Simons JW, and Giannakakou P. 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF. Cancer Cell 2003;3:363-375.
45. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3:721-732.
46. Fang J, Yan L, Shing Y, and Moses MA. HIF-1alpha-mediated up-regulation of vascular endothelial growth factor, independent of basic fibroblast growth factor, is important in the switch to the

angiogenic phenotype during early tumorigenesis. Cancer Res 2001;61:5731-5735.
47. de Jong JS, van Diest PJ, van d, V, and Baak JP. Expression of growth factors, growth-inhibiting factors, and their receptors in invasive breast cancer. II: Correlations with proliferation and angiogenesis. J Pathol 1998;184:53-57.
48. Laughner E, Taghavi P, Chiles K, Mahon PC, and 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;21:3995-4004.
49. Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM, Simons JW, and Semenza GL. Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res 2000;60:1541-1545.
50. Gunaratnam L, Morley M, Franovic A, de Paulsen N, Mekhail K, Parolin DA, Nakamura E, Lorimer IA, and Lee S. Hypoxia inducible factor activates the transforming growth factor-alpha/epidermal growth factor receptor growth stimulatory pathway in VHL(-/-) renal cell carcinoma cells. J Biol Chem 2003;278:44966-44974.
51. Kourembanas S, Hannan RL, and Faller DV. Oxygen tension regulates the expression of the platelet-derived growth factor-B chain gene in human endothelial cells. J Clin Invest 1990;86:670-674.
52. Ulleras E, Wilcock A, Miller SJ, and Franklin GC. The sequential activation and repression of the human PDGF-B gene during chronic hypoxia reveals antagonistic roles for the depletion of oxygen and glucose. Growth Factors 2001;19:233-245.
53. Zhang SX, Gozal D, Sachleben LR, Jr., Rane M, Klein JB, and Gozal E. Hypoxia induces an autocrine-paracrine survival pathway via platelet-derived growth factor (PDGF)-B/PDGF-beta receptor/phosphatidylinositol 3-kinase/Akt signaling in RN46A neuronal cells. FASEB J 2003;17:1709-1711.
54. Giatromanolaki A, Koukourakis MI, Sivridis E, Turley H, Talks K, Pezzella F, Gatter KC, and Harris AL. Relation of hypoxia inducible factor 1 alpha and 2 alpha in operable non-small cell lung cancer to angiogenic/molecular profile of tumours and survival. Br J Cancer 2001;85:881-890.
55. Li J, Shworak NW, and Simons M. Increased responsiveness of hypoxic endothelial cells to FGF2 is mediated by HIF-1alpha-dependent regulation of enzymes involved in synthesis of heparan sulfate FGF2-binding sites. J Cell Sci 2002;115:1951-1959.

Chapter 6

Levels of Hypoxia-Inducible Factor-1α Independently Predict Prognosis in Patients with Lymph Node Negative Breast Carcinoma

Cancer 2003;97:1573-1581

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BACKGROUND. Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that plays an important role in tumor growth and metastasis by regulating energy metabolism and inducing angiogenesis to survive cellular hypoxia. Increased levels of HIF-1α, the O_2-regulated subunit of HIF-1, were noted during breast carcinogenesis. In this study, the prognostic value of HIF-1α expression and its correlation with various clinicopathologic variables in patients with invasive breast carcinoma were investigated.

METHODS. Expression levels of HIF-1α, HER-2/*neu*, estrogen receptor, and progesterone receptor were analyzed in 150 patients with early-stage breast carcinoma by immunohistochemistry. HER-2/*neu* gene amplification was investigated with automated fluorescent in situ hybridization. The mitotic activity index, histologic grade, and tumor type were assessed in hematoxylin and eosin stained specimens. Clinical data included disease-free survival, overall survival, lymph node status, and tumor size. The data were analyzed with two-sided univariate and multivariate tests, with P values < 0.05 considered significant.

RESULTS. High levels of HIF-1α had an association of borderline significance with decreased overall survival (P=0.059) and disease-free survival (P=0.110) that was ascribed completely to the subgroup of women with lymph node negative tumors (n = 81 patients; P=0.008 and P=0.004, respectively). HER-2/*neu* immunoreactivity (P<0.001) and gene amplification (P<0.001), vascular endothelial growth factor expression (P=0.016), and Ki-67 expression (P<0.001) were correlated strongly with HIF-1α positivity, although none of those factors had an independent effect on survival.

CONCLUSIONS. Increased levels of HIF-1α were associated independently with shortened survival in patients with lymph node negative breast carcinoma. Therefore, the use of immunohistochemical assessment of HIF-1α as a new predictor of poor outcome may improve clinical decision-making regarding adjuvant treatment of patients with lymph node negative breast carcinoma.

IT HAS BEEN SHOWN that intratumoral hypoxia is a poor prognostic sign in tumors that are accessible for O_2 measurements, such as carcinoma of the uterine cervix or the head and neck (for an overview, see Hockel and Vaupel [1]). Hypoxic conditions that occur during embryogenesis, cardiovascular disease, and tumor development induce the activity of hypoxia-inducible factor 1 (HIF-1), the master regulator of cellular O_2 homeostasis [2]. The transcriptional activity of a broad spectrum of genes is altered under hypoxic conditions by HIF-1 [3]. Protein products of these downstream genes function to increase O_2 delivery or to activate alternate metabolic pathways that do not require O_2. Thus, HIF-1 stimulates processes like angiogenesis, glycolysis, erythropoiesis, and (if these fail) apoptosis [2]. The transcription factor HIF-1 consists of two subunits: HIF-1α represents HIF-1 activity, because it is overexpressed rapidly in response to hypoxic conditions [4,5,6,7], and HIF-1β is expressed constitutively. HIF-1α protein has a very short half-life under normoxic conditions due to its continuous ubiquitination and proteosome-mediated degradation, which is inhibited by hypoxia [4] and by p53 [8] or von Hippel–Lindau (VHL) [9] tumor-suppressor gene defects, leading to stabilization of the HIF-1α protein. Upon stabilization, HIF-1α protein is transported into the nucleus, where it heterodimerizes with HIF-1β and binds to its DNA recognition sequence, 5'-RCGTG-3', which is present within the hypoxia response element of various target genes [10]. Activation of these genes by HIF-1 provides the cell with mechanisms to adapt to the changed environment, thereby stimulating cell survival [10,11].

By activating transcription of the gene encoding vascular endothelial growth factor (VEGF), HIF-1 plays a critical role in angiogenesis. Direct correlations between HIF-1, VEGF, and tumor angiogenesis have been demonstrated [8,10,12]. HIF-1 expression may have prognostic and therapeutic consequences because angiogenesis is a prognostic indicator [13,14]. Although the precise mechanism, and especially the initiation, of tumor angiogenesis is not understood fully, anti-angiogenic therapies are under rapid development and clinical evaluation [15]. Along with intratumoral microvessel density, increased VEGF expression is associated with a poor prognosis; therefore. several new therapies for malignant disease are based on targeting VEGF [16]. A correlation has been described between the intercapillary distance and the degree of hypoxia in cervical carcinoma, and this surrogate for hypoxia also has been identified as a poor prognostic sign [17]. Otherwise, the clinical importance of tumor metabolism and the intratumoral pH [18] provide further theoretic support for the hypothesis that HIF-1 up-regulation has a negative effect on patient survival. Recently, we found increased levels of HIF-1α during breast carcinogenesis, especially in poorly differentiated lesions [19]. In addition, it has been reported that levels of carbonic anhydrase IX, a downstream product of HIF-1, harbors prognostic potential in human breast carcinoma [20]. Thus, multiple mechanisms point to HIF-1 as a key regulator of tumor survival and progression in breast carcinoma. Based on these observations, we hypothesized that levels of HIF-1α may have prognostic value in patients with invasive breast carcinoma.

Recently, it was shown in a breast carcinoma cell line that a common genetic alteration in breast carcinoma, activation of the HER-2/*neu* oncogene, through the phosphatidylinositol-3-OH (PI-3) kinase pathway leads to such an increase in HIF-1α synthesis that not all HIF-1α produced can be degraded, thus activating HIF-1 [21]. To

date, a few studies, all based on a relatively small number of patients, have investigated the prognostic relevance of HIF-1 in human solid tumors, and none of those studies compared this relevance with that of HER-2/*neu* amplification. In patients with early cervical carcinoma (*n* = 91 patients) [22], oropharyngeal carcinoma (*n* = 98 patients) [23], oligodendrogliomas (*n* = 51 patients) [24], and nonsmall cell lung carcinomas (*n* = 108 patients) [25], high levels of HIF-1α predicted poor prognosis; however, the opposite was reported for lung carcinoma (*n* = 55 patients) [26] and head and neck squamous cell carcinoma (*n* = 69 patients) [27], whereas no effect on survival was noticed in patients with ovarian carcinoma (*n* = 60 patients) [28]. Therefore, studies based on adequate numbers of patients with long-term follow-up are needed to elucidate the prognostic relevance of HIF-1α in specific types of human malignancies. In addition, such studies should try to address the initiator(s) of HIF-1 activity: hypoxia and/or oncogene activation.

In this study, the prognostic value of HIF-1α was investigated for the first time in a large group of 150 patients who had early-stage breast carcinoma with long-term follow-up. HIF-1α was related to other established prognostic factors, such as the proliferation rate (expressed as the mitotic activity index [MAI]) [29,30], histologic grade, lymph node status, and tumor size. In particular, HER-2/*neu* immunoreactivity and gene copy numbers were investigated. Furthermore, levels of HIF-1α were compared with levels of estrogen receptor (ER) and progesterone receptor (PR) as predictors of antihormone treatment response. In addition, the expression of VEGF was investigated as a downstream effector of HIF-1.

MATERIALS AND METHODS

Patients

A representative group of 153 patients with Stage I–II breast carcinoma who were diagnosed between 1985 and 1993 at the VU University Medical Center (Amsterdam, The Netherlands) was comprised as follows: A search in our tumor bank for patients with Stage I–II invasive breast carcinoma yielded 169 patients with paraffin blocks available. From these, adequate follow-up data were available on 153 patients, who were used for the current study. The mean age at the time of diagnosis was 60 years (range, 30–86 years). Anonymous use of redundant tumor material for research purposes is part of the standard treatment agreement with patients in our hospital. Breast-conserving therapy or modified radical mastectomy were the surgical procedures performed on the primary tumors. Axillary dissection, including at least levels I and II, was undergone by 150 of 153 patients. For consistency in statistical analysis, the three patients with unknown lymph node status were excluded from this study. All surgical specimens were fixed in neutral 4% buffered formaldehyde with minimal fixation delay.

Invasive breast carcinomas were classified histologically according to the World Health Organization criteria as ductal (*n* = 129 tumors), lobular (*n* = 11 tumors), mucinous (*n* = 4 tumors), tubular (*n* = 3 tumors), cribriform (*n* = 1 tumor), medullary (*n* = 1 tumor), or metaplastic (*n* = 1 tumor) [31]. Tumors were graded following the criteria of Elston as Grade 1 (*n* = 35 tumors), Grade 2 (*n* = 49 tumors), or Grade 3 (*n* = 66 tumors) [32]. The mean greatest tumor dimension was 2.6 cm (range, 0.7–7.0 cm) ac-

cording to the TNM classification system (45 T_1 tumors, 94 T_2 tumors, and 11 T_3 tumors) [33]. Locally advanced breast carcinomas (TNM Stage III) were excluded. The group included 81 patients (54%) with negative lymph node status and 69 patients (46%) with positive lymph node status. None of the patients received preoperative chemotherapy, radiotherapy, or hormone therapy. Only patients with positive lymph node status underwent axillary radiotherapy when they had four or more positive lymph nodes or extracapsular metastatic growth and received adjuvant chemotherapy (cyclophosphamide, methotrexate, and 5-fluorouracil) when premenopausal or tamoxifen when postmenopausal. The follow-up was 26–174 months (mean, 106 months) for surviving patients. During follow-up, 38 patients developed locoregional recurrence (*n* = 5 patients) and/or distant metastases (*n* = 36 patients), leading to 23 disease-related deaths. Four additional patients died from causes other than breast carcinoma and were censored in the survival analysis.

Immunohistochemistry

Paraffin embedded tumor tissue sections were obtained from the archives of the Department of Pathology. Immunohistochemistry was performed on 4-μm thick sections. After deparaffinization and rehydration, the Catalyzed Signal Amplification System (DAKO, Glostrup, Denmark) was used for detecting HIF-1α, as described previously [19,34]. Briefly, target retrieval solution (DAKO) was used for antigen retrieval with all slides placed in a water bath for 45 minutes at 97 °C. A cooling off period of 20 minutes preceded the incubation of the anti-HIF-1α mouse monoclonal H1α67 at a dilution of 1:500 (Abcam, Cambridge, United Kingdom) [35]. For ER, PR, and VEGF, antigen retrieval was performed in an autoclave with the slides placed in a citrate buffer, pH 6.0. Mouse monoclonal antibodies against ER (1:50 dilution; DAKO), PR (1:50 dilution; Novocastra Laboratories, Newcastle upon Tyne, United Kingdom), HER-2/*neu* (1:10,000 dilution; Dr. M. van der Vijver, Dutch Cancer Institute, Amsterdam, The Netherlands), and a rabbit polyclonal antibody against VEGF (1:40 dilution; R&D systems, Abington, United Kingdom) were used. All primary antibodies were incubated overnight at 4 °C and were detected using a biotinylated rabbit antimouse (or swine antirabbit) antibody (DAKO). The signal was amplified by avidin-biotin complex formation and was developed with diaminobenzidine followed by hematoxylin counterstaining. The sections were dehydrated in alcohol and xylene and were mounted on slides.

Staining was scored by two observers who were blinded to clinical outcome. HIF-1α was assessed with stereology-based, semiautomated immunoquantification with an interactive digitizing video overlay system (QPRODIT; Leica, Cambridge, United Kingdom). For HIF-1α, the 66.7th percentile (≥ 5% HIF-1α) was used as the cut-off value to be in balance with the expected number of events (1 in 3) in this study population. ER and PR status were determined by the Histoscore [36], in which a score ≥ 100 was considered positive. The presence or absence of HER- 2/*neu* membrane staining was scored as positive or negative, respectively. Cytoplasmic VEGF expression as assessed as moderate or strong, as described previously [34]. In the hematoxylin and eosin stained sections, the MAI was counted, as described previously [37], with a traditional cut-off value of 10/ 1.6 mm^2.

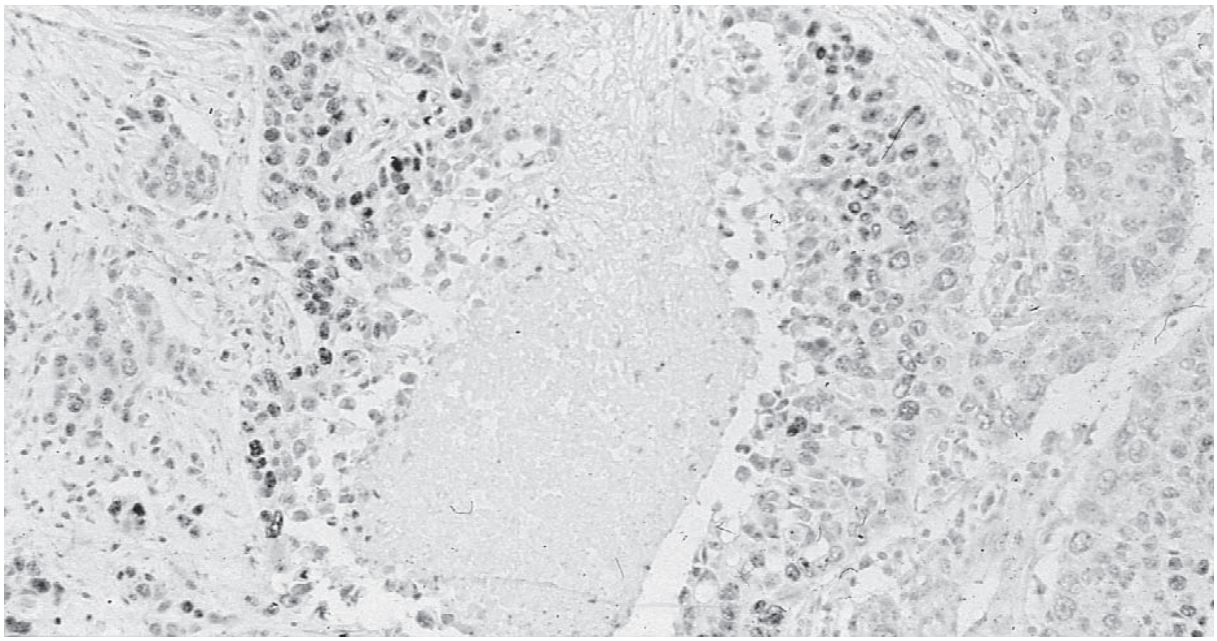

FIGURE 1. An immunohistochemical staining of hypoxia-inducible factor-1α in patients with invasive breast carcinoma. Definite nuclear staining is visible in perinecrotic areas. (for **color plate** see **p.127**)

Fluorescence in Situ Hybridization

The HER-2/*neu* gene copy number was determined by fluorescent in situ hybridization using a United States Food and Drug Administration-approved, automated staining machine (Benchmark, Ventana), following the manufacturer's recommendations in three consecutive rounds with altered digestion times until adequate signal was detected (137 of 150 tumors). Four or fewer copy numbers in single and nonoverlapping nuclei were considered normal, and more copy numbers were considered amplified.

Statistical Methods

For univariate survival analysis (SPSS for Windows, version 9.0.1; SPSS Inc., Chicago, IL), Kaplan–Meier curves were plotted, and differences between the curves were analyzed with the log-rank test. Multivariate survival analysis (Cox regression) was done to identify independent prognostic variables, with MAI, differentiation grade, tumor size, lymph node status, age, and HIF-1α expression entered as covariates. The entire patient cohort was included in this analysis as well as the separate subgroups of patients with lymph node negative disease and lymph node positive disease.

The nonparametric chi-square tests and Mann–Whitney tests were used to evaluate correlations between HIF-1α and tumor size, lymph node status, HER-2/*neu* expression. Two-sided *P* values <0.05 were considered significant.

RESULTS

Detectable levels of HIF-1α (expression in ≥ 1% of cells) were found in 75% of breast carcinomas. The mean level of nuclear staining for HIF-1α was 7% of cells (median, 1%; range, 0–79%). HIF-1α staining often was heterogeneous and was ob-

served especially in viable cells surrounding areas of necrosis (Fig. 1).

Univariate survival analysis for the whole group of patients showed a borderline inverse correlation between high levels of HIF-1α (≥ 5%), disease-free survival (*P*=0.110), and overall survival (*P*=0.059), as shown in Table 1 and Figure 2.

TABLE 1. Univariate analysis of disease-free survival and overall survival results for the entire group of 150 patients with stage I/II invasive breast carcinoma

Variable	N	% disease	% disease-free	*P*	Log rank	% dead	% alive	*P*	Log rank
HIF-1α									
< 5%	99	21	79	0.110	2.55	11	89	0.059	3.56
≥ 5%	51	33	67			24	76		
Mitotic activity index									
≤ 10	87	22	78	0.154	2.03	10	90	0.322	4.59
> 10	63	31	69			22	78		
Lymph node status									
Negative	81	19	81	0.048	3.91	11	89	0.142	2.16
Positive	69	33	67			20	80		
Histologic grade									
Well	35	14	86	0.203	3.19	6	94	0.006	10.27
Moderate	49	26	71			8	92		
Poor	66	31	71			26	74		
Tumor size									
T_1 (0-2 cm)	45	13	87	0.084	4.95	4	96	0.061	5.60
T_2 (2-5 cm)	94	31	69			20	80		
T_3 (> 5 cm)	11	27	73			18	82		
Age									
< 55 years	48	33	67	0.156	2.02	25	75	0.038	4.30
≥ 55 years	102	22	78			11	89		

Multivariate survival analysis identified MAI, tumor size, and HIF-1α (hazard ratio, 1.672; 95% confidence interval [95% CI], 0.882–3.170) as independent prognostic indicators for the whole group of patients for disease-free survival. For overall survival, MAI and levels of HIF-1α (hazard ratio, 2.155; 95%CI, 0.951–4.885) were independent prognostic variables.

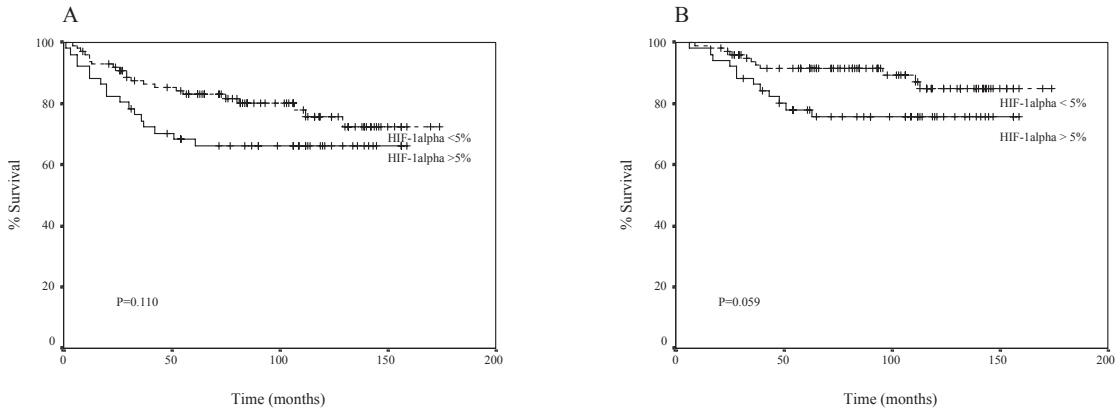

FIGURE 2. Kaplan–Meier survival curves illustrating disease-free survival (A) and overall survival (B) for 150 breast carcinoma patients with low (< 5%) versus high expression levels of HIF-1α (≥ 5%) (mean follow-up, 106 months).

TABLE 2 Association of hypoxia-inducible factor-1α expression with known clinicopathological features in a group of 150 patients with stage I/II invasive breast carcinoma.

	N	HIF-1α level <5%	HIF-1α level ≥5%	*P* (χ^2)
Histologic type				
Ductal	129	83	46	NA
Lobular	11	8	3	
Tubular	3	3	0	
Mucinous	4	3	1	
Metaplastic	1	1	0	
Medullary	1	0	1	
Cribriform	1	1	0	
Histologic grade				
Well	35	26	9	0.011
Moderate	49	38	11	
Poor	66	35	31	
Mitotic Activity Index				
≤ 10	87	65	22	0.008
> 10	63	34	29	
Ki-67				
< 10 %	71	58	13	<0.001
≥ 10 %	79	41	38	
Estrogen receptor				
Negative	98	60	38	0.090
Positive	52	39	13	
Progesterone receptor				
Negative	92	54	38	0.017
Positive	58	45	13	
HER-2/neu				
Negative	126	91	35	<0.001
Overexpressed	24	8	16	
HER-2/neu gene copy number				
1-4	115	84	31	<0.001
> 4	22	6	16	
VEGF expression				
Weak	35	29	6	0.016
Strong	115	70	45	
Tumor size				
T_1 (0-2 cm)	45	33	12	0.355
T_2 (2-5 cm)	94	58	36	
T_3 (> 5 cm)	11	8	3	
Age				
< 55 years	48	35	13	0.220
≥ 55 years	102	64	38	
Lymph node status				
Negative	81	51	30	0.395
Positive	69	48	21	

NA, not applicable

For the whole group of patients, high levels of HIF-1α expression (≥ 5%) were associated significantly with poor histologic grade (*P*=0.011), increased MAI (*P*=0.008), positive HER-2/*neu* protein status (*P*=0.001) and gene amplification (*P*=0.001), strong VEGF expression (*P*=0.016), and loss of PR (*P*=0.017). A weak correlation was found between high levels of HIF-1α and loss of ER (P=0.090). For HIF-1α, no correlation was found with tumor size, lymph node status, age, or menopausal status. The associations between levels of HIF-1α and the clinicopathologic variables described above are shown in Table 2.

In the subgroup of patients with negative lymph node status (n = 81 patients), univariate survival analysis revealed that high levels of HIF-1α were correlated strongly with shortened disease-free survival (P=0.004) and overall survival (P=0.008), as shown in Figure 3 and Table 3. For disease-free survival, MAI, tumor size, and HIF-1α expression (hazard ratio, 4.194; 95% CI, 1.456–12.079) were independently predictive in multivariate analysis for this subgroup; and, for overall survival, both MAI and HIF-1α expression (hazard ratio, 6.365; 95% CI, 1.321–30.666) were independently predictive in multivariate analysis in this subgroup, as summarized in Table 4. In the same subgroup of patients, high levels of HIF-1α were associated significantly with increased histologic grade (*P*=0.003), high MAI (*P*=0.003), positive Ki-67 status (*P*=0.001), and positive HER-2/*neu* protein and gene amplification status (*P*=0.001 for both). The positive association between increased tumor size (*P*=0.054), loss of PR (*P*=0.099), and increased levels of HIF-

1α approached the level of signi-ficance. No correlation was found with menopausal status, VEGF expression (P=0.194), or ER status (*P*=0.383).

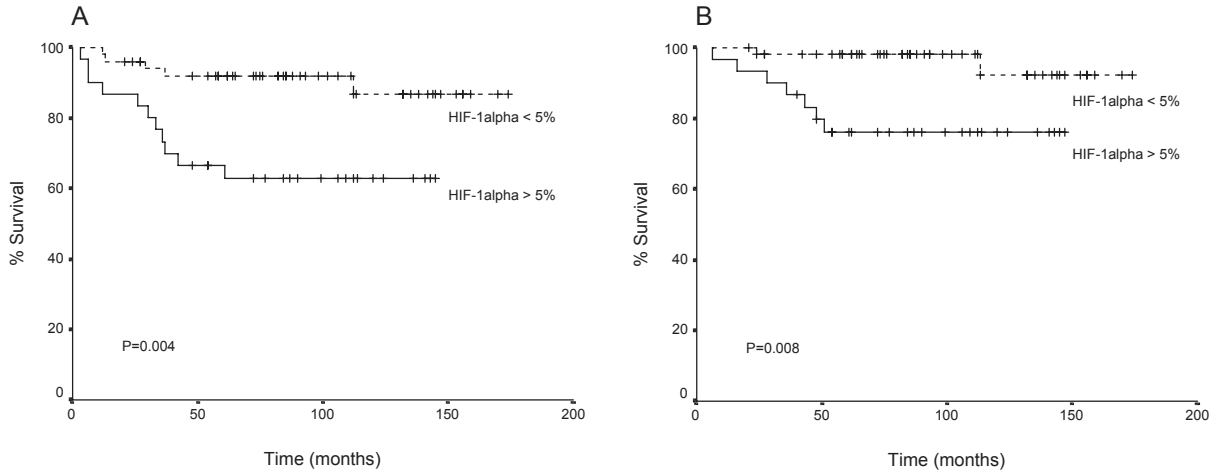

FIGURE 3. Kaplan–Meier survival curves illustrating disease-free survival (A) and overall survival (B) for 81 patients who had lymph node negative breast carcinoma with low expression levels of hypoxia-inducible factor-1α (HIF-1α (< 5%)) versus high expression levels of HIF-1α (≥ 5%) (mean follow-up, 106 months).

TABLE 3. Univariate analysis of disease-free survival and overall survival results for the subgroup of patients with lymph node negative invasive breast carcinoma.

Variable	N	% disease	% disease-free	*P*	Log rank	% dead	% alive	*P*	Log rank
HIF-1α									
< 5%	51	10	90	0.004	8.39	4	96	0.008	7.02
≥ 5%	30	37	63			23	77		
Mitotic activity index									
≤ 10	54	11	89	0.009	6.80	6	94	0.016	5.81
> 10	27	33	67			12	78		
Histologic grade									
Well	28	14	86	0.049	6.03	7	93	0.007	9.80
Moderate	26	8	92			0	100		
Poor	27	33	67			26	74		
Tumor size									
T_1 (0-2 cm)	34	6	94	0.040	6.42	0	100	0.024	7.44
T_2 (2-5 cm)	46	28	72			20	80		
T_3 (> 5 cm)	1	0	100			0	100		
Age									
< 55 years	22	27	73	0.242	1.37	18	82	0.243	1.36
≥ 55 years	59	15	85			8	92		

However, in the subgroup of patients with positive lymph node status, neither univariate analysis nor multivariate analysis established an association of HIF-1α levels with disease-free survival or overall survival. In this subgroup, high levels of HIF-1α were associated with positive Ki-67 status (*P*=0.035) and strong VEGF expression (*P*=0.038). High levels of HIF-1α approached the level of statistical significance in combination with the loss of ER (*P*=0.075) and PR (*P*=0.085) and positive HER-2/*neu* protein status (*P*=0.122): The latter was confirmed by a significant

positive correlation between the presence of HER-2/*neu* gene amplification and high HIF-1α levels (*P*=0.036). In relation to levels of HIF-1α, no correlations were found for histologic grade, high MAI (*P*=0.284), tumor size (*P*=0.700), or menopausal status in patients with positive lymph nodes.

TABLE 4. Hazard ratios, 95% confidence intervals, and *P* values for variables with independent prognostic value in a Cox regression analysis of patients with stage I/II invasive breast carcinoma using hypoxia-inducible factor-1α, the mitotic activity index, differentiation grade, tumor size, lymph node status, and age as covariates

	Disease-free survival			Overall survival		
	HR	95% CI	*P*	HR	95% CI	*P*
Whole group (n = 150)						
MAI	1.580	0.836-2.986	0.159	2.425	1.049-5.607	0.038
Tumor size	1.664	0.964-2.874	0.068	-	-	-
HIF-1α	1.672	0.882-3.170	0.115	2.155	0.951- 4.885	0.066
Negative lymph node status (n = 81)						
MAI	3.114	1.158-8.377	0.025	4.709	1.175-18.881	0.029
Tumor size	2.631	0.962-7.193	0.060	-	-	-
HIF-1α	4.194	1.456-12.079	0.008	6.365	1.321-30.666	0.021
Positive lymph node status (n = 69)						
MAI	0.812	0.350-1.880	0.626	1.341	0.465-3.870	0.587

DISCUSSION

For the first time, the current study showed that levels of HIF-1α were a strong and independent prognostic indicator in patients with invasive breast carcinoma. This confirmed our hypothesis, based on the knowledge that the presence of hypoxia in a tumor reflects a poor prognosis [1], that increased levels of HIF-1α occur especially in patients with poorly differentiated breast lesions [19]. Subgroup analysis showed that this could be explained completely by the group of lymph-node negative patients, as in lymph-node positive patients, levels of HIF-1α were not predictive. This finding suggests that levels of HIF-1α may be used to select highrisk patients with negative lymph node status who would benefit from systemic adjuvant therapy.

Another important finding was the association between overexpression of HER-2/*neu* and HIF-1α that provided clinical confirmation of the recent demonstration that HER-2/*neu* activation induces HIF-1α synthesis in breast carcinoma cells through activation of the PI-3-kinase/protein kinase β (AKT)/FKBP-rapamycin-associated protein (FRAP) signal-transduction pathway [21]. Loss-of-function mutations in the tumor suppressor genes encoding p53, phosphatase and tensin homolog deleted on chromosome 10 (PTEN), and VHL also result in increased HIF-1α expression in human malignancies. Whether levels of HIF-1α represent the degree of cellular hypoxia or mainly point to oncogene activation has not been elucidated to date. In this study, we provided clinical confirmation of the important association between HIF-1α expression and the amplification and overexpression of HER-2/*neu*. It is interesting to note that the survival analysis showed no significance for HER-2/*neu* gene amplification or overexpression (data not shown), suggesting that HIF-1α overexpression in breast carcinoma not only is oncogene-related but probably also is hypoxia-related, because there were more HIF-1α-overexpressing tumors than tumors harboring HER-

2/*neu* amplification.

If levels of HIF-1α reflect reduced intratumoral O_2 concentrations, then hypoxia should be a contributing factor to the poor patient outcome observed in this study. No studies measuring O_2 tension in breast carcinoma in relation to patient survival have been published; however, in patients with cervical carcinoma, it is known that in vivo intratumoral hypoxia predicts poor survival [1]. In the current study and in our previous report [19], HIF-1α staining often was observed in viable cells surrounding areas of necrosis, suggesting that hypoxia contributes to HIF-1α up-regulation in breast carcinoma. Adaptation to hypoxia is mediated by HIF-1, which stimulates angiogenesis (through VEGF) and high and anaerobic metabolism (through glucose transporters and glycolytic enzymes). It has been shown that high microvessel density, VEGF overexpression, and increased lactate production are associated with a poor prognosis in human malignancies [13,14,16]. Similar to the results from our previous study [19], we found that high levels of HIF-1α were correlated with overexpression of VEGF, one of its main downstream effectors, confirming angiogenesis as one of the proposed mechanisms by which HIF-1 activation stimulates tumor growth and metastasis.

Analysis of other clinicopathologic variables in this study confirmed our previous data [19], revealing a positive association between increased proliferation, poor histologic grade, and high levels of HIF-1α. The finding that HIF-1α expression is associated with these markers of tumor aggressiveness is consistent with our demonstration that high HIF-1α levels are associated with a poor prognosis. Furthermore, in our previous study [19], in which we analyzed breast tissue specimens ranging from normal to invasive carcinoma, we reported that levels of both HIF-1α and ER were increased during breast carcinogenesis, but there was no association within the subgroup of patients with invasive disease (unpublished data). In the current study, we only investigated invasive breast carcinoma; and it is interesting to note that we observed a negative association for PR and the same trend for ER status and levels of HIF-1α. In fact, this correlation is consistent with the prognostic value of HIF-1α, because loss of the steroid receptors characterizes tumor aggressiveness.

For patients with breast carcinoma, systemic adjuvant treatment of all patients with negative lymph node status will lead to significant overtreatment and, thus, is rejected by most clinicians. Therefore, accurate identification of patients who are at risk of developing recurrent disease and who may benefit from systemic adjuvant therapy is needed urgently. Our data suggest that high HIF-1α levels may identify these patients with lymph node negative breast carcinoma. This intriguing finding may be explained by the fact that HIF-1 has an important role in the process of metastasis development [3] and, thus, harbors prognostic significance in patients with negative lymph nodes. Agents that inhibit HIF-1 activity, therefore, have potential efficacy as adjuvant therapy. The clinical relevance of targeting HIF-1 is suggested by therapies that target either VEGF specifically or angiogenesis in general. HIF-1α overexpression may explain the resistance of hypoxic tumor cells to chemotherapy or radiation therapy, as demonstrated recently for squamous cell oropharyngeal tumors [23]. Tumor xenograft studies targeting HIF-1, either by disruption of its interaction with coactivators p300 and cyclic AMP regulatory element-binding protein or by administration of antisense HIF-1α plasmids, have provided evidence that this approach, perhaps in combination with conventional treatment, warrants clinical consideration.

This study underlines the rationale for this approach in patients with breast carcinoma.

ACKNOWLEDGEMENTS

Supported by the AEGON International Scholarship in Oncology (R. Bos, P. van der Groep, A. E. Greijer, and A. Shvarts).

Ventana Medical Systems provided consumables and equipment for HER-2/*neu* fluorescent in situ hybridization analysis.

The authors thank Dr. Harm van Tinteren (Dutch Cancer Institute, Amsterdam, The Netherlands) for his help in calculating the hazard ratios.

REFERENCES

1. Hockel M and Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 2001;93:266-276.
2. Semenza GL. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol 2000;88:1474-1480.
3. Semenza GL. Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. Crit Rev Biochem Mol Biol 2000;35:71-103.
4. Huang LE, Arany Z, Livingston DM, and Bunn HF. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. J Biol Chem 1996;271:32253-32259.
5. Jiang BH, Semenza GL, Bauer C, and Marti HH. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. Am J Physiol 1996;271:C1172-C1180.
6. Wang GL, Jiang BH, Rue EA, and Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A 1995;92:5510-5514.
7. Yu AY, Frid MG, Shimoda LA, Wiener CM, Stenmark K, and Semenza GL. Temporal, spatial, and oxygen-regulated expression of hypoxia-inducible factor-1 in the lung. Am J Physiol 1998;275:L818-L826.
8. Ravi R, Mookerjee B, Bhujwalla ZM, Sutter CH, Artemov D, Zeng Q, Dillehay LE, Madan A, Semenza GL, and Bedi A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia- inducible factor 1alpha. Genes Dev 2000;14:34-44.
9. Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, and Ratcliffe PJ. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 1999;399:271-275.
10. Semenza GL. Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 1999;15:551-578.
11. Ratcliffe PJ, O'Rourke JF, Maxwell PH, and Pugh CW. Oxygen sensing, hypoxia-inducible factor-1 and the regulation of mammalian gene expression. J Exp Biol 1998;201:1153-1162.
12. Maxwell PH, Dachs GU, Gleadle JM, Nicholls LG, Harris AL, Stratford IJ, Hankinson O, Pugh CW, and Ratcliffe PJ. Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc Natl Acad Sci U S A 1997;94:8104-8109.
13. Weidner N, Folkman J, Pozza F, Bevilacqua P, Allred EN, Moore DH, Meli S, and Gasparini G. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. J Natl Cancer Inst 1992;84:1875-1887.
14. De Jong JS, van Diest PJ, and Baak JP. Hot spot microvessel density and the mitotic activity index are strong additional prognostic indicators in invasive breast cancer. Histopathology 2000;36:306-312.
15. Harris AL. Antiangiogenesis for cancer therapy. Lancet 1997;349 Suppl 2:SII13-SII15.
16. Linderholm B, Tavelin B, Grankvist K, and Henriksson R. Vascular endothelial growth factor is of high prognostic value in node- negative breast carcinoma. J Clin Oncol 1998;16:3121-3128.
17. West CM, Cooper RA, Loncaster JA, Wilks DP, and Bromley M. Tumor vascularity: a histological measure of angiogenesis and hypoxia. Cancer Res 2001;61:2907-2910.
18. Stubbs M, McSheehy PM, Griffiths JR, and Bashford CL. Causes and consequences of tumour acidity and implications for treatment. Mol Med Today 2000;6:15-19.
19. Bos R, Zhong H, Hanrahan CF, Mommers EC, Semenza GL, Pinedo HM, Abeloff MD, Simons JW, van Diest PJ, and van der Wall E. Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. J Natl Cancer Inst 2001;93:309-314.
20. Chia SK, Wykoff CC, Watson PH, Han C, Leek RD, Pastorek J, Gatter KC, Ratcliffe P, and Harris AL. Prognostic significance of a novel hypoxia-regulated marker, carbonic anhydrase IX, in invasive breast carcinoma. J Clin Oncol 2001;19:3660-3668.
21. Laughner E, Taghavi P, Chiles K, Mahon PC, and 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;21:3995-4004.
22. Birner P, Schindl M, Obermair A, Plank C, Breitenecker G, and Oberhuber G. Overexpression of hypoxia-inducible factor 1alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. Cancer Res 2000;60:4693-4696.
23. Aebersold DM, Burri P, Beer KT, Laissue J, Djonov V, Greiner RH, and Semenza GL. Expression of

hypoxia-inducible factor-1alpha: a novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer. Cancer Res 2001;61:2911-2916.
24. Birner P, Gatterbauer B, Oberhuber G, Schindl M, Rossler K, Prodinger A, Budka H, and Hainfellner JA. Expression of hypoxia-inducible factor-1 alpha in oligodendrogliomas: its impact on prognosis and on neoangiogenesis. Cancer 2001;92:165-171.
25. Giatromanolaki A, Koukourakis MI, Sivridis E, Turley H, Talks K, Pezzella F, Gatter KC, and Harris AL. Relation of hypoxia inducible factor 1 alpha and 2 alpha in operable non-small cell lung cancer to angiogenic/molecular profile of tumours and survival. Br J Cancer 2001;85:881-890.
26. Volm M and Koomagi R. Hypoxia-inducible factor (HIF-1) and its relationship to apoptosis and proliferation in lung cancer. Anticancer Res 2000;20:1527-1533.
27. Beasley NJ, Leek R, Alam M, Turley H, Cox GJ, Gatter K, Millard P, Fuggle S, and Harris AL. Hypoxia-inducible factors HIF-1alpha and HIF-2alpha in head and neck cancer: relationship to tumor biology and treatment outcome in surgically resected patients. Cancer Res 2002;62:2493-2497.
28. Nakayama K, Kanzaki A, Hata K, Katabuchi H, Okamura H, Miyazaki K, Fukumoto M, and Takebayashi Y. Hypoxia-inducible factor 1 alpha (HIF-1 alpha) gene expression in human ovarian carcinoma. Cancer Lett 2002;176:215-223.
29. Page DL. Prognosis and breast cancer. Recognition of lethal and favorable prognostic types. Am J Surg Pathol 1991;15:334-349.
30. Jannink I, van Diest PJ, and Baak JP. Comparison of the prognostic value of four methods to assess mitotic activity in 186 invasive breast cancer patients: classical and random mitotic activity assessments with correction for volume percentage of epithelium. Hum Pathol 1995;26:1086-1092.
31. World Health Organization. Histological typing of breast tumors. Tumori 1982;68:181-198.
32. Elston CW and Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991;19:403-410.
33. Sobin LH, Wittekind CH, and editors. UICC TNM classification of malignant tumors, 5th edition. New York: Wiley-Liss 1997;
34. Bos R, van der Hoeven JJM, van der Wall E, van der Groep P, van Diest PJ, Comans EF, Joshi U, Semenza GL, Hoekstra OS, Lammertsma AA, and Molthoff CF. Biologic correlates of (18)fluorodeoxyglucose uptake in human breast cancer measured by positron emission tomography. J Clin Oncol 2002;20:379-387.
35. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, and Simons JW. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 1999;59:5830-5835.
36. Bosman FT, de Goeij AF, and Rousch M. Quality control in immunocytochemistry: experiences with the oestrogen receptor assay. J Clin Pathol 1992;45:120-124.
37. van Diest PJ, Baak JP, Matze-Cok P, Wisse-Brekelmans EC, van Galen CM, Kurver PH, Bellot SM, Fijnheer J, van Gorp LH, Kwee WS, and . Reproducibility of mitosis counting in 2,469 breast cancer specimens: results from the Multicenter Morphometric Mammary Carcinoma Project. Hum Pathol 1992;23:603-607.

Chapter 7

Biologic correlates of ^{18}Fluorodeoxyglucose uptake in human breast cancer measured by positron emission tomography

J Clin Oncol 2002;20:379-387

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BACKGROUND. Variable uptake of the glucose analog ^{18}fluorodeoxyglucose (FDG) has been noticed in positron emission tomography (PET) studies of breast cancer patients, with low uptake occurring especially in lobular cancer. At present, no satisfactory biologic explanation exists for this phenomenon. This study compared ^{18}FDG uptake in vivo with biomarkers expected to be involved in the underlying biologic mechanisms.

METHODS. Preoperative ^{18}FDG-PET scans were performed in 55 patients. ^{18}FDG activity was assessed visually by three observers using a four point score. Tumor sections were stained by immunohistochemistry for glucose transporter-1 (Glut-1); Hexokinase (HK) I, II, and III; macrophages; hypoxia inducible factor-1-alfa (HIF-1α); vascular endothelial growth factor ($VEGF_{165}$); and microvessels. Mitotic activity index (MAI), amount of necrosis, number of lymphocytes, and tumor cells/volume were assessed.

RESULTS. There were positive correlations between ^{18}FDG uptake and Glut-1 expression (P<0.001), MAI (P=0.001), amount of necrosis (P=0.010), number of tumor cells/volume (P=0.009), expression of HK I (P=0.019), number of lymphocytes (P=0.032), and microvessel density (r=0.373; P=0.005). HIF-1α, $VEGF_{165}$, HK II, HK III, and macrophages showed no univariate correlation with ^{18}FDG. In logistic regression, however, HIF-1α and HK II added value to MAI and Glut-1.

CONCLUSIONS. ^{18}FDG uptake in breast cancer is a function of microvasculature for delivering nutrients, Glut-1 for transportation of ^{18}FDG into the cell, HK for entering ^{18}FDG into glycolysis, number of tumor cells/volume, proliferation rate (also reflected in necrosis), number of lymphocytes (not macrophages), and HIF-1α for upregulating Glut-1. Together, these features explain why breast cancers vary in ^{18}FDG uptake and elucidate the low uptake in lobular breast cancer.

In 1930, Warburg et al [1] discovered that tumors are characterized by the production of lactate (caused by glycolysis) despite the presence of sufficient oxygen, indicating the importance of cell metabolism in tumor biology. A new impulse was given to interest in this phenomenon by Weber [2], who described the role of key isoenzymes in tumor metabolism. This knowledge cumulated in the use of several metabolic isoenzymes (eg, lactate dehydrogenase) for the prediction of prognosis and monitoring of treatment response [3]. At the end of the last millennium, the hypoxia inducible factor-1 (HIF-1) gene was discovered, which gave more insight into the intracellular regulation of oxygen and metabolic homeostasis [4]. Hypoxia was shown to upregulate the HIF-1 complex, a transcription factor, which stimulates the cell to survive hypoxic conditions by inducing glycolysis, angiogenesis, and erythropoiesis [5]. In addition, several oncogenes (eg, v-SRC and h-RAS) were found to upregulate HIF-1, providing a possible genetic explanation for the Warburg effect [6]. Levels of HIF-1-alfa (HIF-1α), one of the two subunits of the HIF-1 complex, are representative of the activity of HIF-1 [5]. It was demonstrated that overexpression of the HIF-1α protein occurs in ductal carcinoma-in-situ and invasive breast cancer [7].

Positron emission tomography (PET) provides the opportunity to study tumor metabolism in vivo [8,9]. Glucose metabolism can be assessed using the glucose analog ^{18}F-2-fluoro-2-deoxy-D-glucose (^{18}FDG) [9]. In breast cancer this technique has been used for tumor detection and staging [10], to obtain long-term prognostic information [11], and to identify tumor response to chemotherapy at an early phase of treatment [12,13,14,15].

Surprisingly, after 20 years of research, the underlying mechanisms for ^{18}FDG uptake in tumors are still a matter of debate. Interestingly, different patterns of ^{18}FDG uptake can be distinguished between different tumor types. For example, the majority of bronchioloalveolar lung carcinomas are PET negative, in contrast to the other histologic types of lung carcinomas, which all show avid uptake [16]. In breast cancer, the degree of ^{18}FDG uptake seems to be more heterogeneous in comparison to many other cancers [10], which has been the main reason for the present investigation of the causal mechanism that determines ^{18}FDG uptake in breast cancer.

Several processes determine glucose and, thus, ^{18}FDG uptake by the cell. Of major importance is the integrity of the vascular network that is necessary for the supply of nutrients, including glucose, and oxygen to the cell [17]. Subsequent cellular uptake of the nutrients is regulated by various transporters at the cell membrane [18]. The most common glucose transporter expressed in all tissues is glucose transporter-1 (Glut-1), which is insulin independent [19]. When glucose has entered the cell, hexokinases (HKs) are necessary to phosphorylate glucose into glucose-6-phosphate. This is the last key regulatory step, and from here glucose follows the process of glycolysis. ^{18}FDG follows the same pathway as glucose, but, after phosphorylation, ^{18}FDG is not further metabolized. The proportion of cellular metabolic rate and oxygen status will further determine whether glycolysis will occur under aerobic or anaerobic conditions. These essential features for glucose metabolism (angiogenesis, transmembrane transport, phosphorylation of glucose, and cellular metabolic rate) are all known to be upregulated in cancer [20]. Upregulation of glucose transporters, HKs, and vascular endothelial growth factor (VEGF), which induces angiogenesis, is also known to be part of the downstream

effects of HIF-1 activation, which, therefore, serves as a promising therapeutic target [21].

In the search for a biologic explanation of the variable ^{18}FDG uptake in breast cancer, several biomarkers representing the above-described biologic model can be considered. We determined several parameters that are involved in the rate of glucose metabolism in the primary tumor of 55 breast cancer patients who underwent PET scanning: Glut-1 as the key glucose membrane transporter; the HKs I, II, and III as key glucose phosphorylators; HIF-1α as upregulator of Glut-1 and VEGF; $VEGF_{165}$ as major angiogenic factor in breast cancer; the intratumoral microvessel density as measure for the microvascular network; mitotic index as a measure of tumor cell proliferation; the presence of necrosis, reflecting that proliferation outnumbers vascular supply; and the presence of macrophages and lymphocytes as possible confounding active metabolic cells. Finally, we measured tumor cell density to correct for cellularity. Defining a correlation between these biomarkers and ^{18}FDG-PET imaging may lead to a better understanding and interpretation of ^{18}FDG-PET scans in breast cancer. To highlight the clinical importance of the variables described above to determine ^{18}FDG uptake, we tested whether the low ^{18}FDG signal usually seen in lobular breast cancers [10] could be explained.

PATIENTS AND METHODS

Patients

The study group comprised 54 women diagnosed with primary breast cancer between 1998 and 2000 at the Amstelveen Hospital (42 patients), Amstelveen, and the Vrije Universiteit Medical Center (12 patients), Amsterdam, the Netherlands. Only patients with cytologically-proven (fine-needle biopsy) or histologically-proven (core biopsy) breast cancer were invited to participate in this study and provided informed consent. No other selection criteria were applied. As PET scans were primarily performed for diagnostic reasons, special ethical approval was not required. One patient had two primary breast cancers, one in each breast. The patients' ages ranged from 34 to 85 years, with a mean of 60.4 years, 10 patients (19%) being premenopausal and 44 (81%), postmenopausal at the time of diagnosis. None of the patients had diabetes mellitus. After PET scanning, excision biopsy or mastectomy with sentinel node guided axillary lymph node dissection were the surgical procedures used for all patients (except for two who did not undergo lymph node dissection).

All specimens were fixed in neutral 4% buffered formaldehyde for at least 12 hours. The group included 29 (54%) lymph node–negative and 24 (43%) lymph node–positive cancers, and the nodal status was not investigated for two cancers. None of the patients received any preoperative chemotherapy, hormonal therapy, or radiotherapy.

The 55 primary tumors were histologically classified as invasive ductal (n = 39); invasive lobular (n = 11); invasive mucinous carcinoma (n = 2); and one each of invasive cribriform, medullary, and papillary carcinoma. The mean tumor diameter was 2.4 cm, ranging from 0.5 to 5.5 cm (after the tumor-node-metastasis system classification: four T_1, 27 T_2, and 24 T_3 tumors).

PET Imaging

Before the operation, patients fasted at least 6 hours before scanning, and serum glucose was measured just before the intravenous administration of 370 MBq ^{18}FDG in the contralateral arm. The ^{18}FDG was produced by Cyclotron BV at Vrije Universiteit. Patients remained supine until the acquisition started, 60 minutes later. The acquisition protocol involved two-dimensional emission scans of the breast and axilla (two bed positions, 15 minutes each), with patients in supine position. Scans were acquired with an ECAT Exact HR+ PET scanner (Siemens/CTI, Knoxville, TN). Emission data were not corrected for attenuation and were reconstructed with filtered back projection. Spatial resolution of the reconstructed images was approximately 7 mm full width at half maximum.

Immunohistochemistry

Table 1 lists all antibodies, dilutions, incubation times, and antigen retrieval methods used. Immunohistochemistry was performed on 4-μm thick tissue sections. After deparaffination and rehydration, endogenous peroxidase activity was blocked for 30 minutes in a methanol solution that contained 0.3% hydrogen peroxide. After antigen retrieval, a cooling-off period of 20 minutes preceded the incubation of the primary antibody. All antibodies were detected with a standard avidin-biotinyl complex method: a biotinylated rabbit antimouse antibody (DAKO, Glostrup, Denmark) and an avidin-biotinyl complex (DAKO), except for CD31, detected with the Labvision kit (DAKO), and HIF-1α, detected with the catalyzed signal amplification kit (DAKO) as described previously [7]. All stainings were developed with diaminobenzidine and counterstained for 30 seconds with hematoxylin and eosin. Appropriate negative (obtained by omission of the primary antibody) and positive controls were used throughout.

TABLE 1 Antibodies, dilution, incubation and detection methods

Antibody*	Company	Dilution	Incubation	Antigen retrieval	Detection
HIF-1α	Semenza	1/500	30' 20 °C	WB TRS 95 °C 45'	CSA
CD31	DAKO	1/40	60' 20 °C	AC Citr. 122 °C 20'	Labvision
$VEGF_{165}$	R&D	1/40	o/n 4 °C	MW Citr. 95 °C 10'	ABC
Glut-1	DAKO	1/200	60' 20 °C	MW Citr. 95 °C 10'	ABC
Hk I	Chemicon	1/100	60' 20 °C	MW Citr. 95 °C 10'	ABC
Hk II	Chemicon	1/5000	60' 20 °C	MW Citr. 95 °C 10'	ABC
Hk III	Chemicon	1/200	60' 20 °C	MW Citr. 95 °C 10'	ABC
CD68	DAKO	1/400	60' 20 °C	MW Citr. 95 °C 10'	ABC

Abbreviations: Semenza, G.L. Semenza, Institute of Genetic Medicine, Johns Hopkins University School of Medicine, USA; R&D, R&D systems, Abingdon, United Kingdom; Chemicon, Chemicon International, Inc. Temecula, CA, USA; o/n, overnight; WB, waterbath; AC, autoclave; MW, microwave; TRS, target retrieval solution (DAKO); Citr, citrate buffer pH 6.0; CSA, catalyzed signal amplification; ABC, avidin-biotinylated peroxidase complex.
*All antibodies are mouse monoclonal, except $VEGF_{165}$ which is goat polyclonal

Data Analysis

PET images were analyzed visually, using 10 slices in axial, coronal, and sagittal axes, by three independent observers blinded to clinical history and outcome. Scoring was graded as negative (grade 0), weak (grade 1), moderate (grade 2), or intense (grade 3) for the primary tumor. The score of the three observers were summed to obtain a score between 0 and 9. The interobserver correlation was high (intraclass coefficient, 0.92; 95% confidence interval, 0.88 to 0.98).

On the basis of the median PET intensity score of all patients, patients were regarded to have high ^{18}FDG accumulation when all three observers independently scored the maximum intensity (grade 3); all other patients were classified as having low ^{18}FDG accumulation. For statistical tests based on nominal values, the sum of the three scores were used.

Also, tumor to nontumor (T/N) ratios were determined by image analysis as follows. The hot spot of the tumor was interactively marked in the plane where the tumor was best visible, and regions of interest were then automatically set, using a region-growing algorithm, around the tumor area and in the corresponding normal tissue in the contralateral breast in all the planes where the tumor was found. The three-dimensional volumes of interest included all pixels within a 50% isocontour of the maximum pixel value of the tumor. The automatic results were interactively corrected if necessary. A good correlation (χ^2, P<0.001, or r=0.63) between visual analysis and T/N ratios was demonstrated (visually low, T/N 5 $\pm$ 1.5 SE; visually high, T/N 16 $\pm$ 2.1 SE). We preferred visual scoring because of the absence of attenuation correction.

The fraction of nuclei with expression of HIF-1α was estimated as described before [7], only regarding homogenously and darkly stained nuclei as positive. Within the CD31-stained slides, the microvessel hotspot was identified, and microvessels were counted at a $\times$40 magnification in four adjacent fields of vision representing 0.6 mm^2, according to Weidner et al [22,23] as described before [24,25], expressing counts as microvessels per millimeter squared. Also, a global microvessel density per millimeter squared was assessed in 25 systematic adjacent diagonal fields of the tumor. Tumor cytoplasm was scored for $VEGF_{165}$ and HK isoforms I, II, and III as negative, weak, positive, and strong positive, ignoring nuclear staining, which was frequently noted for all HKs. The percentage of cells with Glut-1 membrane staining was estimated, and its presence was interpreted as overexpression. The number of CD68-positive macrophages was semiquantitatively scored as none, occasional, moderately frequent, or frequent.

In the hematoxylin-eosin–stained sections, the percentage of necrotic tumor volume was estimated, and the degree of lymphocyte infiltration was semiquantitatively scored as none, slight, moderate, or severe. In the same slide, the MAI was assessed as described before [26], by counting the total number of mitoses in 10 adjacent high-power fields (1.6 mm^2 in total). Tumor cell density was semiautomatically assessed with an interactive digitizing video overlay system (QPRODIT; Leica, Cambridge, United Kingdom) as described before [27]. Using a four-point Weibel grid overlaid on the microscope image of the computer screen, grid points that overlaid tumor epithelium or stroma were registered in 100 fields, systematically spread over the whole tumor area, and the area percentage tumor epithelium was calculated. All cases were scored with researches blinded to all other biomarkers and ^{18}FDG-uptake.

Statistical Methods

For statistical evaluation (SPSS for Windows v9.0.1; SPSS Inc, Chicago, IL), the nonparametric χ^2 test for determining correlations between ^{18}FDG accumulation (grouped as low *v* high) and the other variables were performed. Median values were

used as a cutoff level, except for the MAI (cutoff, 10) and HIF-1α (cutoff, 1%), for which previously established values were chosen [7]. *P* values less than 0.05 were regarded as significant. In addition, correlations between the nominal PET score (0-9) and the other nominal variables were tested using the bivariate Pearson correlation test. Finally, stepwise logistic regression analysis was performed to investigate which combination of parameters best explained ^{18}FDG uptake.

RESULTS

Variable ^{18}FDG uptake was seen between patients, as shown by the distribution of nominal PET values in Fig 1. In 24 of 55 cancers, intense ^{18}FDG uptake was noted. Overexpression of Glut-1 and HIF-1α was observed both around necrosis and also heterogeneously in some areas of the tumor. The intensity of expression of the HKs and $VEGF_{165}$ was sometimes heterogeneous within the tumor, but most tumors had a rather homogeneous expression throughout the tumor. In Tables 2 and 3, the descriptive statistics for all variables are shown.

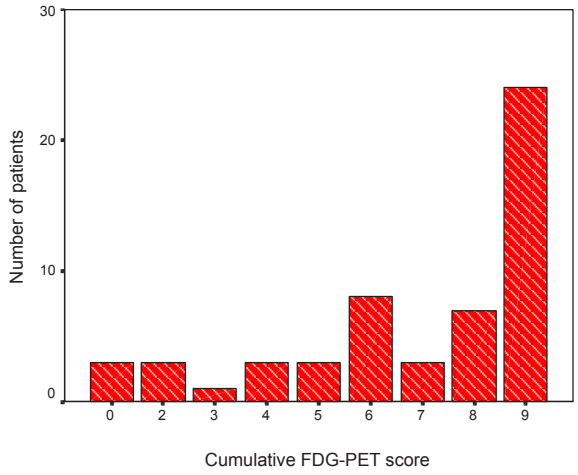

FIGURE 1. Distribution of the cumulative ^{18}FDG PET (0-3) scores as assessed by 3 independent observers (0 being negative according to all, 9 having the maximum signal according to all three observers).

TABLE 2 Biomarkers, studied in 55 breast cancers: nominal variables

Biomarker	Range	Mean
HIF-1α positivity, %	0-60	6
Glut-1 positivity, %	0–5	1
Microvessel density		
Hot spot, vessels/mm^2	32-231	110
Global, vessels/mm^2	28-195	69
MAI, mitosis/1.6 mm^2	1-68	15
Area necrosis positivity, %	0-25	3
Area tumor epithelium positivity, %	12-70	33

TABLE 3 Biomarkers, studied in 55 breast cancers: categorical variables

Biomarker	No of cases
$VEGF_{165}$	
Negative	2
Weak	8
Positive	40
strongly positive	5
Hexokinase I	
Negative	13
Weak	33
Positive	7
strongly positive	2
Hexokinase II	
Negative	4
Weak	36
Positive	14
strongly positive	1
Hexokinase III	
Negative	3
Weak	34
Positive	18
strongly positive	0
Macrophages	
None	3
Occasional	8
moderate	16
Frequent	28
Lymphocytic infiltrate	
none	1
slight	10
moderate	23
severe	21

The results of the statistical comparison between ^{18}FDG uptake (low *v* high) and the biomarkers are described in Table 4.

TABLE 4 Relationship between ^{18}FDG uptake as measured by PET scanning and different variables using χ^2.

Biomarker	^{18}FDG uptake low	^{18}FDG uptake high	χ^2, *P*
Glut-1			
Negative	28	10	< 0.001
Positive	3	14	
MAI			
≤ 10/1.6mm^2	20	5	0.001
> 10/1.6mm^2	11	19	
Area tumor epithelium			
≤ 31%	20	7	0.009
> 31%	11	17	
Area necrosis			
< 5 %	29	16	0.010
≥ 5 %	2	8	
HK I			
Negative	11	2	0.019
Positive	20	22	
Lymphocytic infiltrate			
None	23	11	0.032
Present	8	13	
Histologic classification			
Ductal	18	21	0.036
Lobular	9	2	
HK II			
Negative	20	20	0.120
Positive	11	4	
HIF1α			
≤ 1%	20	12	0.279
> 1%	11	12	
Microvessel density			
Hot spot			
≤ 99/mm^2	17	10	0.333
> 99/mm^2	14	14	
Global			
≤ 66/mm^2	14	12	0.721
> 66/mm^2	17	12	
VEGF$_{165}$			
Weak	7	3	0.336
Strong	24	21	
Macrophages			
None	15	12	0.906
Present	16	12	
HK III			
Negative	21	16	0.993
Positive	10	8	

MAI (*P*=0.001), Glut-1 membrane staining (*P*=0.001), presence of necrosis (*P*=0.010) as well as tumor cell density (*P*=0.009), the intensity of cytoplasmic HK I

(*P*=0.019), and the presence of lymphocytic infiltrate (*P*=0.032) all yielded a statistically significant positive correlation. Using χ^2 tests, the intensity of HK II, HK III, HIF-1α expression, $VEGF_{165}$ expression, the microvessel density (both hot spot and global counts), and the presence of macrophages were not significantly correlated with ^{18}FDG uptake. In Table 5, the correlation coefficients of nominal variables compared with nominal PET scores are shown. This test confirmed the previously found significant associations in χ^2 tests. More interestingly, a significant (although weak) positive correlation between microvessel density (hotspot count) and PET score (*r*=0.373, *P*=0.005) was found, as shown in Fig 2.

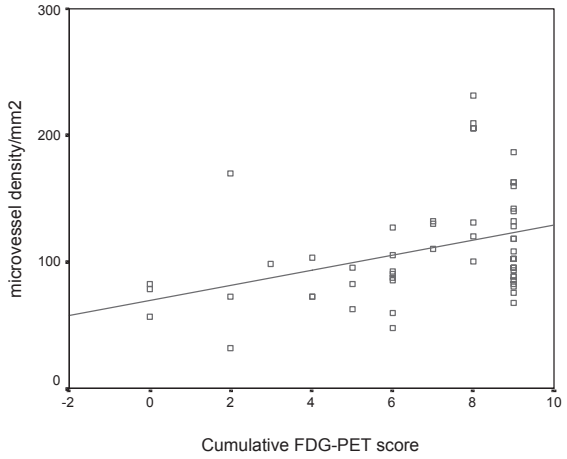

FIGURE 2. Correlation between the nominal ^{18}FDG-PET score and the microvessel density per mm^2 (hotspot count) (r = .373, *P* = .005).

TABLE 5 Relationship between ^{18}FDG uptake as measured by PET scanning and different biomarkers using Pearson correlation test.

Biomarker	r	*P*
MAI	0.488	< 0.001
Area tumor epithelium	0.475	< 0.001
Glut-1	0.336	0.006
Microvessel density		
Hot spot	0.373	0.005
Global	0.216	0.057
Area necrosis	0.288	0.017
HIF-1α	0.012	0.466

In stepwise logistic regression, the MAI, Glut-1, HIF-1α, and HK II seemed to be the strongest combination (*P*=0.001) of variables to predict ^{18}FDG positivity (85.5% accuracy). Analyzing the bioprofile of lobular cancer (Table 6), the ability to detect PET seemed to depend especially on Glut-1, MAI, tumor cell density, and necrosis.

DISCUSSION

This is the most comprehensive study so far to address the role of various biomarkers that were expected to be involved in ^{18}FDG uptake in human breast cancer, which confirmed the role for Glut-1 and HK I in tumor biology in vivo.

The results indicate, however, that uptake of glucose by breast cancer is also related to proliferation rate; tumor cell density; and the presence of necrosis, lymphocytes, angiogenesis, and, to some degree, HIF-1α. In this study, we combined the rate of metabolism, angiogenesis, proliferation, and histologic architecture of the tumor, with ^{18}FDG uptake shown by PET scanning in breast cancer. The significant correlation between several parameters of metabolism and ^{18}FDG uptake explains the variability in PET scanning results between breast cancer patients and resolves why invasive lobular breast cancers in general have low ^{18}FDG uptake.

TABLE 6 Relationship between ^{18}FDG uptake as measured by PET scanning and different variables in 11 lobular breast cancers

Biomarker	^{18}FDG uptake	
	low	high
Glut-1		
Negative	9	1
Positive	0	1
MAI		
≤ 10/1.6mm^2	6	0
> 10/1.6mm^2	3	2
Area tumor epithelium		
≤ 31%	7	0
> 31%	2	2
Area necrosis		
< 5 %	9	1
≥ 5 %	0	1
Lymphocytic infiltrate		
None	7	1
Present	2	1
Microvessel density		
Hot spot		
≤ 99/mm^2	5	0
> 99/mm^2	4	2
Global		
≤ 66/mm^2	5	1
> 66/mm^2	4	1
Macrophages		
None	5	0
Present	4	2
VEGF$_{165}$		
Weak	3	0
Strong	6	2
HK I		
Negative	2	0
Positive	7	2
HK II		
Negative	6	2
Positive	3	0
HK III		
Negative	7	2
Positive	2	0
HIF1α		
≤ 1%	7	2
> 1%	2	0

Facilitated transport of glucose through the cellular membrane is mainly carried out by Glut-1. In 1974, Hatanaka [28] had already reported an increase of glucose transporters as a possible explanation of the Warburg effect. This finding was confirmed in vitro [29] and in vivo [30]. In the present study, 32% of the 55 breast cancers were Glut-1 positive, which is in agreement with previous reports. Younes et al [31] showed Glut-1 positivity in 42% of 118 breast cancers, and Avril et al [32] found Glut-1 positivity in 30% of 46 patients. Only in the smaller study of Brown and Wahl [33] were

all 12 breast cancers and eight lymph node metastases Glut-1 positive. Differences in expression can be explained by differences in antibodies (monoclonal or polyclonal) and detection methods used. More importantly, special attention should be paid to the method of quantification of biomarkers. In the present study Glut-1 activity was only based on membrane staining because this is the location of biologically active Glut-1. Brown et al [34] showed that tritiumfluorodeoxyglucose uptake was correlated to Glut-1 expression in rat breast cancer, and Higashi et al [16] showed the same for lung cancer. In concordance with these findings, we found in the present study a strong positive correlation between presence of Glut-1 and uptake of ^{18}FDG. Interestingly, Avril et al [32] could not detect such a correlation between ^{18}FDG and Glut-1. This could be a result of the fact that they used intensity of cytoplasmic staining rather than membrane staining for quantifying Glut-1. Differences in PET methodology might also play a role. Avril et al [32] used a semiquantitative method, standardized uptake value, that reflected the mean uptake in the tumor of ^{18}FDG. We applied a reproducible visual scoring for the present study, which primarily depended on the maximal contrast between tumor and surrounding tissue, demonstrated by assessment of T/N ratios.

Increased cell proliferation demands energy and, thus, glucose. The proliferation rate was assessed using mitotic activity. Presence of glycolytic metabolism has already been linked to proliferation in vitro, and it has been proposed that the switch from oxidative to glycolytic metabolism serves as an alternative route during mitogenstimulated proliferation to avoid the formation of DNAdamaging reactive oxygen species [35]. The results confirmed the importance of proliferation for glucose metabolism because the MAI strongly correlated with ^{18}FDG uptake in agreement with previous reports [32,36].

An HK phosphorylates glucose when it enters the cell. Of the four isoforms, HK II is said to be the most prominent one associated with cancer [37]. It can be upregulated at the transcriptional and posttranslational level [38]. Its property to bind to the outer membrane of the mitochondria results in a higher affinity for glucose and a reduced feedback inhibition by glucose-6-phosphate [39]. The involvement of HK II in ^{18}FDG uptake was, however, limited compared with the rate of proliferation and Glut-1 expression, although in logistic regression, HK II added value to proliferation and Glut-1, and the univariate relation between HK I and ^{18}FDG was much stronger. In fact, many tumors showed expression of HK I. To the best of our knowledge, this is the first time overexpression of HK I is reported in breast cancer. Further research to confirm these results and to explore the role of HKs in breast cancer is, therefore, warranted.

Tumor cell density was measured because the need for glucose and, therefore, ^{18}FDG uptake correlated linearly with the number of tumor cells per unit volume of tissue (in case of an equal glucose need per cell). In fact, a study in astrocytomas showed such a relationship [40]. In the present study, a large variation in tumor cell density was found in breast cancer (from 12% to 70% epithelial cell density), with a strong positive correlation indeed with ^{18}FDG uptake. Further, it was investigated whether the presence of lymphocytes, macrophages, or necrosis could explain ^{18}FDG accumulation. Little is known about their influence on ^{18}FDG uptake in humans. Lymphocytes and macrophages were investigated because inflammatory cells may

have a major impact on ^{18}FDG uptake [41,42], abscesses and infections being pitfalls in the clinical diagnostic use of PET [43,44]. Indeed, in this study, the number of lymphocytes was correlated positively with ^{18}FDG uptake, in contrast to the presence of macrophages. Studying syngeneic rat mammary cancers grown in immunocompetent rats, Brown et al [45] found a low tritiumfluorodeoxyglucose uptake in lymphocytes and macrophages in contrast to the strong uptake by breast cancer cells. Kubota et al [41] noted the opposite and found that 29% of glucose utilization was non–tumorassociated in breast cancer–bearing mice.

Angiogenesis may promote ^{18}FDG uptake by enhanced tracer delivery. Surprisingly, no correlation between glucose metabolism and the expression of the angiogenesis inducing growth factor $VEGF_{165}$ could be detected. So, although VEGF is an important prognostic indicator in breast cancer, its presence (ie, a basic level) seems to be sufficient and doesn't rate limiting in breast tumor metabolism [46]. In contrast, a direct correlation between ^{18}FDG uptake and the intratumoral microvessel density was found, a correlation also seen in human gliomas [47].

Tumors that grow too rapidly or have a deficient vascular system are characterized by the formation of necrosis. Necrosis reflects cell death caused by hypoxia, and hypoxia increases the ^{18}FDG uptake in vitro [48]. It is, therefore, not surprising that a positive correlation between the presence of necrosis and ^{18}FDG uptake was found, which confirmed the results of a previous study that showed that prenecrotic changes in cells do correlate positively with ^{18}FDG uptake [49]. Further evidence for this relation can be found in the predominant presence of Glut-1 and HIF-1 around necrosis seen in the present study and reported previously [7,50].

Overexpression of HIF-1α has been found in most common cancers, and we have demonstrated its upregulation during breast carcinogenesis before [7,51]. In the present study, HIF-1 was studied because it is known to stimulate the transcription of glycolytic enzymes and VEGF. The involvement of HIF-1 in ^{18}FDG accumulation was limited to only some additional value compared with features like the rate of proliferation and Glut-1 expression. Apparently, the downstream effect of HIF-1 by upregulating Glut-1 [52] is more important to explain ^{18}FDG uptake than the upregulation of HIF-1 itself. $VEGF_{165}$ had no impact on ^{18}FDG uptake, indicating that, as a downstream effect of HIF-1, it is less important than Glut-1 with respect to ^{18}FDG uptake.

Analysis of the profile of the biomarkers described above with ^{18}FDG uptake for all lobular cancers confirmed the associations already described for all cases. However, here, tumor cell density in particular played an important role.

Intuitively, one can wonder if prescan biopsies might influence PET signal intensity, ie, ^{18}FDG uptake. We are not aware of any direct scientific data to test this hypothesis. However, the combination of already known facts (mean tumor volume of a core biopsy) and our own observations (no histologic reactivity to biopsy in operation specimens) provides some decent clues. Krebs et al [53] described that the mean volume of a 14-gauge needle ranged from 9.9 to 17.9 mm^3, which is approximately 1/600 of the mean tumor volume (diameter, 2.4 cm; $[0.5 \times 2.4]^3 \times [4\pi:3]$ = 7238 mm^3) as described in our study. So, we argue that removal of the tumor tissue by a biopsy can by itself in no way lead to a significant loss of tumor volume and, thus, of PET signal. On the other hand, enhancement of ^{18}FDG uptake could,

theoretically, be a result of a biopsy-caused hematoma or infection, but we did not observe any such reactions in the operation specimens, nor were there any clinical signs for this. The frequency of such complications is indeed low (1 in 1,000), according to Parker et al [54].

In conclusion, the amount of ^{18}FDG uptake in breast cancer is determined by the presence of several credible biologic variables: microvessels that provide glucose, Glut-1 that transports ^{18}FDG into the cell, HKs to enter ^{18}FDG into glycolysis, number of tumor cells per unit volume, rate of tumor cell proliferation (also reflected in necrosis), amount of inflammatory cells within the tumor, and, to some extent, HIF-1α that is upregulated by hypoxia and induces Glut-1 expression and angiogenesis. These data are in conflict with the conclusion of Avril et al [32], which stated that ^{18}FDG-PET imaging may not be used to estimate the tumor biologic behavior of breast cancer. In contrast, our results show that these features can explain why ^{18}FDG uptake is so variable in breast cancer, which may lead to a more rational use of PET scanning in breast cancer patients.

ACKNOWLEDGEMENTS

Supported by AEGON International Scholarship in Oncology, an exchange program for scholars in oncology between the Johns Hopkins Oncology Center, Baltimore, MD, and the Vrije Universiteit Medical Center, Amsterdam, The Netherlands (R.B. and P.vdG.).

We thank Avi Shvarts, PhD, Department of Medical Oncology, Vrije Universiteit Medical Center for critical reading of the manuscript. We also thank R.P.A. Boom, MD, and D. van Geldere, MD, Department of Surgery, Amstelveen Hospital, and S. Meijer, MD, PhD, Department of Surgery, Vrije Universiteit Medical Center, for referring patients and M. van der Vijver, MD, PhD, and J.L. Peterse, MD, PhD, Department of Pathology, the Netherlands Cancer Institute, who provided tumor material from some of the patients.

REFERENCES

1. Warburg O, Wind F, and Negalein E. The metabolism of tumours in the body. J Physiol 1927;8:519-530.
2. Weber G. Enzymology of cancer cells (first of two parts). N Engl J Med 1977;296:486-492.
3. Csako G, Magrath IT, and Elin RJ. Serum total and isoenzyme lactate dehydrogenase activity in American Burkitt's lymphoma patients. Am J Clin Pathol 1982;78:712-717.
4. Semenza GL and Wang GL. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol 1992;12:5447-5454.
5. Semenza GL. Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 1999;15:551-578.
6. Dang CV and Semenza GL. Oncogenic alterations of metabolism. Trends Biochem Sci 1999;24:68-72.
7. Bos R, Zhong H, Hanrahan CF, Mommers EC, Semenza GL, Pinedo HM, Abeloff MD, Simons JW, van Diest PJ, and van der Wall E. Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. J Natl Cancer Inst 2001;93:309-314.
8. Hoekstra CJ, Paglianiti I, Hoekstra OS, Smit EF, Postmus PE, Teule GJ, and Lammertsma AA. Monitoring response to therapy in cancer using [18F]-2-fluoro-2-deoxy-D- glucose and positron emission tomography: an overview of different analytical methods. Eur J Nucl Med 2000;27:731-743.
9. Phelps ME. Inaugural article: positron emission tomography provides molecular imaging of biological processes. Proc Natl Acad Sci U S A 2000;97:9226-9233.
10. Avril N, Rose CA, Schelling M, Dose J, Kuhn W, Bense S, Weber W, Ziegler S, Graeff H, and Schwaiger M. Breast imaging with positron emission tomography and fluorine-18 fluorodeoxyglucose: use and limitations. J Clin Oncol 2000;18:3495-3502.
11. Oshida M, Uno K, Suzuki M, Nagashima T, Hashimoto H, Yagata H, Shishikura T, Imazeki K, and Nakajima N. Predicting the prognoses of breast carcinoma patients with positron emission tomography using 2-deoxy-2-fluoro[18F]-D-glucose. Cancer 1998;82:2227-2234.
12. Jansson T, Westlin JE, Ahlstrom H, Lilja A, Langstrom B, and Bergh J. Positron emission tomography studies in patients with locally advanced and/or metastatic breast cancer: a method for early therapy evaluation? J Clin Oncol 1995;13:1470-1477.
13. Schelling M, Avril N, Nahrig J, Kuhn W, Romer W, Sattler D, Werner M, Dose J, Janicke F, Graeff H, and

Schwaiger M. Positron emission tomography using 18F-Fluorodeoxyglucose for monitoring primary chemotherapy in breast cancer. J Clin Oncol 2000;18:1689-1695.
14. Smith IC, Welch AE, Hutcheon AW, Miller ID, Payne S, Chilcott F, Waikar S, Whitaker T, Ah-See AK, Eremin O, Heys SD, Gilbert FJ, and Sharp PF. Positron emission tomography using 18F-fluorodeoxy-D-glucose to predict the pathologic response of breast cancer to primary chemotherapy. J Clin Oncol 2000;18:1676-1688.
15. Wahl RL, Zasadny K, Helvie M, Hutchins GD, Weber B, and Cody R. Metabolic monitoring of breast cancer chemohormonotherapy using positron emission tomography: initial evaluation. J Clin Oncol 1993;11:2101-2111.
16. Higashi K, Ueda Y, Sakurai A, Wang XM, Xu L, Murakami M, Seki H, Oguchi M, Taki S, Nambu Y, Tonami H, Katsuda S, and Yamamoto I. Correlation of Glut-1 glucose transporter expression with. Eur J Nucl Med 2000;27:1778-1785.
17. Carmeliet P and Jain RK. Angiogenesis in cancer and other diseases. Nature 2000;407:249-257.
18. Thorens B. Facilitated glucose transporters in epithelial cells. Annu Rev Physiol 1993;55:591-608.
19. Watson RT and Pessin JE. Intracellular organization of insulin signaling and GLUT4 translocation. Recent Prog Horm Res 2001;56:175-193.
20. Pauwels EK, Ribeiro MJ, Stoot JH, McCready VR, Bourguignon M, and Maziere B. FDG accumulation and tumor biology. Nucl Med Biol 1998;25:317-322.
21. Kung AL, Wang S, Klco JM, Kaelin WG, and Livingston DM. Suppression of tumor growth through disruption of hypoxia-inducible transcription. Nat Med 2000;6:1335-1340.
22. Weidner N, Semple JP, Welch WR, and Folkman J. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. N Engl J Med 1991;324:1-8.
23. Weidner N, Folkman J, Pozza F, Bevilacqua P, Allred EN, Moore DH, Meli S, and Gasparini G. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. J Natl Cancer Inst 1992;84:1875-1887.
24. Belien JA, Somi S, De Jong JS, van Diest PJ, and Baak JP. Fully automated microvessel counting and hot spot selection by image processing of whole tumour sections in invasive breast cancer. J Clin Pathol 1999;52:184-192.
25. De Jong JS, van Diest PJ, and Baak JP. Hot spot microvessel density and the mitotic activity index are strong additional prognostic indicators in invasive breast cancer. Histopathology 2000;36:306-312.
26. van Diest PJ, Baak JP, Matze-Cok P, Wisse-Brekelmans EC, van Galen CM, Kurver PH, Bellot SM, Fijnheer J, van Gorp LH, Kwee WS, and . Reproducibility of mitosis counting in 2,469 breast cancer specimens: results from the Multicenter Morphometric Mammary Carcinoma Project. Hum Pathol 1992;23:603-607.
27. Jannink I, van Diest PJ, and Baak JP. Comparison of the prognostic value of four methods to assess mitotic activity in 186 invasive breast cancer patients: classical and random mitotic activity assessments with correction for volume percentage of epithelium. Hum Pathol 1995;26:1086-1092.
28. Hatanaka M. Transport of sugars in tumor cell membranes. Biochim Biophys Acta 1974;355:77-104.
29. Yamamoto T, Seino Y, Fukumoto H, Koh G, Yano H, Inagaki N, Yamada Y, Inoue K, Manabe T, and Imura H. Over-expression of facilitative glucose transporter genes in human cancer. Biochem Biophys Res Commun 1990;170:223-230.
30. Younes M, Lechago LV, Somoano JR, Mosharaf M, and Lechago J. Wide expression of the human erythrocyte glucose transporter Glut1 in human cancers. Cancer Res 1996;56:1164-1167.
31. Younes M, Brown RW, Mody DR, Fernandez L, and Laucirica R. GLUT1 expression in human breast carcinoma: correlation with known prognostic markers. Anticancer Res 1995;15:2895-2898.
32. Avril N, Menzel M, Dose J, Schelling M, Weber W, Janicke F, Nathrath W, and Schwaiger M. Glucose metabolism of breast cancer assessed by 18F-FDG PET: histologic and immunohistochemical tissue analysis. J Nucl Med 2001;42:9-16.
33. Brown RS and Wahl RL. Overexpression of Glut-1 glucose transporter in human breast cancer. An immunohistochemical study. Cancer 1993;72:2979-2985.
34. Brown RS, Leung JY, Fisher SJ, Frey KA, Ethier SP, and Wahl RL. Intratumoral distribution of tritiated-FDG in breast carcinoma: correlation between Glut-1 expression and FDG uptake. J Nucl Med 1996;37:1042-1047.
35. Brand KA and Hermfisse U. Aerobic glycolysis by proliferating cells: a protective strategy against reactive oxygen species. FASEB J 1997;11:388-395.
36. Minn H, Joensuu H, Ahonen A, and Klemi P. Fluorodeoxyglucose imaging: a method to assess the proliferative activity of human cancer in vivo. Comparison with DNA flow cytometry in head and neck tumors. Cancer 1988;61:1776-1781.
37. Mathupala SP, Heese C, and Pedersen PL. Glucose catabolism in cancer cells. The type II hexokinase promoter contains functionally active response elements for the tumor suppressor p53. J Biol Chem 1997;272:22776-22780.
38. Mathupala SP, Rempel A, and Pedersen PL. Aberrant glycolytic metabolism of cancer cells: a remarkable coordination of genetic, transcriptional, post-translational, and mutational events that lead to a critical role for type II hexokinase. J Bioenerg Biomembr 1997;29:339-343.
39. Smith TA. Mammalian hexokinases and their abnormal expression in cancer. Br J Biomed Sci 2000;57:170-178.
40. Herholz K, Pietrzyk U, Voges J, Schroder R, Halber M, Treuer H, Sturm V, and Heiss WD. Correlation of glucose consumption and tumor cell density in astrocytomas. A stereotactic PET study. J Neurosurg 1993;79:853-858.
41. Kubota R, Yamada S, Kubota K, Ishiwata K, Tamahashi N, and Ido T. Intratumoral distribution of fluorine-18-fluorodeoxyglucose in vivo: high accumulation in macrophages and granulation tissues studied by microautoradiography. J Nucl Med 1992;33:1972-1980.
42. Bakheet SM, Powe J, Kandil A, Ezzat A, Rostom A, and Amartey J. F-18 FDG uptake in breast infection and inflammation. Clin Nucl Med 2000;25:100-103.
43. Lewis PJ and Salama A. Uptake of fluorine-18-fluorodeoxyglucose in sarcoidosis. J Nucl Med 1994;35:1647-1649.
44. Tahara T, Ichiya Y, Kuwabara Y, Otsuka M, Miyake Y, Gunasekera R, and Masuda K. High [18F]-

fluorodeoxyglucose uptake in abdominal abscesses: a PET study. J Comput Assist Tomogr 1989;13:829-831.
45. Brown RS, Leung JY, Fisher SJ, Frey KA, Ethier SP, and Wahl RL. Intratumoral distribution of tritiated fluorodeoxyglucose in breast carcinoma: I. Are inflammatory cells important? J Nucl Med 1995;36:1854-1861.
46. Linderholm B, Tavelin B, Grankvist K, and Henriksson R. Vascular endothelial growth factor is of high prognostic value in node- negative breast carcinoma. J Clin Oncol 1998;16:3121-3128.
47. Aronen HJ, Pardo FS, Kennedy DN, Belliveau JW, Packard SD, Hsu DW, Hochberg FH, Fischman AJ, and Rosen BR. High microvascular blood volume is associated with high glucose uptake and tumor angiogenesis in human gliomas. Clin Cancer Res 2000;6:2189-2200.
48. Clavo AC, Brown RS, and Wahl RL. Fluorodeoxyglucose uptake in human cancer cell lines is increased by hypoxia. J Nucl Med 1995;36:1625-1632.
49. Kubota R, Kubota K, Yamada S, Tada M, Ido T, and Tamahashi N. Active and passive mechanisms of [fluorine-18] fluorodeoxyglucose uptake by proliferating and prenecrotic cancer cells in vivo: a microautoradiographic study. J Nucl Med 1994;35:1067-1075.
50. Brown RS, Fisher SJ, and Wahl RL. Autoradiographic evaluation of the intra-tumoral distribution of 2-deoxy-D-glucose and monoclonal antibodies in xenografts of human ovarian adenocarcinoma. J Nucl Med 1993;34:75-82.
51. Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, and Simons JW. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 1999;59:5830-5835.
52. Chen C, Pore N, Behrooz A, Ismail-Beigi F, and Maity A. Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. J Biol Chem 2001;276:9519-9525.
53. Krebs TL, Berg WA, Severson MJ, Magder LS, Goldberg PA, Campassi C, and Sun CC. Large-core biopsy guns: comparison for yield of breast tissue. Radiology 1996;200:365-368.
54. Parker SH, Burbank F, Jackman RJ, Aucreman CJ, Cardenosa G, Cink TM, Coscia JL, Jr., Eklund GW, Evans WP, III, Garver PR, and . Percutaneous large-core breast biopsy: a multi-institutional study. Radiology 1994;193:359-364.

Chapter 8

Summary and general discussion

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SUMMARY AND GENERAL DISCUSSION:

HIF-1α in breast cancer
BCL-6 in breast cancer
PET and breast cancer
Conclusions

SUMMARY AND GENERAL DISCUSSION:

THIS THESIS IS primarily focused on the previously hardly explored role of HIF-1 in breast cancer. HIF-1 is a transcription factor induced by hypoxia, but also by some oncogenes, tumor suppressor genes and growth factors. Activated HIF-1 can induce angiogenesis, glycolysis, erythropoiesis, and other processes allowing cells to survive during hypoxia or hypoglycaemia. These processes are also involved in the development of breast cancer and subsequent formation of metastases. Thus, HIF-1 could be suspected to play an important role in breast cancer. If so, HIF-1 would be a potential therapeutic target because of its broad regulatory impact.

We therefore tried to unravel the role of HIF-1 in breast carcinogenesis, its interaction with cell cycle proteins, growth factors and steroid receptors. In addition, we determined the role of HIF-1 as a prognosticator.

From a clinical point of view, we tried to answer the question why different breast cancer patients show such a broad variety in ^{18}FDG (radioactively labeled glucose analogue) signal intensity as measured by positron emission tomography (PET). We composed a model with most important determinants of quantitative glucose metabolism in breast cancer, assuming a role of HIF-1 in the variable uptake of ^{18}FDG.

Further, we hypothesized that the cell cycle regulating protein BCL-6 is not only involved in lymphomas, but might also be present in breast cancer. In fact, our data not only showed the presence of BCL-6 in breast cancer, but, also an association of BCL-6 with HIF-1.

HIF-1α in breast cancer

In **chapter 2**, different stages of human breast carcinogenesis were studied for expression of HIF-1α. As the angiogenic switch is recognized as an essential event during tumor development, unraveling its initiation would be most valuable. In breast cancer, the angiogenic switch occurs at the stage of carcinoma in situ. We noted the overexpression of HIF-1α in such early lesions as well as in invasive cancer specimens. In contrast, we did not detect increased levels of HIF-1α in tissue specimens of normal breast nor in areas with ductal hyperplasia.

Levels of HIF-1α increased as the degree of malignancy increased, and were most pronounced in poorly differentiated lesions. These data fit with our current model of breast cancer progression; well-differentiated cancers arising from well differentiated precursor lesions and poorly differentiated cancers from poorly differentiated precursor lesions. Thus, the observed increased levels of HIF-1α overexpression in poorly differentiated DCIS may indicate a higher likelihood that such lesion will acquire invasive properties and that the resulting poorly differentiated invasive lesions may have a poorer prognosis. Therefore, HIF-1α Is a potential prognosticator in pure carcinoma in situ lesions. However, it will be difficult to compose a large group of pure DCIS cases with adequate follow-up data to proof this hypothesis.

Further, it is interesting to note the occasional finding of nuclei with HIF-1α positivity in usual ductal hyperplasia adjacent to invasive cancers and areas of atypical ductal hyperplasia. This phenomenon was attributed to “malignancy

associated changes". In addition to our study, similar results were found in a study with precursor lesions of prostate cancer [1,2]. For other types of cancer, no such studies have as yet been published.

We observed that an increased level of HIF-1α was associated with a high degree of proliferation and ER-positivity. In addition, an angiogenesis-inducing role for HIF-1α during breast carcinogenesis seems likely in view of the positive association between increased levels of HIF-1α and both VEGF and microvessel density (the latter only in the subgroup of DCIS patients).

Since HIF-1α-positive cells are especially seen around areas of necrosis, we postulate that the HIF-1 overexpression is mainly hypoxia related. Thus, hypoxia-induced HIF-1 overexpression seems to occur in breast cancer *in vivo*. This is in line with presence of hypoxia in breast cancer as witnessed by the presence of necrosis and actual *in vivo* low oxygen measurements in breast cancer [3]. Using *in situ* hybridization, previous studies showed that tumors of hepatoma wild type cells expressing HIF-1 also expressed high mRNA levels of VEGF and Glut-3 in viable cells surrounding areas of necrosis. This was not seen in HIF-1 non-expressing sub-clones [4]. These and our data suggest that physiologic upregulation of HIF-1 contributes to angiogenesis and tumor growth. However, some cases with diffuse overexpression unrelated to hypoxia were noted, which might be oncogene/tumor suppressor gene related. Indeed, for the first time a positive association between HER-2/*neu* and HIF-1α was shown on the protein level as a putative explanation of non-hypoxia related HIF-1 induction.

In **chapter 3**, 150 invasive breast cancers were evaluated for aberrant expression of cell cycle related proteins and their association with levels of HIF-1α. In this study, we confirm that HIF-1α overexpression is associated with increased proliferation (cyclin A and Ki-67).

These associations were primarily seen in the subgroup of ER negative tumors. In the ER positive tumors, only p21, a known downstream target of HIF-1, was associated with HIF-1α, in contrast to proliferation. Therefore, we hypothesized that ER might be necessary for p21 induction, because p21 is known to block cell cycle progression. This was further confirmed by the fact that VEGF and p53 likewise lacked any association with HIF-1α in the ER positive subgroup, which was, in contrast to the data found in the ER-negative subgroup. Thus, the different associations in both ER-subgroups could not solely be attributed to a well-known difference in proliferation rate.

In addition, the results seemed to be independent from p53 status. We were able to show for the first time, that p53 overexpression was associated with increased levels of HIF-1α in human breast cancer. Further, increased levels of cyclin D_1 corresponded to high p21, but not to HIF-1α. Since p21 is orchestred by HIF-1, we postulate that cyclin D_1 and HIF-1 may both be stimulated by the same mechanism (hypoxia or oncogenes).

Another result of this study was the inverse association between HIF-1α and BCL-2 (an inhibitor of apoptosis). Loss of BCL-2 is associated with tumor aggressiveness in breast cancer, and fits to our model in which upregulation of HIF-α is associated with breast cancer aggressiveness. In contrast to (precursor) lesions of

oral cavity cancer [5], we found a positive association between HIF-1α and the apoptotic index [6]. Although we do not have the data to prove so, we propose that this association is epigenetic, because in aggressive breast cancer a high rate of both proliferation and apoptosis co-exist.

In **chapter 5**, in 45 invasive breast cancers the association between HIF-1α and several important growth factors, growth inhibiting factors and their receptors is described. In summary, we found that HIF-1α is significantly associated with expression of growth factors PDGF-BB and bFGF, and growth factor receptor EGFR. In prostate cancer, androgens have been shown to increase HIF-1 activity via stimulating EGF, which in turn stimulates HIF-1 [7]. In this way, the use of antiandrogens might inhibit HIF-1. These and our data contribute to the rationale for blocking EGFR and HER-2/*neu* in breast cancer patients, because we think that these growth factor inhibitory therapies may diminish HIF-1 activity. However**,** the exact mechanisms responsible for the association of HIF-1α with EGFR, bFGF and PDGF are still unknown. Most likely, the growth factors and receptors induce HIF-1 activation in an autocrine fashion or via a mechanism similar to HER-2/*neu.*

Surprisingly, in these 45 patients only a trend for a positive association between HIF-1α and VEGF (*P*=0.109) was found, while significant associations were found for HIF-1α and VEGF in chapters 2 and 7.

Finally, the finding of an association of the microvessel density (as a degree of angiogenesis) with HIF-1α levels in human breast cancer is described for the first time. This is in line with data from other tumors types [8,9,10,11] that also show a positive association between HIF-1α and angiogenesis. Since in breast cancer high levels of HIF-1α as well as a high microvessel density are associated with a poor prognosis [12], and because both factors are interrelated, we argue that targeting HIF-1 might have anti-angiogenesis effects in breast cancer.

In **chapter 6**, the long-term survival of 150 early stage breast cancer patients has been related to the protein levels of HIF-1α. For the first time it was shown that high levels of HIF-1α independently predict poor prognosis in patients with invasive breast carcinoma, especially in the subgroup of lymph-node negative patients. When these data can be confirmed, it suggests that levels of HIF-1α may be used to select high risk patients with negative lymph node status who would benefit from systemic adjuvant therapy. Obviously, before its introduction in daily practice, the predictive value of HIF-1α has to be investigated, and a large prospective international study is required to prove so. Further, before clinical implementation, additional research should try to answer the question whether the combined overexpression of HIF-1α and its downstream targets better predicts prognosis in breast cancer than HIF-1α alone. In fact, in a non-small-cell lung cancer study, the combined value of HIF-1α and LHD-5 was suggested to reflect a functional HIF-1 pathway, and harbored stronger prognostic significance than both markers separately. Also, the use of surrogate markers of HIF-1α expression (i.e. downstream targets, which only represent HIF-1 activity) should be investigated, in order to bypass the relatively difficult detection method for detecting HIF-1α.

Further, in the same 150 patients we provided clinical confirmation of the impor-

tant association between HIF-1α expression and the amplification and overexpression of HER-2/*neu* found *in vitro* [13]. This implies that *in vivo* HIF-1α-overexpression may also be due to HER-2/*neu* amplification and overexpression. In addition, besides EGFR and HER-2/*neu*, HIF-1α staining might be considered as a predictor (and perhaps follow up marker) for response to anti-HER-2/*neu* and anti-EGFR therapies. This is supported by Koukourakis et al., who noted that the difference in clinical response to trastuzumab treatment in two locally recurrent HER-2/*neu* positive breast carcinomas could be explained by a difference in expression of HIF-1. Special attention should be paid to genetic alterations which can induce HIF-1 activity. In fact, both Cowden disease and Li Fraumeni syndrome, based on loss of both PTEN alleles or due to a p53 mutation, respectively, are associated with breast cancer. These patients could benefit even more from forthcoming adjuvant anti-HIF-1 therapy, and clinicians should consider such treatment for such patients.

In accordance with the results of chapter 2, we found that high levels of HIF-1α were correlated with overexpression of VEGF, increased proliferation, and poor histologic grade. However, in contrast to chapter 2, we observed a negative association between levels of HIF-1α and PR, and ER. This observation is consistent with the prognostic value of HIF-1α, because loss of the steroid receptors characterizes tumor aggressiveness. This discrepancy may be explained by the fact that Chapter 5 was confined to invasive breast cancer without (as in chapter 2) the inclusion of normal breast tissue. Additional analysis on the separate group of invasive breast cancers of chapter 2 indeed revealed no association between overexpression of HIF-1α and ER.

Whether future anti-HIF therapeutics, such as 2-methoxyestradiol (2ME2) or YC-1 [14,15], will be beneficial in practice, should be awaited from the first trials that are ongoing [16]. In the meantime however HIF-1 has substantially increased our current understanding of multiple pathways in breast cancer, and has contributed to more knowledge of the mechanism of other therapies, such as the tyrosine kinase inhibitors and anti-angiogenesis therapies.

BCL-6 in breast cancer

In **chapter 4**, we were the first to recognize BCL-6 protein expression in breast cancer. Thus, the dogma that BCL-6 overexpression is a unique feature of lymphomas was discarded. Increased BCL-6 nuclear expression was seen in 35% of invasive breast cancers and BCL-6 protein overexpression was found in 16% of cases. Also, the recognized breast cancer precursor lesions ductal hyperplasia and DCIS sometimes showed increased expression. Genetic alterations are the main reason for tumor biological activity of BCL-6 in lymphomas. Our data imply that BCL-6 gene amplification could, in part, be responsible for protein overexpression, because by analyzing 86 breast cancers with comparative genomic hybridization (CGH) we found in 16% of patients a gain at the BCL-6 locus, 25% of these having BCL-6 protein overexpression. More research to unravel the exact mechanism of BCL-6 overexpression is however needed.

Further, BCL-6 nuclear overexpression was significantly associated with overexpression of HIF-1α. This association was unexpected and a functional relationship between these genes has not been established. We also observed concerted overex-

pression of HIF-1α and BCL-6 in diffuse large cell B-cell lymphomas to exclude a breast cancer restricted phenomenon. Interestingly, BCL-6 aberrations in lymphomas have been associated with high LDH levels, which indirectly support our HIF-1 association with BCL-6 [17,18]. Further research is definitely required to unravel whether this association has any functional meaning.

BCL-6 nuclear overexpression was also significantly associated with p53 and cyclin D_1 overexpression. This association between BCL-6 and cyclin D_1 might be important since many investigators have searched for the cause of cyclin D_1 upregulation in breast cancer, which is a common phenomenon and just partly explained by a genetic alteration (amplification, translocation) and ER expression. BCL-6 therefore seems to qualify as a next upregulator of cyclin D_1.

In lymphomas presence of BCL-6 translocation at certain locations is known to harbor a relative good prognostic sign [19,20,18]. However, we could not detect prognostic value for BCL-6 in breast cancer (unpublished data).

PET and breast cancer

In **chapter 7**, we showed that a model with multiple parameters for delivery of glucose can explain the differential uptake of ^{18}FDG in PET scanning of breast cancer patients. This model consists of several credible biologic variables: microvessels that provide glucose, Glut-1 that transports ^{18}FDG into the cell, hexokinases to enter ^{18}FDG into glycolysis, number of tumor cells per unit volume, rate of tumor cell proliferation (also reflected in necrosis), amount of inflammatory cells within the tumor, and, to some extent, HIF-1α that is upregulated by hypoxia and induces Glut-1 expression and angiogenesis.

With this study, we were the first to report overexpression of hexokinase I in breast cancer. In fact, HK I overexpression was associated with high ^{18}FDG uptake. HK II has traditionally more often been related to glucose uptake, but in our study to a much lesser extent and only significant in multivariate analysis. Further, it was investigated whether the presence of lymphocytes, macrophages, or necrosis could explain ^{18}FDG accumulation. Indeed, in this study, the number of lymphocytes was correlated positively with ^{18}FDG uptake, in contrast to the presence of macrophages. Also, a positive correlation between the presence of necrosis and ^{18}FDG uptake was found. Evidence for this relation can be found in the predominant presence of Glut-1 and HIF-1 around necrosis as noted in this study. No correlation between glucose metabolism and the expression of the angiogenesis inducing growth factor $VEGF_{165}$ could be detected. However, the involvement of HIF-1 in ^{18}FDG accumulation was limited to some additional value compared with features like the rate of proliferation and Glut-1 expression. In contrast, a direct correlation between ^{18}FDG uptake and the intratumoral microvessel density was found. Also, a large variation in tumor cell density was found in breast cancer (from 12% to 70% cell density), with a strong positive correlation with ^{18}FDG uptake.

In summary, this study resolves why invasive lobular breast cancers in general have low ^{18}FDG uptake, and why there is such a large variety of ^{18}FDG uptake in other types of breast cancer.

Conclusions

1. Both cellular hypoxia and oncogenes are considered main activators of HIF-1 activity in breast cancer, with a minor role for immune modulators. Unraveling whether hypoxia, oncogenes or growth factors are responsible for HIF-1 activation will identify potential therapeutic targets. In the present thesis, we link both HER-2/*neu*, EGFR and PDGF with HIF-1 activation.
2. Blocking HIF-1 seems to be attractive in breast cancer treatment since several downstream effects of HIF-1 (such as VEGF production, formation of angiogenesis, and stimulation of glycolysis) all potentiate aggressiveness of breast cancer.
3. As a consequence, levels of HIF-1α represent tumor aggressiveness and predict prognosis. This is especially valuable to identify high risk lymph node negative breast cancer patients, and might be used for further studies to assess the risk of pure DCIS recurrence.
4. BCL-6 protein is overexpressed in breast cancer, possibly due to gene amplification, and is associated with p53, cyclin D1 and HIF-1α.
5. The different intensity of FDG-PET scanning between breast cancer patients can be explained by a biologic model of glucose transport and rate of proliferation per tumor volume.

REFERENCES

1. Huss WJ, Hanrahan CF, Barrios RJ, Simons JW, and Greenberg NM. Angiogenesis and prostate cancer: identification of a molecular progression switch. Cancer Res 2001;61:2736-2743.
2. Zhong H, Semenza GL, Simons JW, and De Marzo AM. Up-regulation of hypoxia-inducible factor 1alpha is an early event in prostate carcinogenesis. Cancer Detect Prev 2004;28:88-93.
3. Vaupel P, Schlenger K, Knoop C, and Hockel M. Oxygenation of human tumors: evaluation of tissue oxygen distribution in breast cancers by computerized O2 tension measurements. Cancer Res 1991;51:3316-3322.
4. Maxwell PH, Dachs GU, Gleadle JM, Nicholls LG, Harris AL, Stratford IJ, Hankinson O, Pugh CW, and Ratcliffe PJ. Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc Natl Acad Sci U S A 1997;94:8104-8109.
5. Costa A, Coradini D, Carrassi A, Erdas R, Sardella A, and Daidone MG. Re: Levels of hypoxia-inducible factor-1alpha during breast carcinogenesis. J Natl Cancer Inst 2001;93:1175-1177.
6. Bos R, van Diest PJ, and van der Wall E. RESPONSE: Re: Levels of Hypoxia-Inducible Factor-1 During Breast Carcinogenesis. J Natl Cancer Inst 2001;93:1177-1177.
7. Mabjeesh NJ, Willard MT, Frederickson CE, Zhong H, and Simons JW. Androgens stimulate hypoxia-inducible factor 1 activation via autocrine loop of tyrosine kinase receptor/phosphatidylinositol 3'-kinase/protein kinase B in prostate cancer cells. Clin Cancer Res 2003;9:2416-2425.
8. Birner P, Gatterbauer B, Oberhuber G, Schindl M, Rossler K, Prodinger A, Budka H, and Hainfellner JA. Expression of hypoxia-inducible factor-1 alpha in oligodendrogliomas: its impact on prognosis and on neoangiogenesis. Cancer 2001;92:165-171.
9. Kuwai T, Kitadai Y, Tanaka S, Onogawa S, Matsutani N, Kaio E, Ito M, and Chayama K. Expression of hypoxia-inducible factor-1alpha is associated with tumor vascularization in human colorectal carcinoma. Int J Cancer 2003;105:176-181.
10. Sivridis E, Giatromanolaki A, Gatter KC, Harris AL, and Koukourakis MI. Association of hypoxia-inducible factors 1alpha and 2alpha with activated angiogenic pathways and prognosis in patients with endometrial carcinoma. Cancer 2002;95:1055-1063.
11. Takahashi R, Tanaka S, Hiyama T, Ito M, Kitadai Y, Sumii M, Haruma K, and Chayama K. Hypoxia-inducible factor-1alpha expression and angiogenesis in gastrointestinal stromal tumor of the stomach. Oncol Rep 2003;10:797-802.
12. de Jong JS, van Diest PJ, and JP AB. Hot spot microvessel density and the mitotic activity index are strong additional prognostic indicators in invasive breast cancer. Histopathology 2000;36:306-312.
13. Laughner E, Taghavi P, Chiles K, Mahon PC, and 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;21:3995-4004.
14. Mabjeesh NJ, Escuin D, LaVallee TM, Pribluda VS, Swartz GM, Johnson MS, Willard MT, Zhong H, Simons JW, and Giannakakou P. 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF. Cancer Cell 2003;3:363-375.
15. Yeo EJ, Chun YS, Cho YS, Kim J, Lee JC, Kim MS, and Park JW. YC-1: a potential anticancer drug targeting hypoxia-inducible factor 1. J Natl Cancer Inst 2003;95:516-525.
16. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3:721-732.
17. Jerkeman M, Aman P, Cavallin-Stahl E, Torlakovic E, Akerman M, Mitelman F, and Fioretos T. Prognostic implications of BCL-6 rearrangement in uniformly treated patients with diffuse large B-cell

lymphoma--a Nordic Lymphoma Group study. Int J Oncol 2002;20:161-165.
18. Vitolo U, Botto B, Capello D, Vivenza D, Zagonel V, Gloghini A, Novero D, Parvis G, Calvi R, Ariatti C, Milan I, Bertini M, Boccomini C, Freilone R, Pregno P, Orsucci L, Palestro G, Saglio G, Carbone A, Gallo E, and Gaidano G. Point mutations of the BCL-6 gene: clinical and prognostic correlation in B-diffuse large cell lymphoma. Leukemia 2002;16:268-275.
19. Artiga MJ, Saez AI, Romero C, Sanchez-Beato M, Mateo MS, Navas C, Mollejo M, and Piris MA. A short mutational hot spot in the first intron of BCL-6 is associated with increased BCL-6 expression and with longer overall survival in large B-cell lymphomas. Am J Pathol 2002;160:1371-1380.
20. Lossos IS, Jones CD, Warnke R, Natkunam Y, Kaizer H, Zehnder JL, Tibshirani R, and Levy R. Expression of a single gene, BCL-6, strongly predicts survival in patients with diffuse large B-cell lymphoma. Blood 2001;98:945-951.

Nederlandse samenvatting

Voor niet-ingewijden

HIF-1 in borstkanker
BCL-6 in borstkanker
PET en borstkanker
Algemene conclusies

IN DIT PROEFSCHRIFT (dissertatie) worden de wetenschappelijke gegevens gebundeld die in zes verschillende wetenschappelijke tijdschriften zijn gepubliceerd. Deze zes artikelen worden voorafgegaan door een inleiding en afgesloten met een discussie. Hiermee krijgt de lezer de onderliggende medische kennis, de reden van het onderzoek, de resultaten en suggesties voor vervolgonderzoek.

In **hoofdstuk 1,** de introductie, wordt uitgelegd dat dit proefschrift met name over de rol van HIF-1 in borstkanker gaat. Tot voor kort (1999) was hier nog nooit onderzoek naar gedaan. In 1999 waren wij, samen met onze collega's in Baltimore (De Verenigde Staten), de eerste onderzoeksgroep die hier interesse voor kregen. Tot dan toe was van geen enkele kankersoort bekend welke rol HIF-1 zou kunnen spelen. Wij kozen ervoor om de rol van HIF-1 bij borstkanker te onderzoeken omdat borstkanker in Nederland de meest voorkomende vorm van kanker is. Per jaar krijgen in Nederland 11000 vrouwen te horen dat zij borstkanker hebben en dit aantal neemt nog elk jaar toe.

HIF-1 is de afkorting van Hypoxie induceerbare factor-1. Hypoxie staat voor zuurstof (O_2) gebrek. In 1995 werd deze factor ontdekt door Prof.Gregg Semenza, werkzaam in het Johns Hopkins Kanker Centrum in Baltimore. Hierna werd snel duidelijk dat HIF-1 een belangrijk eiwit is, die in alle zoogdiercellen aanwezig is en nauwlettend de leefomstandigheden van een cel in de gaten houdt. Indien een cel te weinig zuurstof of energie (glucose) krijgt dan wordt deze overlevingsfactor actief, omdat dan het bestaan van de cel wordt bedreigd. HIF-1 werkt als een overlevingsfactor omdat het bepaalde genen kan dwingen tot stoppen met werken of juist kan activeren. Hierdoor gaan er meerdere reddingsmechanismen werken, zoals de aanmaak van nieuwe bloedvaatjes (voor energie- en zuurstoftoevoer). Ook worden andere manieren van energieproductie gestart.

HIF-1 kan al deze processen reguleren omdat het alleen actief is bij zuurstof- of energietekort, dan komen twee eiwitten (HIF-1α en HIF-1β) bij elkaar om het actieve eiwitcomplex HIF-1 te vormen. Omdat HIF-1β altijd aanwezig is, maar HIF-1α alleen bij zuurstof- of energiegebrek, wordt dit eiwit als de zuurstof- en energiemeter van de cel beschouwd.

Omdat van veel kankersoorten, zoals borstkanker, bekend is dat bij het ontstaan van kanker nieuwe bloedvaatjes worden aangemaakt en andere vormen van energieproductie worden gebruikt, veronderstelden wij dat HIF-1 aan deze processen ten grondslag zou kunnen liggen.

HIF-1 in borstkanker

Daarom onderzochten we, zoals beschreven in **hoofdstuk 2,** in een model voor het ontstaan van borstkanker of en wanneer het eiwit HIF-1α aanwezig is. Inderdaad bleek HIF-1α aantoonbaar, met behulp van een herkenningsstof (antilichaam) van het afweersysteem. Dit antilichaam herkent specifiek HIF-1α en is zichtbaar te maken met behulp van een kleurstof, op heel dunne plakjes van het operatiemateriaal van borstkanker patiënten (deze techniek heet immuno-histochemie). HIF-1α was niet aantoonbaar in normaal weefsel en goedaardige borstafwijkingen. Wel vonden we HIF-1α in het voorstadium van borstkanker en in borstkanker zelf en dan

met name in de snelst groeiende tumoren.

Tevens vonden we dat de aanwezigheid van HIF-1α met name in tumoren met veel celdelingen, dus bij de agressievere vormen van borstkanker. In het weefsel van vrouwen met een voorstadium van kanker werd een overeenkomst tussen HIF-1α en de aanmaak van nieuwe bloedvaatjes gevonden. Verder zagen we dat HIF-1α voornamelijk aangetoond kon worden in kankergebieden rondom dode kankercellen. Verondersteld wordt dat dit wijst op de productie van HIF door cellen in de nabijheid van gebieden met zuurstoftekort. Een andere verklaring voor de aanwezigheid van HIF-1α in borstkanker zou ook een genetische verandering kunnen zijn die met borstkanker te maken heeft zoals de overmatige aanwezigheid van het HER-2/*neu* gen.

Om de overeenkomst tussen HIF-1α en veel celdelingen (en dus snelle tumorgroei) verder te onderzoeken hebben wij, zoals uiteengezet in **hoofdstuk 3**, bij 150 borsttumoren verschillende eiwitten, die bekend zijn om hun betrokkenheid bij de celdeling, onderzocht op hun associatie met HIF-1α.

In deze studie werd duidelijk dat de aanwezigheid van HIF-1α samengaat met een hoge celdelingsactiviteit (cycline A en Ki-67), waarmee onze eerdere observatie bevestigd kon worden

Echter, theoretisch klopt een associatie tussen celdeling (welke veel energie verbruikt) en HIF-1α (is er alleen bij bedreigde cel in tijden van verminderde energie en zuurstof) niet. Daarom twijfelen wij of er een directe relatie is tussen beiden. Dit wordt mede gesteund door het feit dat deze associatie alleen gevonden wordt in tumoren zonder de hormoonreceptor, oestrogeen receptor, (OR). In de groep tumoren met wel een OR is er juist sprake van een associatie met p21 en niet met celdeling. p21 is één van de vele genen die door HIF-1 wordt geactiveerd. Van p21 is bekend dat het de celdeling remt. Omdat al veel langer bekend is dat OR positieve cellen veel minder celdelingen hebben, vergeleken we in de beide OR groepen ook 2 andere factoren, VEGF en p53, met HIF-1α. Hieruit blijkt dat in de OR negatieve groep de verwachte associatie met HIF-1α en VEGF en p53 bestaat, maar niet in de OR positieve groep. Dit sterkt ons vermoeden dat er een essentieel verschil is tussen OR status en de interactie met HIF-1 en dat deze niet alleen berust op een verschil in celdeling. Wij stellen de hypothese dat OR nodig is om via HIF-1/p21 de celdeling te remmen. Meer onderzoek op dit gebied lijkt ten zeerste aanbevolen.

Verder toonden we in deze studie aan dat een hoge mate van aanwezigheid van p53 (een belangrijk eiwit dat betrokken is bij herstel van schade aan de genen en hiermee de celdeling bewaakt en indien dit mislukt tot celdood kan aanzetten) geassocieerd is met aanwezigheid van HIF-1α.

Tevens werd een verhoogde aanwezigheid van cycline D_1 (eiwit betrokken bij celdeling) geassocieerd met p21, maar niet met HIF-1α. Omdat p21 door HIF wordt aangestuurd en mogelijk dus ook een relatie heeft met cycline D_1, stellen we de hypothese dat beiden door hetzelfde mechanisme zouden kunnen worden geactiveerd (zuurstof gebrek of genetische veranderingen).

Als laatste van deze studie dient BCL-2 genoemd te worden. Van BCL-2 is bekend dat het celdood tegen kan gaan. Bij borstkanker geldt dat hoe agressiever de tumor hoe minder BCL-2. En inderdaad, wij vonden hoe meer HIF-1α, des te minder

BCl-2. Dit onderschrijft dan toch weer onze eerdere bevindingen dat HIF-1α met name bij snel groeiende tumoren tot uiting komt.

Bloedvatnieuwvorming, angiogenese, is een bekend fenomeen bij borstkanker. Hierdoor kunnen de tumorcellen beter en sneller groeien en eventueel uitzaaien naar elders in het lichaam. Inmiddels zijn er al verscheidene geneesmiddelen die de bloedvatnieuwvorming remmen. Om een beter inzicht te krijgen in de rol van HIF-1 bij de totstandkoming van nieuwe bloedvaten bij borstkanker bestudeerden we 45 tumoren in **hoofdstuk 5**. Hierbij keken we naar belangrijke groeifactoren die de bloedvatnieuwvorming stimuleren of juist remmen, ook werd de hoeveelheid bloedvaten per tumor geteld als maat voor de aanwezigheid van bloedvatnieuwvorming. De groeifactoren PDGF-BB en bFGF bleken geassocieerd met de aanwezigheid van HIF-1α. Hetzelfde geldt voor EGFR, een andere groeifactor receptor. Onduidelijk is nog hoe deze factoren op HIF-1 ingrijpen, maar mogelijk wordt HIF-1 door deze factoren gestimuleerd (dus zonder zuurstof- of energiegebrek). Op deze manier zal ook een genetische afwijking of een groeifactor, geproduceerd door de tumor, HIF-1 kunnen activeren. Momenteel zijn er al nieuwe medicijnen die sommige van deze groeifactoren kunnen blokkeren. Mogelijk hebben deze nieuwe middelen een goed anti-tumor effect doordat ze ook HIF-1 onderdrukken.

Van VEGF (een belangrijke groeifactor die bloedvatvorming stimuleert en aangestuurd wordt door HIF-1) konden we in deze groep van 45 patiënten geen statistische relatie vinden met HIF-1, terwijl we dat wel konden aantonen in hoofdstuk 2 en 7 (bij grotere groepen patiënten).

Vele onderzoeken hebben laten zien dat de levensverwachting van een patiënt met borstkanker slechter is als er veel bloedvaatjes in de tumor zijn. Wij vonden in deze studie dat de aanwezigheid van veel bloedvaatjes samengaat met HIF-1 activiteit.

Wij suggereren dat de nieuwe zogenaamde anti-angiogenese middelen, medicijnen tegen bloedvaatjes, eigenlijk vervangen zouden moeten worden door geneesmiddelen die tegen HIF-1 gericht zijn.

Omdat we nu weten dat HIF-1α in borstkanker voorkomt, was onze volgende vraag of de aanwezigheid van HIF-1α iets zegt over de levensverwachting van borstkanker patiënten. Daarom hebben we in **Hoofdstuk 6** het operatiemateriaal van een groep van 150 vrouwen met borstkanker uit de jaren 1987-1993 onderzocht op de aanwezigheid van HIF-1α. Door de overleving van deze patiënten te vergelijken met de hoeveelheid HIF-1α in hun tumoren bleek dat vrouwen met veel HIF-1α eerder uitzaaiingen krijgen en/of eerder dood gaan. Met name in de groep vrouwen die geen uitzaaiingen in de okselklieren (lymfeklieren =Ln) hadden bleek dat HIF-1α een voorspeller is van de levensverwachting. Dat is een bijzonder interessante bevinding omdat juist bij deze categorie patiënten bekend is dat een deel van hen een goede levensverwachting heeft en een deel een grote kans heeft op het ontstaan van uitzaaiingen. Echter, tot op heden is er geen sluitende methode om de patiënten met een goede en zij met een slechte levensverwachting van elkaar te onderscheiden, en dus is onvoldoende bekend wie je wel en wie je niet moet nabehandelen met bv chemotherapie Er is recent veel aandacht voor nieuwe methoden die op basis van zogenaamde genetische profielen van de borsttumor een onderscheid kunnen maken in vrouwen met een goede en zij met een minder goede levensverwachting.

Het Nederlands Kanker Instituut in Amsterdam speelt hierin een voortrekkers rol. Het zal nog even duren voordat deze methoden routinematig gebruikt kunnen gaan worden. Het lijkt erop dat HIF-1α in de tussentijd een vergelijkbare rol zou kunnen vervullen. Echter, voordat dit in de praktijk gebruikt kan worden dient eerst in een grotere groep patiënten deze resultaten herhaald te worden. Verder onderzoek is ook nodig om uit te maken of HIF-1α gecombineerd met andere tumorkarakteristieken een nog betere voorspeller is van de levensverwachting.

Tevens vonden we in dit onderzoek een sterke samenhang tussen HIF-1α en HER-2/*neu*. Dit betekent dat, naast zuurstof en energietekort, inderdaad ook genetische afwijkingen kunnen lijden tot activatie van HIF-1. HER-2/*neu* is een eiwit dat, als gevolg van een genetische afwijking, in overmaat aanwezig is op de celmembraan van tumorcellen en voorkomt bij tumoren van 20-30% van de borstkanker patiënten. Er is inmiddels een medicijn beschikbaar, trastuzumab, dat specifiek het HER-2/*neu* in haar werking blokkeert. Mogelijk dat een deel van de effectiviteit van trastuzumab te verklaren is door een effect op HIF-1.

Zodra er medicijnen beschikbaar zijn die tegen HIF-1 gericht zijn, moeten deze met name ingezet worden bij bepaalde genetische (Li Fraumeni en Cowden) oorzaken van borstkanker. Omdat bij deze vormen van borstkanker HIF-1 door een gendefect continu gestimuleerd wordt (dus zonder dat er een zuurstof tekort is).

In overeenstemming met de resultaten van hoofdstuk 2, vonden we ook bij dit onderzoek dat HIF-1α geassocieerd was met snel groeiende tumoren en de groeifactor VEGF. Echter, in tegenstelling tot hoofdstuk 2 vonden we in dit onderzoek een associatie tussen HIF-1α en het ontbreken van de oestrogeen- en progesteronreceptor (in plaats van het aanwezig zijn daarvan). Waarschijnlijk komt deze tegenstelling doordat we in Hoofdstuk 2 ook goedaardige afwijkingen in de analyse hebben meegenomen.

Bcl-6 in borstkanker

Van een andere hoek vanuit het kankeronderzoek, namelijk kanker van lymfeklieren (non-hodgkin lymfoom), kwamen we op het idee om te kijken of BCL-6 ook in borstkanker aanwezig is. Deze gedachte is gebaseerd op het feit dat BCL-6 een belangrijke rol bij de regulatie van celdeling en celdood bleek te hebben. Tot nog toe werd aangenomen dat BCL-6 alleen bij lymfomen voorkwam. Bij deze tumoren zijn ook genetische afwijkingen beschreven die de aanwezigheid van BCL-6 verklaren. Vanwege de algemene betrokkenheid bij de celdeling veronderstelden wij dat BCL-6 ook bij borstkanker een rol bij de celdeling zou kunnen spelen. In **hoofdstuk 4**, vonden we inderdaad dat BCL-6 ook bij borstkanker aanwezig kan zijn. Door tumoren van 86 borstkanker patiënten genetisch te onderzoeken, bleek dat bij hen bij wie veel BCL-6 eiwit aanwezig was inderdaad genetische afwijkingen aangetoond konden worden in de buurt van het BCL-6 gen. Mogelijk zijn er bij borstkanker dus ook genetische afwijkingen die leiden tot BCL-6 activatie. Echter meer onderzoek naar de oorzaak van BCL-6 aanwezigheid bij borstkanker is nodig. In tegenstelling tot de aanwezigheid van HIF-1α lijkt de aanwezigheid van BCL-6 niets over de levensverwachting van een borstkankerpatiënt te zeggen (niet gepubliceerde gegevens).

Verrassend genoeg bleek dat de aanwezigheid van BCL-6 samenging met de aanwezigheid van HIF-1α. Of en hoe beide factoren elkaar beïnvloeden is ons niet

duidelijk. Om meer zekerheid over onze bevinding te krijgen keken we ook naar de aanwezigheid van HIF-1α in lymfomen, deze ging inderdaad samen met BCL-6. Verder is al eerder beschreven dat lymfomen met BCL-6 veel LDH produceren. LDH wordt onder andere gestimuleerd door HIF-1, dus een directe relatie tussen beide lijkt zeker niet uitgesloten. Ook hier geldt dat meer onderzoek nodig is.

Verder vonden we ook dat BCL-6 geassocieerd was met p53 en cycline D_1. Cycline D_1 is een celdelingseiwit welke vaak verhoogd aanwezig is bij borstkanker, waarom is tot nog toe onduidelijk. Mogelijk dat een genetische afwijking in BCL-6 dus ook leidt tot cycline D_1 activatie.

PET en borstkanker

Positron emissie tomografie (PET) is een geavanceerde techniek om met behulp van nucleair geladen deeltjes een bepaalde stof in het lichaam te vervolgen door de vrijgekomen energie van deze deeltjes te meten. Daar waar de gemarkeerde stof in het lichaam zich ophoopt zal op de scan een vlekje te zien zijn. Op het gebied van kankeronderzoek wordt vooral gebruik gemaakt van nucleair geladen glucose (de eenvoudigste vorm van suiker). Door een patiënt deze glucose in te spuiten zal daar waar veel energie (=suiker) nodig is een vlekje op de scan ontstaan. Op deze manier kunnen sommige uitzaaiingen al worden gezien die niet door reguliere CT-scans worden gezien. Uiteraard is het zichtbaar worden van een vlekje ook afhankelijk van de grootte van een tumor.

Bij borstkanker komt het soms voor dat bij een patiënt geen aankleuring wordt gezien terwijl er bij operatie wel een grote tumor in de borst gevonden is. Ook het omgekeerde komt voor. Dit heeft bij veel nucleair geneeskundigen vraagtekens opgeroepen, zonder ooit een duidelijk antwoord te krijgen. Omdat HIF-1 betrokken is bij zowel de vorming van glucose als het aanmaken van bloedvaatjes (aanvoer van glucose), veronderstelden wij dat wij middels HIF vele vraagtekens konden beantwoorden. Daarom onderzochten wij in **hoofdstuk 7** welke factoren een belangrijke rol spelen bij het wel of niet zichtbaar worden van een borstkanker tumor op een PET scan. Daartoe stelden we een model op van verschillende factoren die van belang zouden kunnen zijn om het verschil in glucoseopname te verklaren. Deze factoren werden bij tumoren van 55 borstkanker patiënten bepaald nadat ze een PET scan hadden gehad en waren geopereerd. Bij patiënten met een sterk PET signaal kwamen de volgende factoren naar voren; grote aantallen bloedvaatjes in de tumor (aanvoer glucose), aanwezigheid van Glucose transporter-1 (een eiwit dat glucose vanuit het bloed de cel in vervoerd), van hexokinases (enzymen die nodig zijn voor verbranding van glucose tot energie), de hoeveelheid tumorcellen per volume oppervlak (er is een groot verschil qua compositie tussen verschillende borstkankers), een hoge celdeling, veel aanwezige afweercellen en indirect ook de aanwezigheid van HIF-1α (stimuleert de bloedvaatjesgroei, productie van Glut-1 en verbranding van glucose).

Hiermee waren wij de eerste die een duidelijke verklaring hadden voor de verschillen op een PET scan tussen patiënten onderling. Ook was nog nooit de aanwezigheid van hexokinase I in borstkanker gerapporteerd. Tot nog toe werd juist van macrofagen (opruimcellen) aangenomen dat ze veel glucose gebruiken en dus een hoger PET signaal geven, echter wij konden dit niet bevestigen. Nieuw was ook dat

we een associatie vonden tussen de bloedvaatjesdichtheid en een sterk PET signaal. Een overeenkomst tussen VEGF en het PET signaal konden we vreemd genoeg niet vinden. Wat ook opviel was dat Glut-1 met name in nog levende cellen rond resten van dode cellen te zien was. Hoogst waarschijnlijk is deze glut-1 door zuurstoftekort (HIF-1) geactiveerd.

Een bepaald type borstkanker (lobulair) heeft vaak een zwak PET signaal. Dit kunnen we nu goed begrijpen aan de hand van boven beschreven factoren, omdat lobulaire tumoren meestal niet snel delen en niet compact of bolvormig, maar juist heel diffuus en dun bezaaid groeien.

Algemene conclusies

1. Zowel zuurstoftekort als ook genetische veranderingen kunnen HIF-1 activeren in borstkanker. In dit proefschrift vinden we aanwijzingen dat HER-2/*neu*, EGFR en PDGF kunnen zorgen voor HIF-1 activatie.
2. Therapie tegen HIF-1 lijkt een aantrekkelijk nieuw doelwit omdat daarmee meerdere onderliggende processen worden aangepakt (VEGF productie, bloedvat-nieuwvorming, energievorming) die allen ten grondslag liggen aan agressieve kankersoorten.
3. De hoeveelheid HIF-1α geeft de agressiviteit van een tumor weer en voorspelt de levensverwachting van een patiënt. Hiermee zou met name bij patiënten zonder lymfeklieruitzaaiingen bepaald kunnen worden wie er wel en wie geen aanvullende chemotherapie nodig heeft. Mogelijk kunnen ook de ‘slechte’ voorstadia van borst-kanker onderscheiden worden van de relatief gunstige voorstadia.
4. BCL-6 eiwit is ook bij borstkanker aanwezig, hetgeen mogelijk door genetische afwijkingen verklaard kan worden. Tevens is, bij borstkanker, BCL-6 geassocieerd met p53, cyclin D_1 en HIF-1α.
5. Het verschil in PET signaal tussen verschillende borstkanker patiënten kan verklaard worden door een biologisch model waarin het glucose transport, de celde-lingsnelheid en het aantal tumorcellen per oppervlak van groot belang zijn.

Curriculum Vitae

REINHARD BOS (1975) was born in Rotterdam, grew up in Papendrecht and studied one year physics in Delft. In 1995 he started as a medical student at the Free University Amsterdam (now named Free University Medical Center, VUmc). In addition to his study he worked from his first till his third study year at the MS-MRI Center as a MRI image processor at the department of Radiology (Prof dr. F. Barkhof). He measured, with a computerized method, the volume of Multiple Sclerosis lesions on MRI brain images. Thereafter, he worked as a medical administrative employer for a general practitioner in Amsterdam (1999-2000). During this period he compiled and inserted the medical history of 1200 patients in a computerized general practitioner information system.

Meanwhile, also next to his study, in October 1998 Reinhard started his first laboratory investigations in the field of breast cancer under the auspice/ supervision of Prof. dr. P.J. van Diest (pathologist). In 1999, Prof dr E. van der Wall (oncologist) joined the project, which soon expanded into a PhD program in combination with his medical study. In June 1999, Reinhard followed a rotation in HIF-1α immunohistochemistry at the Johns Hopkins University School of Medicine, USA (Prof. dr. J.W. Simons).

After his marriage with Nannie Schous and fulfilling his masters degree in 1999, Reinhard decided to postpone his medical study for two years to fully dedicate himself to basic scientific research. Posters with research data of Reinhard Bos were presented at several international conferences including the 23rd San Antonio Breast Cancer Symposium in San Antonio, TX USA (2000), for which he achieved a personal travel scholarship by the Royal Dutch Scientific Academy (Van Walree Foundation).

From 2001 to 2003 he followed his internship in the VU medical center and hospitals affiliated to the VUmc. In October 2003 he passed his MD examination. Thereafter, he completed his breast cancer research as described in this thesis. From February 2004 he started with his residency in internal medicine at the Academic Hospital Groningen (Prof. dr. R.O.B. Gans and Prof. dr. M.H. van Rijswijk).

REINHARD BOS WERD geboren in Rotterdam op 1 februari 1975. Hij groeide op in Papendrecht, alwaar hij ook zijn middelbare school periode volgde aan de Prins Bernard Mavo. Havo 4 en VWO 5 en 6 doorliep hij ook in Papendrecht aan de Lage Waard Scholengemeenschap. Daarna studeerde hij een jaar technische natuurkunde in Delft.

In 1995 begon hij met de studie geneeskunde aan de Vrije Universiteit Amsterdam (VUmc). Vanaf zijn eerste tot derde studiejaar kwam hij in aanraking met het doen van onderzoek bij het MS-MRI Center van de afdeling Radiologie (Prof dr. F. Barkhof). Met een gecomputeriseerde methode mat hij het volume van Multiple Sclerose laesies op MRI beelden. Daarna werkte hij bij een Amsterdamse huisartsenpraktijk (1999-2000) om de medische voorgeschiedenis van ca 1200 patiënten samen te vatten en in de computer in te voeren.

Daarnaast (en naast zijn studie) startte hij in 1998 met zijn eerste laboratorium onderzoek naar borstkanker bij Prof dr. P.J. van Diest (patholoog). Door de samenwerking met Prof dr E. van der Wall (oncoloog) werd HIF-1α het studieobject. Hiervoor volgde Reinhard in juni 1999, een training in HIF-1α immunohistochemie in het Johns Hopkins in Baltimore (Prof. dr. J.W. Simons).

Na zijn huwelijk met Nannie Schous en zijn afstuderen in 1999, besloot Reinhard om twee jaar fulltime onderzoek te gaan doen. Met de in dit boekje beschreven resultaten bezocht hij meerdere internationale congressen onder andere het 23rd San Antonio Breast Cancer Symposium in San Antonio, (Texas, USA 2000) met geld verstrekt door het Van Walree Fonds.

Nadien volgde hij van 2001 tot 2003 zijn co-schappen in het VUmc en omliggende perifere ziekenhuizen. Na het behalen van zijn artsexamen begon hij in februari 2004 met de (voor)opleiding interne geneeskunde in het Academisch Ziekenhuis Groningen (Prof. dr. R.O.B. Gans en Prof. dr. M.H. van Rijswijk).

List of publications

1. R. Bos, H. Zhong, C. F. Hanrahan, E. C. Mommers, G. L. Semenza, H. M. Pinedo, M. D. Abeloff, J. W. Simons, P. J. van Diest, and E. van der Wall. Levels of hypoxia-inducible factor-1alpha during breast carcinogenesis. ***J.Natl.Cancer Inst***. 93 (4):309-314, 2001.

2. R. Bos, P. J. van Diest, and E. van der Wall. RESPONSE: Re: Levels of Hypoxia-Inducible Factor-1 During Breast Carcinogenesis. ***J Natl Cancer Inst*** 93:1177, 2001.

3. R. Bos, J. J. Van der Hoeven, E. van der Wall, P. van der Groep, P. J. van Diest, E. F. Comans, U. Joshi, G. L. Semenza, O. S. Hoekstra, A. A. Lammertsma, and C. F. Molthoff. Biologic correlates of (18)fluorodeoxy-glucose uptake in human breast cancer measured by positron emission tomography. ***J.Clin.Oncol***. 20 (2):379-387, 2002.

4. I. L. Tan, R. A. van Schijndel, M. A. van Walderveen, M. Quist, R. Bos, P. J. Pouwels, P. Desmedt, H. J. Ader, and F. Barkhof. Magnetic resonance image registration in multiple sclerosis: comparison with repositioning error and observer-based variability. ***J Magn Reson.Imaging*** 15 (5):505-510, 2002.

5. R. Bos, P. van der Groep, A. E. Greijer, A. Shvarts, S. Meijer, H. M. Pinedo, G. L. Semenza, P. J. van Diest, and E. van der Wall. Levels of hypoxia-inducible factor-1alpha independently predict prognosis in patients with lymph node negative breast carcinoma. ***Cancer*** 97 (6):1573-1581, 2003.

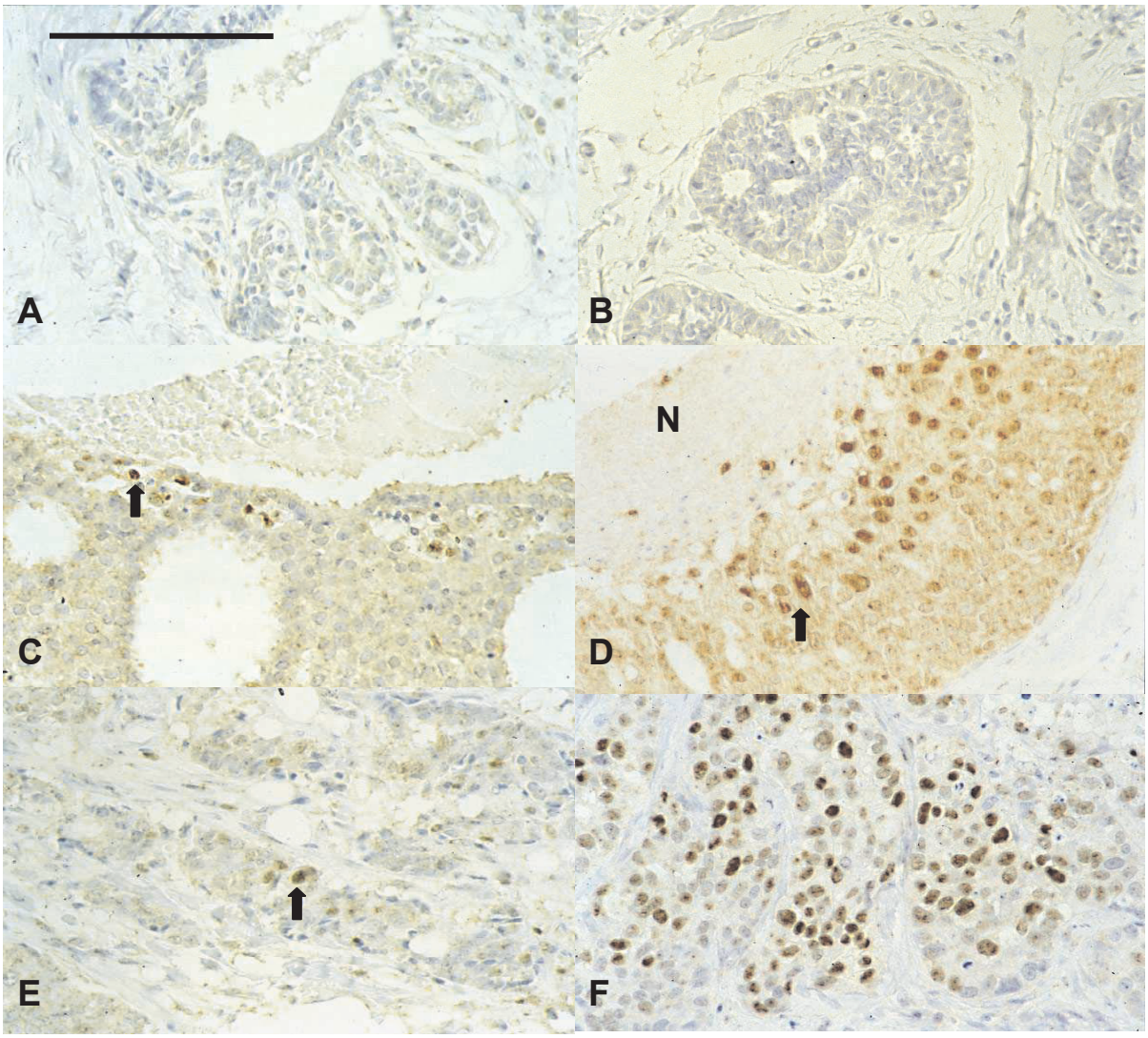
6. R. Bos, P. J. van Diest, P. van der Groep, A. E. Greijer, M. A. Hermsen, I. Heijnen, G. A. Meijer, J. P. Baak, H. M. Pinedo, E. van der Wall, and A. Shvarts. Protein expression of B-cell lymphoma gene 6 (BCL-6) in invasive breast cancer is associated with cyclin D1 and hypoxia-inducible factor-1alpha (HIF-1α). ***Oncogene*** 22 (55):8948-8951, 2003.

7. R. Bos, P. J. van Diest, P. van der Groep, A. Shvarts, A. E. Greijer, and E. van der Wall. Expression of hypoxia-inducible factor-1alpha and cell cycle proteins in invasive breast cancer are estrogen receptor related. ***Breast Cancer Res***. 6 (4):R450-R459, 2004.

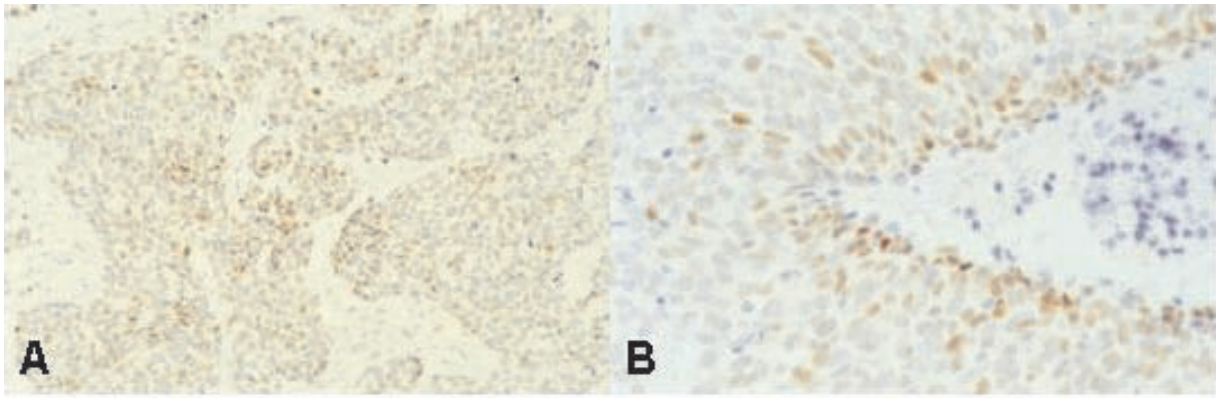
8. M. M. Vleugel, R. Bos, P. van der groep, A. E. Greijer, A. Shvarts, H. V. Stel, E. van der Wall, and P. J. van Diest. Lack of lymphangiogenesis during breast carcinogenesis. ***J.Clin.Pathol***. 57 (7):746-751, 2004.

9. R. Bos, P. J. van Diest, J. S. de Jong, P. van der Groep, P. van de Valk, and E. van der Wall. Hypoxia-Inducible Factor-1α is associated with angiogenesis, and expression of bFGF, PDGF-BB, and EGFR in invasive breast cancer. ***Histopathology***: in press, 2004.

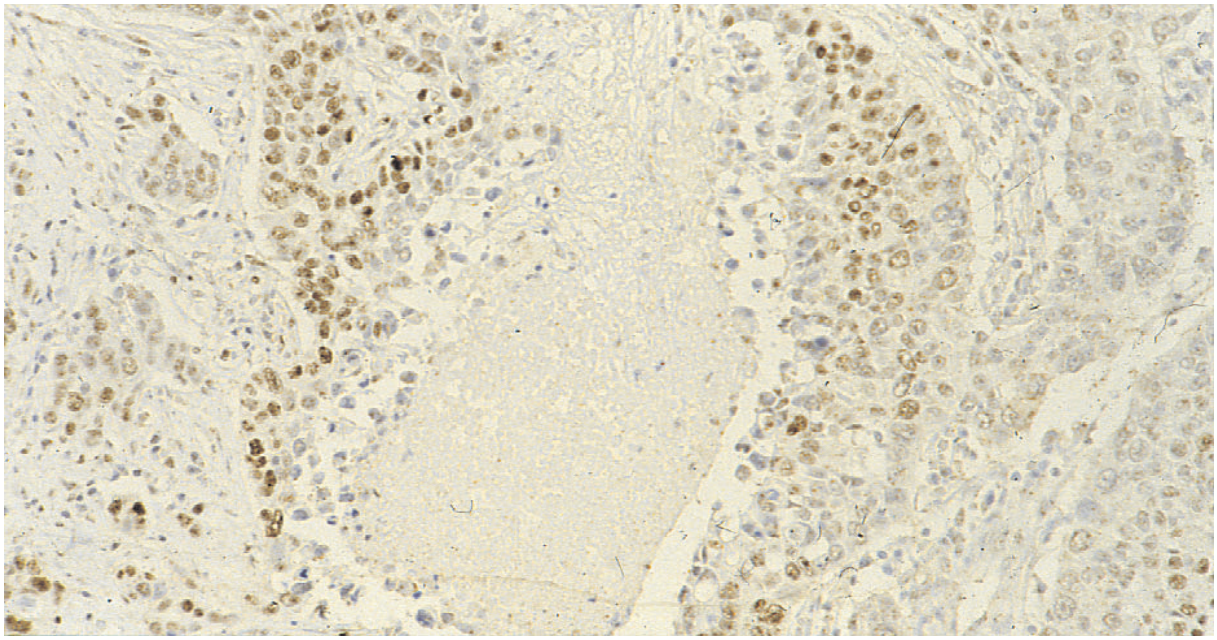
Color plates



CHAPTER 2, FIGURE 1. Immunohistochemical analysis of hypoxia-inducible factor-1α in normal breast tissue (**A**) and in hyperplasia (**B**) shows no increase in HIF-1α. Well-differentiated ductal carcinoma in situ (DCIS) (**C**) and poorly differentiated DCIS (D) show a striking pattern of increased HIF-1α around necrosis (N). A well-differentiated ductal carcinoma shows HIF-1α positivity (**E**). A poorly-differentiated medullary breast carcinoma shows increased regional levels of HIF-1α (**F**). Scale bar = 100μm



CHAPTER 4, FIGURE 1 Invasive ductal breast cancer showing diffuse (A) and perinecrotic (B) nuclear BCL-6 immunohistochemical staining (microscope magnification ×20).



CHAPTER 6, FIGURE 1. An immunohistochemical staining of hypoxia-inducible factor-1α in patients with invasive breast carcinoma. Definite nuclear staining is visible in perinecrotic areas.

Dankwoord

BIJ HET SCHRIJVEN van dit dankwoord wil ik allereerst stilstaan bij de mensen die als patiënt betrokken waren bij dit onderzoek, helaas zijn teveel van hen inmiddels overleden…

Nannie, ondanks alle lasten die je van mijn onderzoek hebt moeten dragen, ben je toch mijn vrouw geworden!! Ik wil je voor beiden danken. Misschien kunnen we nu alle avonden, weekenden en gemiste vakanties in gaan halen…

Mijn wetenschappelijke loopbaan begon op een zaterdagmiddag, want alleen op zaterdagmiddagen kan je Paul van Diest rustig spreken. Midden in een veel te kleine kamer vol met coupes, artikelen, correspondentie en afgrijselijke muziek was hij de boel aan het opruimen! Paul; ik ben blij dat je nu een secretaresse hebt die dat doet, want in onderzoek doen ben je veel beter. Allereerst bewonder ik je voor het respect en vertrouwen waarmee jij studenten weet te benaderen en te enthousiasmeren. Verder wil ik je vooral bedanken voor je eeuwig optimisme, onbegrensde energie, onconventionele aanpak en alle (groei)ruimte die je gaf.

Ook door Elsken van der Wall heb ik meerdere fantastische ervaringen mogen meemaken. Elsken; wat ik vooral in jou bewonder zijn je uitgesproken 'patiënten-voor-alles' mentaliteit, die je combineert met een overweldigende inzet en gracieuze oprechtheid. Niet alleen bedankt voor al jouw steun, onderzoekservaring, leiderschap en communicatietrainingen, maar ook bedankt voor je persoonlijke noot.

Arno Kuijper, jij bent de volgende in het rijtje (datum nog onbekend?). Arno bedankt voor het fungeren als paranimf en spiegel. Er was geen probleem dat ik tegenkwam of jij was hem al tegengekomen of omgekeerd. Maar ja, wie is er dan ook zo dom om vriendin, onderzoek, en een bijbaantje te willen combineren met een studie? Juist, wij dus. Gelukkig bestaat er zoiets als humor, anders hadden wij het uitgevonden…

Petra van der Groep, ook jij bedankt voor het fungeren als paranimf. Maar, veel belangrijker, bedankt voor alle kleuringen die je samen met mij voor dit onderzoek hebt gedaan. Jouw ziel en zaligheid ligt bij het doen van onderzoek. Tot op heden ben ik maar weinig mensen tegen gekomen die dat met meer nauwkeurigheid en doorzettingsvermogen doen dan jij. Ik wacht rustig ook jouw promotieboekje af...

Ellen Mommers; bedankt voor al je tijd (alleen al voor je geduld totdat ik met twee ogen door een microscoop kon kijken…), uitleg en introductie in elk aspect van het AiO-schap en borstkanker onderzoek in het bijzonder.

Angelique Leonhart, jou wil ik bedanken voor de steun en begeleiding bij mijn eerste schreden in het lab. Zo wil ik ook Thea Tadema en Wim Vos bedanken voor al hun meedenken bij mijn afschuwelijke zoektocht naar een goed werkend protocol voor HIF-1α. Natuurlijk wil ik ook de andere medewerkers van de afdeling immuno-histochemie bedanken voor hun prettig gezelschap.

Terug naar mijn thuisbasis; de kwantitatieve pathologie. Natuurlijk wil ik iedereen die ik in de afgelopen jaren daar ben tegengekomen bedanken voor hun altijd gezellige collegialiteit en vele sociale activiteiten (gemiddeld één –*bijna verplichte*-bioscoopavond per week). Dus Jeroen Belien, Gerrit Meijer, Mario & Marta Hermsen, Jane Verhees, Mark Broekaert, Nicole van Grieken, Cindy Postma, Janneke Weiss,

Mireille Vervaart, Marije Vleugel, *David* Shvarts, Astrid Greier en Ingrid Heijnen jullie allen bedankt voor de mooie tijd samen!

Ook de opperhoofden Prof dr. C.J.L.M. Meijer (hoofd pathologie VUmc) en Prof dr. J.P.A. Baak (voormalig hoofd kwantitatieve pathologie VUmc) wil ik bedanken voor hun (stilzwijgende) goedkeuring dat ik op hun goed gefaciliteerde afdeling mocht werken.

Natuurlijk wil ik ook Prof dr. H.M. Pinedo (medische oncologie VUmc) bedanken. Dankzij hem hebben we contacten met het Johns Hopkins kunnen leggen, tevens hebben zijn stimulerende opmerkingen ons onderzoek voortgang gegeven.

De 'PET-club' bestaande uit dr. J.J.M. van der Hoeven (Koos), dr. O.S. Hoekstra (Otto), dr. C.F.M. Molthoff, Prof. dr. A.A Lammertsma (Adriaan) en drs N.C. Krak (Nanda) wil ik graag danken voor hun wetenschappelijke inbreng en goede samenwerking.

Further, I want to thank prof dr. J. W. Simons, dear Jonathan, thank you for your invitation and hospitality when I came over to your lab. Also thank you for your respect and trust given. I was really inspired by the attitude and atmosphere at The Johns Hopkins. Likewise, I want to thank Dr. Hua Zhong and Colleen Hanrahan for their enthusiasm and delicate scientific tricks, which helped me to get the HIF-1α staining work.

Prof dr. G.L. Semenza, dear Gregg, thank you for discovering HIF-1, it was able to keep me busy for a few years. Of course, I also want to thank you for your collaboration and your visit to Amsterdam (plus your regular email correspondence) during which you learned me much more about HIF-1. I am honoured by your very polite words in the preface of this thesis.

De lees- en promotiecommissie, bestaande uit Prof dr. E. van der Wall (promotor), Prof dr. P.J. van Diest (promotor), Prof dr. E.E. Voest, Prof dr. B.G. Wouters, Prof dr. V.W.M. van Hinsbergh, Prof dr. I.H.M. Borel Rinkes en Prof dr. E.G.E. de Vries, wil ik bedanken voor de tijd en aandacht die zij aan de beoordeling van dit proefschrift hebben besteed.

Doroté Evers en Ruud Sies wil ik heel hartelijk danken voor hun professionele vormgeving van de omslag van dit proefschrift.

Als laatste (maar niet de minste) wil ik mijn ouders, boers, zus en schoonfamilie danken voor al hun geduld en begrip wanneer ik weer eens niet kwam opdraven…