Aus dem Institut für Anatomie und Experimentelle Morphologie

des Zentrums für Experimentelle Medizin

des Universitätsklinikums Hamburg-Eppendorf

(Direktor Prof. Dr. Udo Schumacher)

Expression of HIF-1α and GLUT-1 in human xenograft tumors in immundeficient mice

Dissertation

zur Erlangung des Grades eines Doktors der Zahnmedizin dem Fachbereich
der Medizin der Universität Hamburg vorgelegt von

Nils Christian Pfeffer

aus Hamburg

Hamburg 2013

Angenommen von der Medizinischen Fakultät

der Universität Hamburg am 16.07.2013

Veröffentlicht mit der Genehmigung der

Medizinischen Fakultät Hamburg

Prüfungsausschuss, der/die Vorsitzende: **Prof. Dr. Udo Schumacher**

Prüfungsausschuss, 2. Gutachter/in: **Prof. Dr. Janos Mester**

1. Hypothesis and Question

Hypoxia-inducible-factor 1α (HIF-1α) is a transcriptional factor which plays central role in adaptation of cells to hypoxia. Through the mediation of HIF-1α the expression of many micro-milieu modulating proteins is mediated. Some of the HIF-1 target genes are carbonic anhydrase 9 and 12 (CA IX and XII), vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT-1), erythropoietin (EPO). These few molecules are only a small sample for the more than 100 probable HIF-1α target genes.[1,2]

The HIF-1 transcriptional factor is the central regulator for adapting to the hypoxic conditions by stimulating the expression of many genes making the survival under these specialized conditions possible.[3] Malignant tumors with distinctive hypoxia are known to be more resistant to radio- or chemotherapy and show stronger invasiveness than malignant tumors without pronounced hypoxia.

The knowledge about the biological behavior of a malignant tumor is of importance for choosing the appropriate therapy and to determine the prognosis of the patient. Target molecules of HIF-1 such as GLUT-1 have been used as endogenous markers for tumor hypoxia.[4] [5] However, whether these target molecules are reliable marker of hypoxia remains controversial.[6]

The aim of our study was to investigate HIF-1α and GLUT-1 expression in malignant tumors. To achieve this aim several tumor entities were investigated in order to analyze whether the expression is limited to certain tumor entities or not. Therefore a wide variety of tumor samples were investigated for HIF-1α and GLUT-1 expression using immunohistochemistry. We further wanted to investigate if there are characteristic expression pattern of both proteins with reference to different tumor entities.

2. Introduction

2.1 Hypoxia

Hypoxia in solid malignant tumors is the result of an oxygen supply that does not meet the oxygen consumption. Hypoxia is defined as low O_2 partial pressure under critical level of about 8-10 mm Hg.[7]

Different forms of hypoxia are known which are caused by different reasons. Höckel and Vaupel classified hypoxia as follows:[7] a) as result of a low pO_2 in the blood caused either by low oxygen availability in the lungs or limited intake of oxygen in the lung (hypoxic hypoxia); b) caused by reduced transport capacity of the blood in an anemic situation, because of carbon monoxide poisoning, or because of methemoglobin formation (anemic hypoxia); c) reduced tissue perfusion generalized or local (circulatory or ischemic hypoxia); d) deterioration of the diffusion geometry such as increased perfusion distances, concurrent versus countercurrent blood flow within microvessels (diffusional hypoxia); e) inability of cells to use oxygen because of intoxication (histotoxic hypoxia); it also has to be regarded if these cases of hypoxia are acute or chronic.

Causative for hypoxia in solid malignant tumors seems to be the problem of O_2 delivery. In normal tissue a higher oxygen consumption can easily be served by increasing the delivery. A sharp division between hypoxia and normoxia does not generally exist because there are different tolerances and O_2 consumption levels in different tissues. The reasons for an oxygen level below critical threshold in many solid malignant tumors might be a combination of abnormal blood vessel architecture, inappropriate diffusion geometry, disturbed
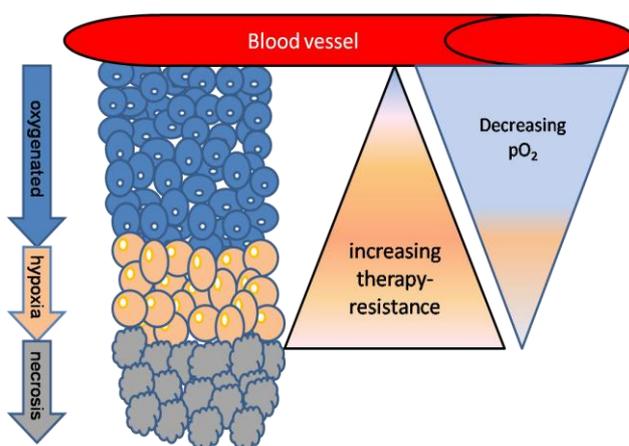
Fig.1 Connection between oxygenation status and vessel distance. The displayed therapy resistance of cells is also influenced by decreasing nutrition and drug delivery.

3

microcirculation, physical limitations such as compression of vessels. This is why normoxic areas can be found directly next to hypoxic areas in solid tumors.[7]

The presence of tumor hypoxia is especially important for radio- and or radio chemotherapy. Radiotherapy is less effective in hypoxic tissue which is in addition to other reasons a result of lower DNA damage through free O_2 radicals caused by ɣ- or x-ray called oxygen enhancement.[8] The effectiveness of ionizing radiation is two and a half to three times higher in normoxic tissue than in hypoxic tissue.[9]

The resistance to chemotherapeutic agents might have several reasons. One reason might be a reduced and unbalanced distribution of therapeutic agents. There are agents like carboplatin, cyclophosphamid and doxorubicin that have shown to be oxygen dependent. Another way of inducing resistance through hypoxia is probably the loss of apoptotic potential.[10]

2.2 Measuring Hypoxia

Up to now different methods are used to measure the oxygenation status of tissues. These methods include invasive, non-invasive as well as direct and indirect methods.

Polarographic needle electrodes are a possibility to measure the oxygenation status of a solid tumor in vivo. The way of direct measuring with a polarographic oxygen sensor such as the KIMOC-6650 (Eppendorf GmbH) is one of the most common methods. This commercial system has a hypodermic needle with a diameter of 300 μm and is covered by an O_2 permeable membrane. It measures the O_2 level through a correlation of passing oxygen and flowing current in the needle.[11] This method of measuring the oxygenation is limited by its invasiveness and access to tumor location *in vivo*. An evaluation of archived material is therefore impossible. This technique is regarded as the gold standard in hypoxia determination although a precise needle placement is very difficult to impossible because of the needle size and lack of position control. A hypoxia measurement on cellular and individual basis is also not possible.

Exogenous hypoxia markers are chemicals that have to be delivered to the organism. They are reducible under hypoxic conditions. The most common exogenous hypoxia markers are 2-nitromidazoles such as Pimonidazol (PIM) and EF5. They offer the possibility to visualize low oxygen tensions (< 10 mm Hg) in tissues. PIM has to be applied *in vivo* several hours before biopsy so that the drug can be metabolized and bind to thiol groups of molecules with an oxygen tension below 10 mm Hg. A detection is carried out by using commercial anti-PIM/-EF5 antibodies.[12] This way of measurement allows to evaluate connections between hypoxia and tumor architecture. In some experiments comparing the binding of PIM and direct measurement with pO_2 sensitive electrodes only a poor correlation between direct electrode and indirect PIM measurements is reported. [13] Another disadvantage of PIM is the lack of retrospective analysis because the marker has to be applied *in vivo.* Again, an analyses of archived tissue is therefore not possible.

Endogenous hypoxia markers are generally the target genes of HIF-1 such as GLUT-1, VEGF, CA IX and HIF-1α itself. These target genes are amongst others all over expressed in hypoxic tissue. As they are target genes of HIF-1 there is the problem that HIF-1 can be stabilized in tissues that are normoxic by the influence of other factors such as gene defects and oncogenes so that the HIF-1α protein is over expressed despite normoxia. Hence there arises the problem how to correlate the occurrence of these markers with hypoxia *in vivo* as endogenous hypoxia markers can only be detected in biopsy material sections.

Radiologic imaging techniques such as Positron-Emission-Tomography (PET) and Magnetic Resonance Imaging (MRI) have also be used to detect hypoxia. These techniques are non-invasive. The most commonly used metabolite to be detected by PET scan is 18F-fluorodeoxyglucose. The glucose utilization is only indirectly linked to the oxygenation of solid tumors. This procedure is unable to define an assessment to the oxygenation status and has its function in general detection of metabolic active processes. Another weakness is the low spatial resolution of analyzed structures. In experiments a lack of correlation with pO_2 sensors carried out by polarographic testing was found.[14]

The dynamic contrast-enhanced (DCR) MRI is a another way of imaging tumors. In this method gadolinum-chelates are used as contrast agents. The MRI is able to visualize vascularisation, blood flow and permeability of blood vessels in solid malignant tumors.[15] So this way also gives an overview of tumor perfusion, but is unable to detect oxygenation levels on a cellular basis. In addition these measures also showed a lack of correlation with pO_2 measurement carried out by polarographic testing.[16]

2.3 HIF-1α and the target molecule GLUT-1

When cells are exposed to hypoxia an adaption to the new condition is imparted by systemic changes. The hypoxia-inducible factor HIF-1α seems to play a key role in this adaption. Hypoxia-inducible factor 1α is a transcription factor consisting of an alpha and beta- subunit. Three different alpha subunits are known up to now of which the subunit 1α (HIF-1-alpha) seems to play the most important role in adaption to hypoxia. It was first named in literature in 1992 by Semenza and Wang[17] as an protein involved in the expression of the EPO-gene under hypoxia. The HIF-1β protein forms a subunit in different transcriptional factors while HIF-1α can only be found in the HIF-1 complex.

HIF-1α is constantly, slightly and ubiquitary expressed in human cells but under normoxic conditions it gets rapidly degraded. This degradation is mediated by prolyl-hydroxylation in position 402 and 564. The hydroxylation is managed by three different isoenzymes called PHDs (prolyl-hydroxylase-domains). These PHDs belong to 2-oxoglutarate- and iron-dependent dioxygenase family which need O_2 as a co-substrate together with iron and ascorbate and 2-oxyglutarate as co-factors. So this pathway of HIF-1α degradation is O_2 dependent.[18]

Hydroxylation makes HIF-1α targetable for the Hippel-Lindau tumor suppressor protein (pVHL).[19] [20] HIF-1α is recognized by E3 ubiquitin ligase complex and degraded by the proteasome.[21]
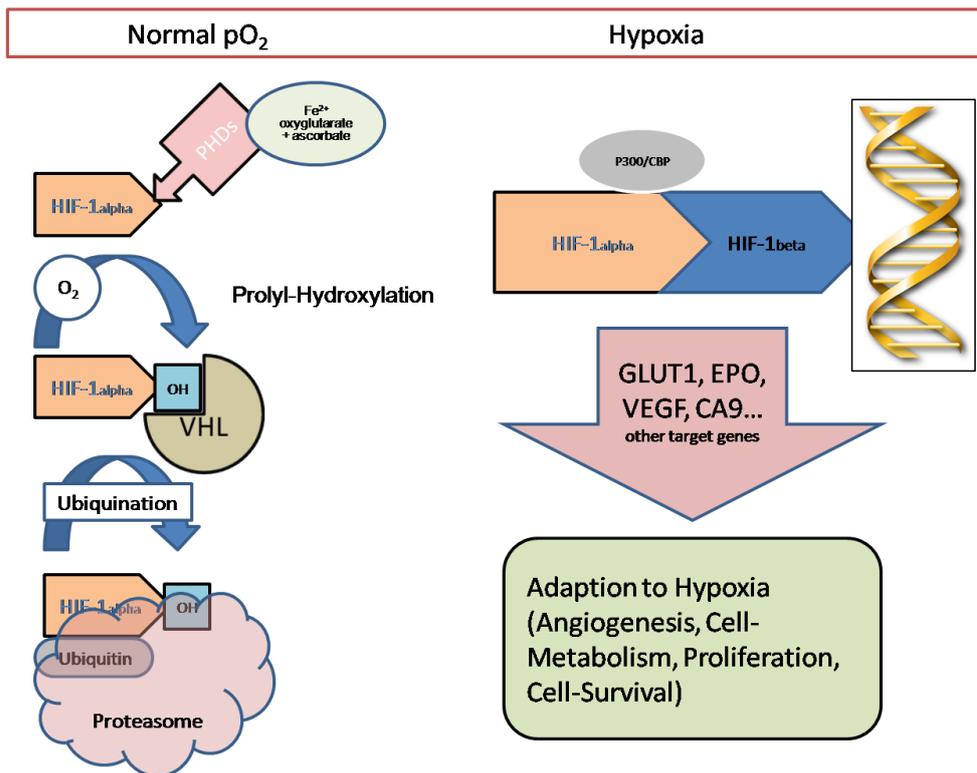
Fig. 2 Different behavior of HIF-1α under normoxic and hypoxic conditions. As explained in the text there are also other ways for stabilization of HIF-1 even under normoxic conditions.

A further inactivation pathway of HIF-1α is the O_2 dependent hydroxylation of asparagin which inhibits the interaction to co-activator p300. The inhibitor is called FIH (factor inhibiting HIF).[22]

Damages or inactivation of p53 or pVHL can lead to HIF-1 stabilization even under normoxia. In normal tissues HIF-1α degradation is so fast that immunhistochemical detection of HIF-1α is not possible. The half-life period in post hypoxic cells of HIF-1α is less than 5 minutes.[23]

Under hypoxia the named ways of degradation are limited by hypoxia and the HIF-1α complex is transferred into the nucleus.[24,25] In the nucleus HIF-1alpha binds to the constantly discharged HIF-1β subunit, which is constantly present in the nucleus.

This HIF-1α-HIF-1β complex is now able to bind to hypoxia response elements (HRE) on hypoxia inducible genes. There is a great variety of transcriptional targets for the HIF-1 protein. The targets include vascular growth an regulation factors, iron metabolism and erythropoiesis, glucose uptake and metabolism, collagen matrix formation, regulation of HIF-1 transactivation,

proliferation/differentiation/cell viability, catecholamine biosynthesis, regulation of extracellular pH-number.[26,27,22]
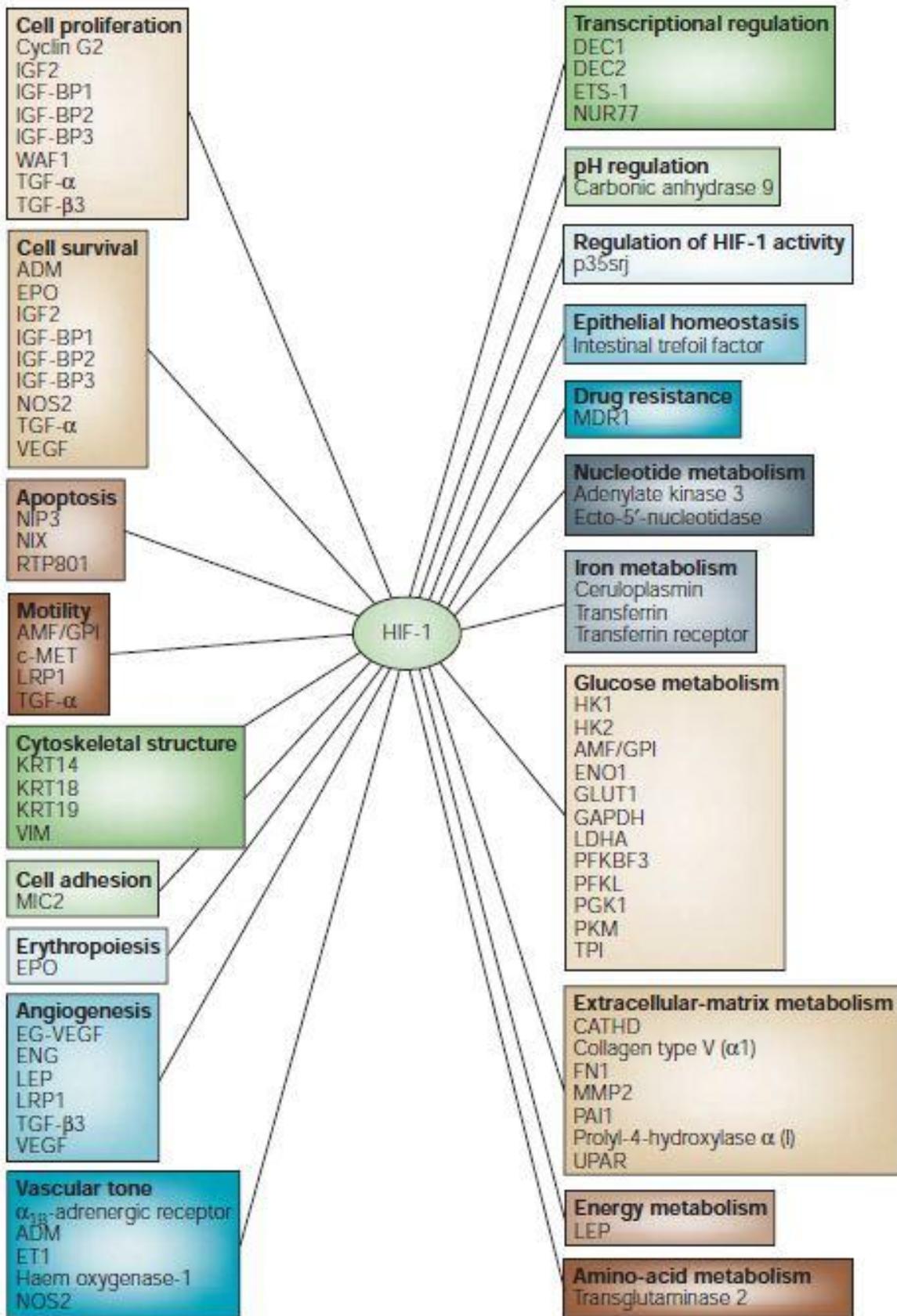
Fig. 3 Genes that are transcriptionally activated by HIF-1;   Figure by G. Semenza[1]

A splice variant of HIF-1α was reported which can be stabilized under normoxic conditions mediated by the Ras-oncogene, phorbol esters or growth factors such as endothelial growth factor (EGF) and insulin-like growth factor (IGF).[28] This stabilization through mutations in RAS proteins or other growth factors emphasizes the role of HIF-1α in the process of tumor progression and aggressiveness.

In conclusion, the HIF-1 complex can modulate a great variety of cellular functions under hypoxia. The control of gene expression through HIF-1α is not only oxygen-dependent imparted by the cell-own O_2-sensor in form of PHDs. Damages or mutation of pVHL or p53 also lead to higher accumulation and thus activation of HIF-1α under normoxic conditions through reduced degradation and higher availability of co activator p300. Mutations in growth factors and splice variants of HIF-1α promote tumor growth. HIF-1α plays a central role for tumor survival such as adaption to hypoxia, angiogenesis and glucose metabolism.

The GLUT-1 transporter is a trans-membrane protein encoded by the SLC2A1 gene in humans. The GLUT-1 transport system is expressed in erythrocytes and in the cell membrane of endothelial cells of the blood-brain barrier. This glucose transporter is essential for the supply of glucose to erythrocytes and the brain but it also occurs in many other cells. Its function is the transfer of glucose through the cell membrane. However, it also transports pentose, hexose and ascorbic acid to the cytoplasm. The GLUT-1 transporter is an insulin independent uniport system. The integration of this transport protein into the cell membrane facilitates glycolysis under anaerobic conditions.[29] The expression of GLUT-1 under hypoxic conditions seems to be controlled by the HIF-1α pathway.[30,31]

Hypoxic tumors are known to be less responsive to radiotherapy and or radio-chemotherapy. It was found that in hypoxic tumors also an over-expression of GLUT-1 could be found. Malignant tumors have a high glucose consumption and generate their energy mainly through anaerobic glycolysis. The high expression of GLUT-1 is linked to HIF-1α as a master regulator in adaption to hypoxia. Several authors postulate GLUT-1 occurrence correlates with tumor hypoxia. [32]

The appearance of high HIF-1α levels and though its target genes does not have to be dependent on occurring hypoxia. As shown before there are splice variants stimulated by the RAS oncogenes, phorbol esters and growth factors such as EGF and IGF.[33] Higher levels of HIF-1α can also be caused through reduced degradation through mutation or damage in p53 and pVHL. Regarding HIF-1α level and as a result of mutation or protein damage the level of its target genes do not have to correlate with tumor hypoxia.

In our study we compared HIF-1 and GLUT-1 expression because GLUT-1 is often discussed as marker for tumor hypoxia.

3. Materials an Methods

The following cell lines were used in our study: SKNSH, LOX, 5061, MV3, 5072, MCF7, KELLY, T47D, PC3, FEMX, MEWO, OH1 (for their tissue of origin see figure 4). Primary tumors from the named human cell lines xenografted into immundeficient mice were analyzed for their HIF-1α and GLUT-1 expression. The tissue blocks for this investigation were drawn from the archives of the Institute of Anatomy and Experimental Morphology.

Cell line	Tumor tissue derived from
SKNSH (SK-N-SH)	neuroblastoma
KELLY (Kelly)	neuroblastoma
LOX	melanoma
FEMX	melanoma
MEWO	melanoma
MV3	melanoma
5061	adenocarcinoma pancreas
5072	adenocarcinoma pancreas
PC3	adenocarcinoma prostate
T47D	breast ductal carcinoma
MCF7	breast ductal carcinoma
OH1	small cell lung carcinoma

Fig.4 Tissue origin of the malignant tumor cell lines.

3.1 Staining Procedure

The tissue sections were deparaffined according to the following protocol:

3 x 7 minutes Xylene

2 x 5 minutes absolute ethanol

2 x 5 minutes ethanol 96%

2 x 5 minutes ethanol 70%

1 x 5 minutes ethanol 35 %

Deparaffined sections were placed into de-ionized water with 0,1% Tween 20 until they were placed into the cuvette with Target-Retrieval-Solution liquid at 100°C. The cuvette is then placed into pressure a cooker and incubated for 2 minutes at highest level, then left for fourteen minutes without activated heating. After cooling down procedure the sections were transferred to Phosphate Buffered Saline Tween (PBST) pH 7.4 and left for 5 minutes.

Sections were washed twice for five seconds with PBST. They were then incubated in Biotin Blocking System Nr.1 (DAKO cat X0590) for 10 minutes and in Biotin Blocking System Nr. 2 (10 minutes) and Protein Block (15 minutes/ Dako serum free protein block). Each cycle was followed by a washing step consisting of washing 2 x 5 seconds with PBST. Now the mouse monoclonal antibody is applied for 60 minutes at room temperature followed by a washing-cycle. For detection of HIF-1 H1alpha 67 antibody (ab1-250; conc. 1:1000) and for the detection of GLUT1 the antibody SPM498 (ab40084; conc. 1:1000) was used. The biotinylated anti-mouse antibody was applied for 15 minutes. After another washing-cycle the already prepared streptavidin-biotin complex was applied for another 15 minutes. The biotinyl tyramid amplification reagent (NEN Lifesciences Product Renaissance TSA-Plus System) was diluted 1:10 in TNB-Blocking Buffer and applied for 15 minutes. DAKO CSA system procedures were followed. The system K 1500 by DAKO (Patent NEN Life Science Products) was used. After washing Streptavidin-Peroxidase was applied (15 min) followed by a washing cycle PBST pH 8.0. DAB substrate liquid was incubated for 40 seconds and staining with Mayers haematoxylin followed for two seconds. The sections were blued under tap water for 10 minutes. Finally the sections were mounted with Aquatex.

3.2 Digitalization of immunostained sections

Stained sections were digitalized using a MIRAX MIDI slide Scanner (Carl Zeiss Microimaging GmbH) with Mirax Control software (Carl Zeiss Microimaging GmbH). As analyzing-/viewer-software Pannoramic Viewer software by 3DHISTECH (version 1.12.00.53) and ImageScope software by Aperio Technologies (version 11.1.2.760) were used to view the digitalized sections on the computer screen.

This approach allowed a comparison of HIF-1α and GLUT-1 staining expression in one image.

4. Results

In this overview-figure immunohistochemistry HIF-1α staining is always displayed on the left and the corresponding GLUT-1 immunhistochemistry is represented on the right side.
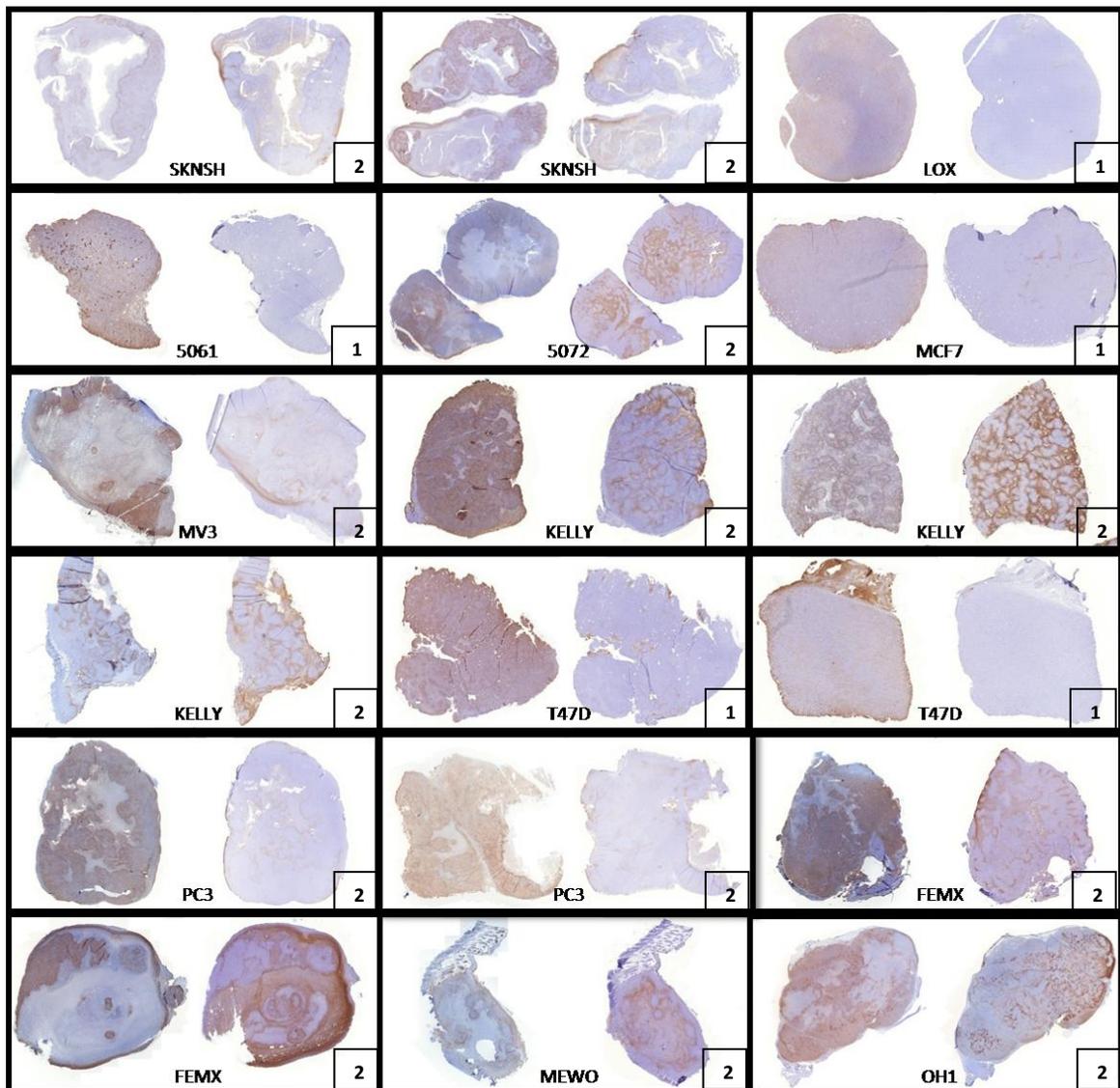
Fig. 5 **Each box contains a low power magnification of an entire tumor. On the left side HIF-1α and on the right GLUT-1 stained sections are shown. The tumors represent the following entities: neuroblastoma (SKNSH/KELLY), melanoma (LOX/FEMX /MEWO/MV3), adenocarcinoma pancreas (5061/5072), adenocarcinoma prostate (PC3), ductal breast carcinoma (T47D) and small cell lung carcinoma (OH1). This overview already shows fundamental differences in immunhistochemistry pattern of HIF-1 and GLUT1 and between different tumor entities, marked as 1 (pattern I) and 2 (pattern II) on the bottom-right side of each box.**

4.1 SKNSH primary neuroblastoma xenograft
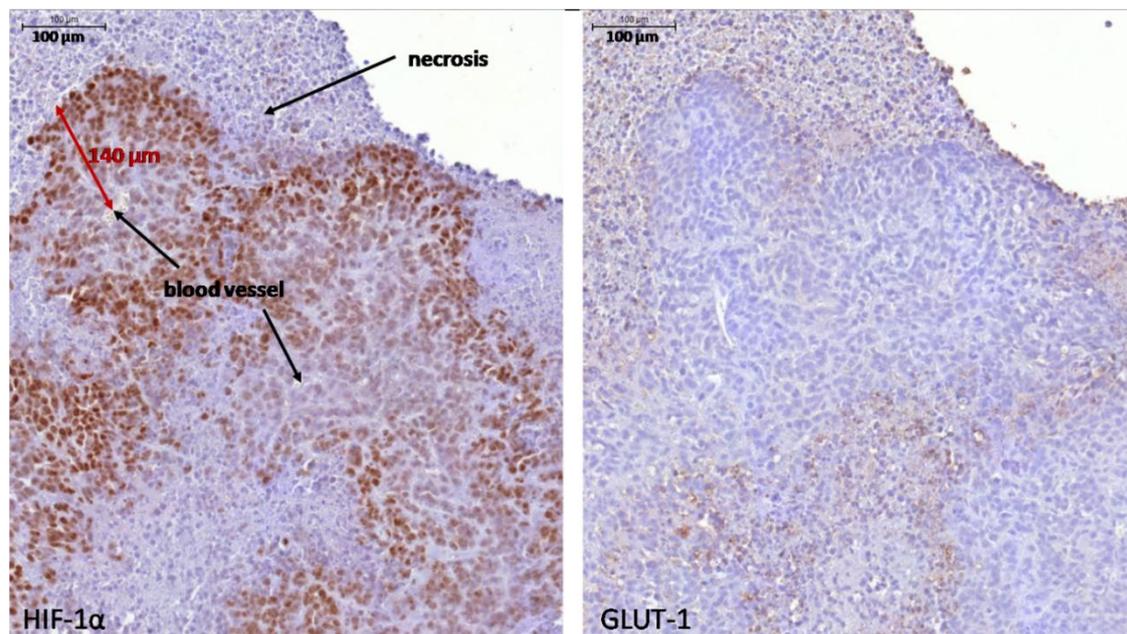
Fig. 6 SKNSH primary neuroblastoma xenograft
Corresponding areas of SKNSH neuroblastoma xenograft grown in a scid mouse. HIF-1α immunohistochemistry on the left side. GLUT-1 immunohistochemistry on the right side. While HIF-1α is intensively stained up to 140 µm around the blood vessel, little GLUT-1 immunoreactivity is seen.
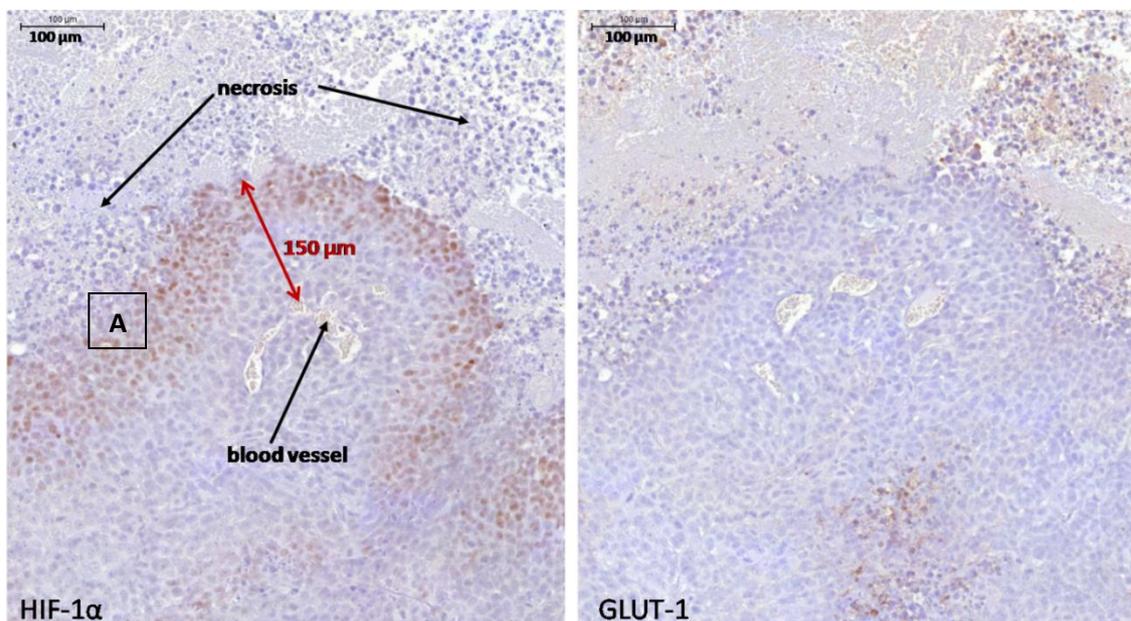
Fig. 7 SKNSH primary neuroblastoma xenograft
Corresponding areas of primary SKNSH tumor xenograft grown in a scid mouse. HIF-1α immunohistochemistry is displayed on the left and the corresponding tumor area with GLUT-1 on the right side. HIF-1α immunoreactivity can be observed up to about 150 µm around a blood vessel while GLUT-1 immunoreactivity is low. (A) Note the HIF-1α staining at the border region between peripheral zone around the blood vessel and the necrotic area.

In neuroblastoma cell-line SKNSH different distribution of HIF-1α and GLUT-1 immunoreactivity is observed. The HIF-1α staining is mostly present in the nuclei of neuroblastoma cells in border zone of the vital tumor tissue to the surrounding connective tissue and towards areas of intratumoral necrosis. In these areas strongest staining can be observed. Characteristic for both neuroblastoma cell lines is the increasing staining intensity proportional to the blood-vessel distance. The cells adjacent to a blood vessel show no staining or only very weak staining. The number of cell layers before noticeable staining varies from one to seven layers of nuclei.

The GLUT-1 staining does not show the same immunoreactivity pattern. Generally the staining is not as intensive as the HIF-1α staining. The pattern of the GLUT-1 staining does not match the observed HIF-1α staining pattern. The GLUT-1 staining, located in cell membranes, is only overlapping with a few HIF-1α positive cell layers. Strong but non-specific GLUT-1 staining is observed in areas of necrosis.

4.2 KELLY primary neuroblastoma xenograft
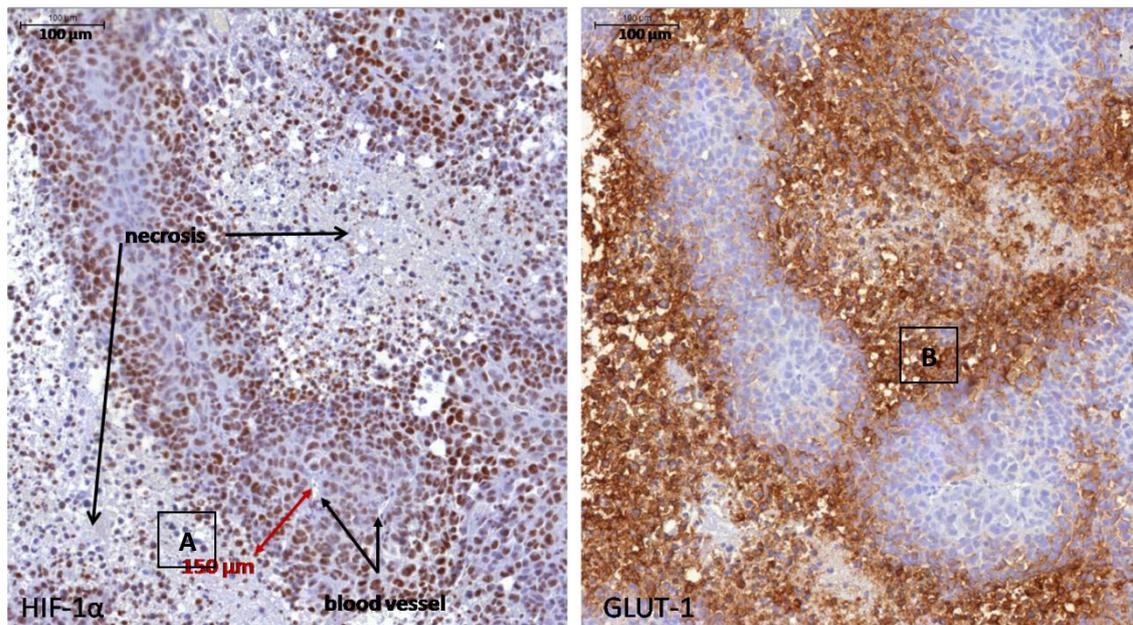
Fig. 8 KELLY primary neuroblastoma xenograft
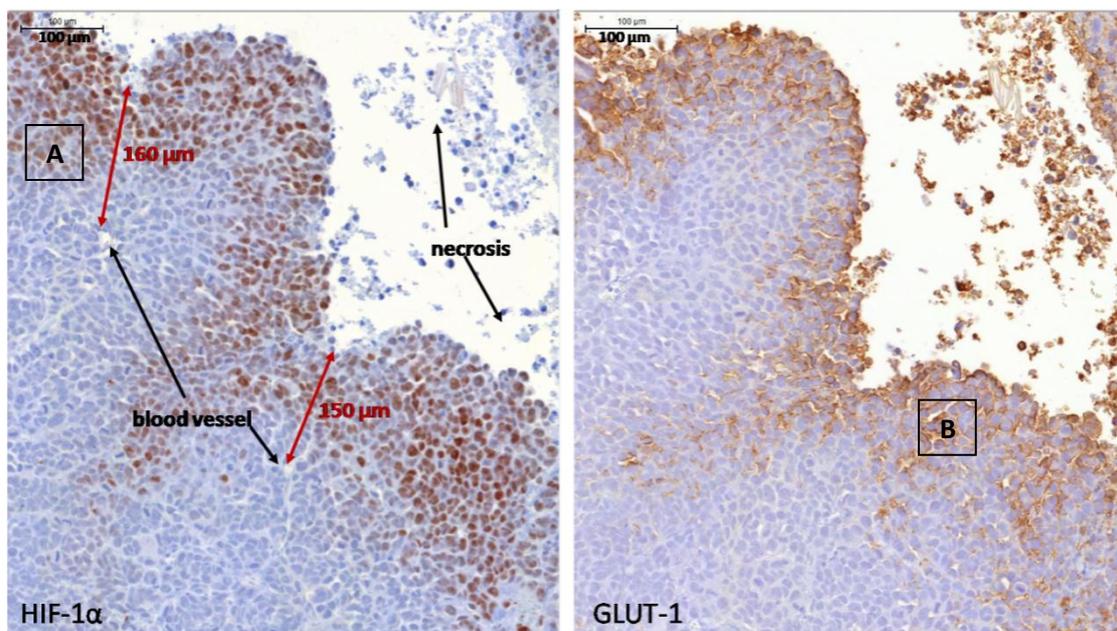
Fig. 9 KELLY primary neuroblastoma xenograft
Corresponding areas of primary KELLY neuroblastomas xenograft grown in scid mice. HIF-1α immunohistochemistry is displayed on the left and the corresponding tumor area with GLUT-1 immunohistochemistry on the right side. (A) HIF-1α immunoreactivity can in this case be observed up to about 150 to 160 µm around a blood vessel. (B) GLUT-1 Immunoreactivity can be observed in necrotic areas and in cell membranes of cells in a small marginal zone between vital tumor tissue and necrosis

In neuroblastoma cell line KELLY a moderate to intense HIF-1α immunoreactivity in the nuclei can be observed.

The HIF-1α staining intensity increases with growing distance from blood-vessels. Highest staining intensity is observed in the zone between necrosis and peripheral zone around blood vessels.

The GLUT-1 staining is very intense in this tumor and marks the border between vital tumor tissue and necrosis. As GLUT-1 is a trans-membrane protein staining is observed in cell membranes in opposite to HIF-1α staining which is located in the nucleus. In vital tumor areas adjacent to necrosis we see weak staining of cell membranes. The staining of HIF-1α and GLUT-1 does not correlate in this cell line.

4.3 LOX primary melanoma xenograft
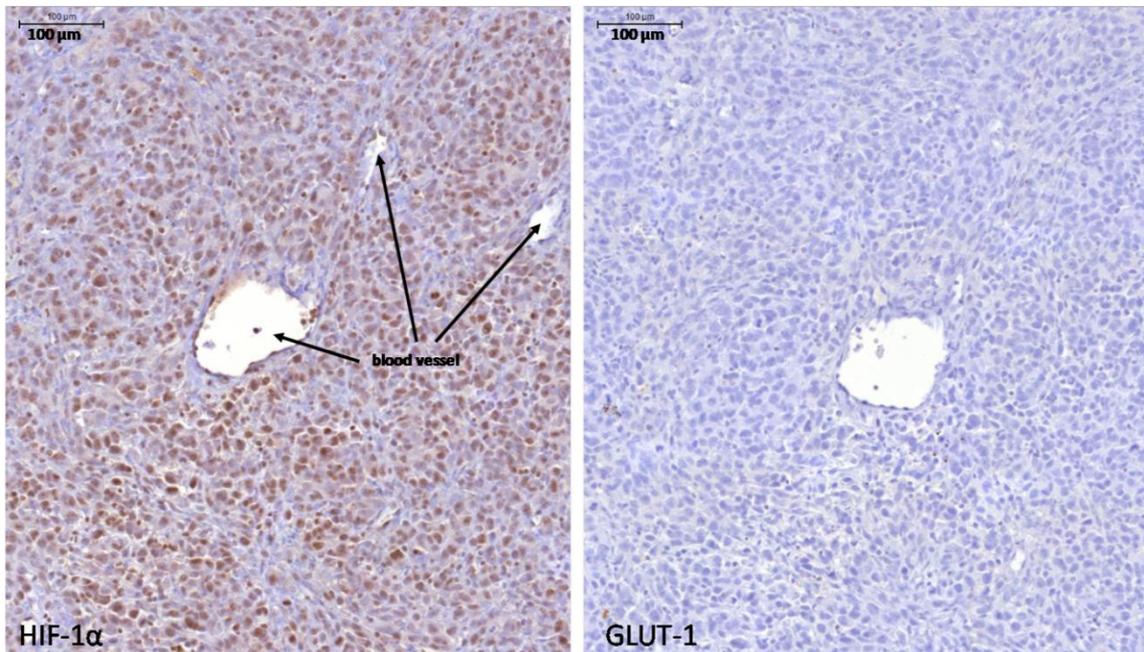
Fig. 10 LOX primary melanoma xenograft
Corresponding areas of primary LOX melanoma xenograft grown in scid mice are displayed. HIF-1α immunohistochemistry on the left and GLUT-1 on the right side. Note the moderate to intense HIF-1α staining throughout the hole tumor while no GLUT-1 immunoreactivity can be observed.

In melanoma cell-line LOX the HIF-1α staining can be found throughout the whole tumor. The staining intensity is nearly regular distributed throughout the whole tumor with moderate to strong intensity. Even cells that are directly adjacent to a blood vessel show an obvious HIF-1α staining. The vessel size does not influence the staining intensity.

LOX primary tumors do not show GLUT-1 immunoreactivity. Hence no correlation between HIF-1α and GLUT-1 immunoreactivity exists. No noteworthy necrosis can be observed.

4.4 FEMX primary melanoma
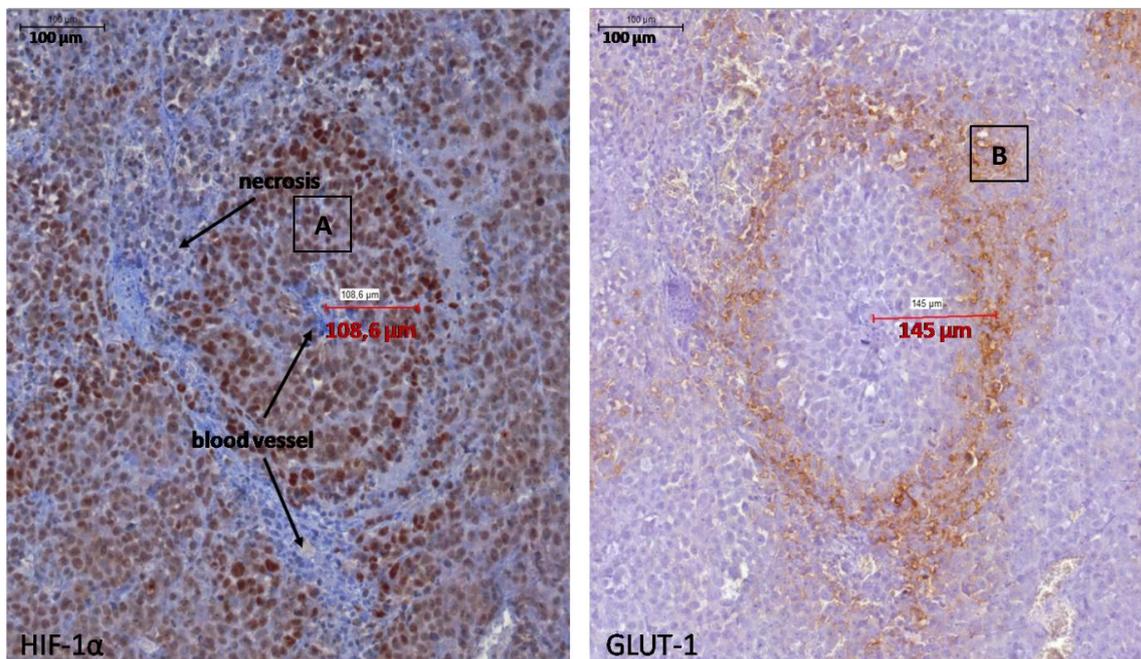
Fig. 11 FEMX primary melanoma xenograft
 Corresponding areas of primary FEMX melanoma xenograft grown in scid mice are displayed. The HIF-1α immunoreactivity is displayed on the left side and the corresponding GLUT-1 immunoreactivity on the right side. HIF-1 staining does not correlate with GLUT-1 staining. As (A) HIF-1α immunoreactivity starts directly next to a central vessel (B) GLUT-1 immunohistochemistry is not detectable in cell membranes of vital tumor cells.

HIF-1α immunoreactivity in a FEMX primary melanoma. The immunoreactivity is evenly distributed in the nuclei of the tumor, even in regions directly adjacent to a blood vessel. The intensity of HIF-1α immunoreactivity in the nuclei is moderate to strong.

In contrast GLUT-1 immunoreactivity is detected in cell membranes in a marginal zone between a blood vessel and necrotic areas only overlapping with a few HIF-1α positive cell layers.

The immunoreactivity of HIF-1α and GLUT-1 does not correlate. GLUT-1 staining marks the border of cell vitality and in this tumor entity also the border of HIF-1α staining.

4.5 MV3 primary melanoma xenograft
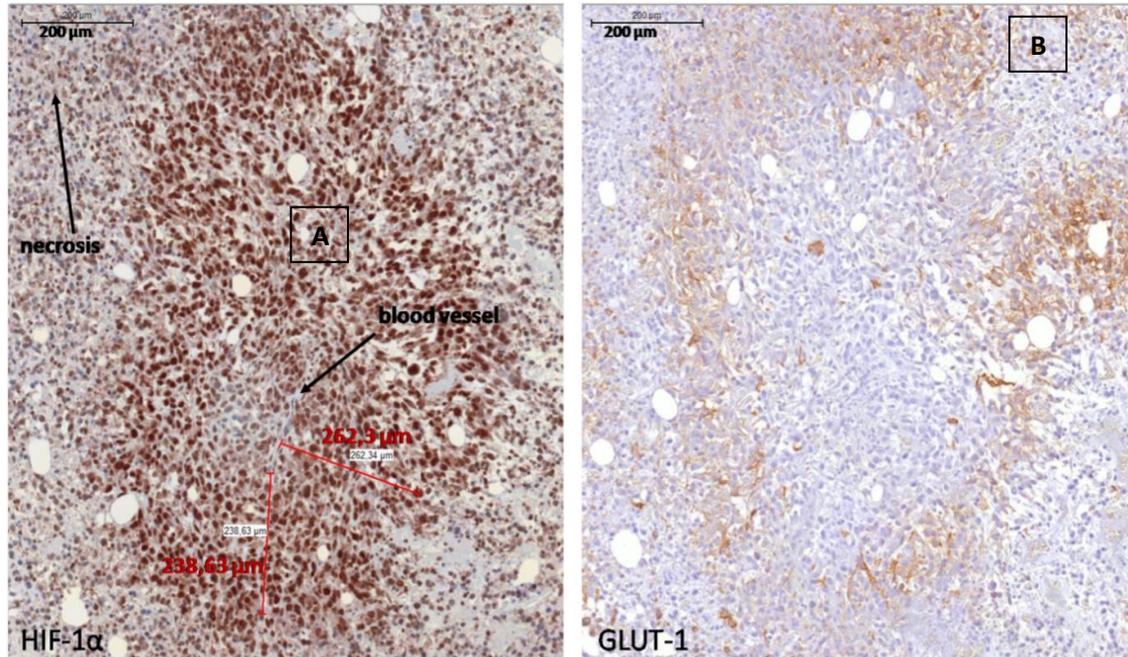
Fig. 12 MV3 primary melanoma xenograft
Corresponding areas of primary melanoma xenograft MV3 grown in scid mice are shown in this figure. HIF-1α immunoreactivity is displayed on the left and GLUT-1 immunoreactivity on the right side. (A) HIF-1α immunoreactivity is moderate to strong throughout whole tumor. (B) GLUT-1 immunoreactivity of cell membranes can be found at the margin to necrosis.

MV3 primary tumors grown in scid mice show HIF-1α immunoreactivity in the nuclei throughout whole vital tumor tissue with moderate to strong staining. The intensity of detectable HIF-1α immunoreactivity increases with growing distance to a blood vessel. GLUT-1 immunhistochemistry is present in cell membranes of tumor cells at the margin to necrotic areas only overlapping with only a few HIF-1α positive cell layers. The detectable GLUT-1 immunoreactivity in cell membranes does not correlate with the distribution of HIF-1α immunoreactivity in MV3 tumor entity.

4.6 MEWO primary melanoma xenograft
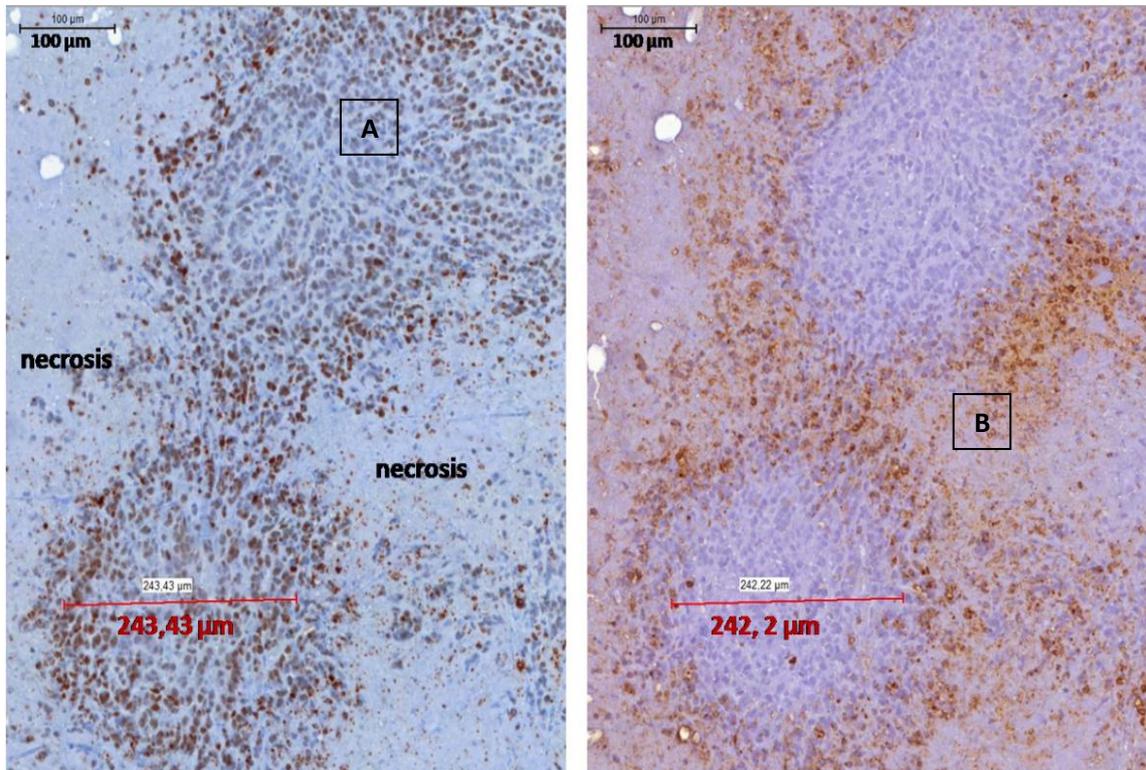
Fig. 13 MEWO primary melanoma xenograft
Corresponding areas of primary melanoma xenograft MEWO grown in a scid mouse is displayed. The left side displays HIF-1α immunoreactivity and the right side GLUT-1 immunoreactivity. (A) Moderate to strong HIF-1α immunoreactivity can be observed throughout whole vital tumor. (B) GLUT-1 immunoreactivity can be observed in cell membranes at margin to necrosis and in necrotic areas.

In melanoma cell line MEWO the detectable HIF-1α immunoreactivity of the nuclei is present throughout the whole vital tumor cells. The staining intensity increases proportional to blood vessel distance.

The GLUT-1 immunoreactvity of the corresponding tumor area is detected in cell membranes of tumor cells at the marginal zone between vital tumor cells and necrosis and in necrosis itself. The overlapping of HIF-1α positive cells with GLUT-1 positive stained cells includes only a few cell layers.

The immunoreactivity of HIF-1α and GLUT-1 does not correlate in primary melanoma of the MEWO cell line.

4.7 5061 primary pancreatic adenocarcinoma xenograft
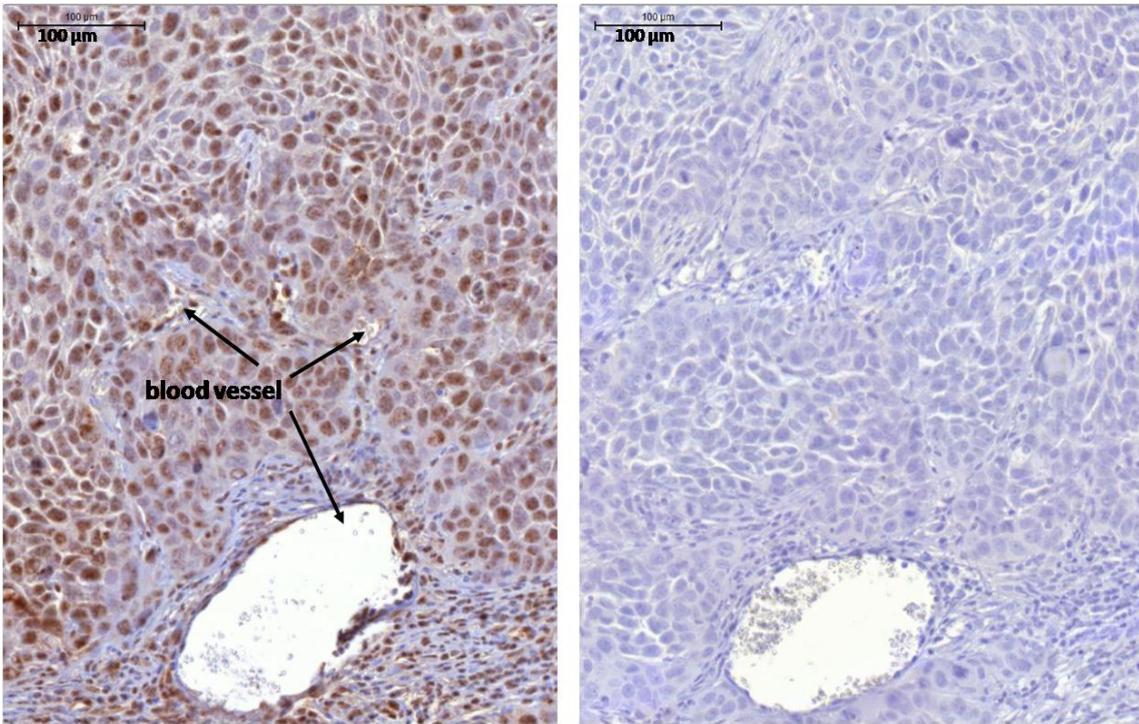
Fig. 14   5061 primary pancreatic adenocarcinoma xenograft
Corresponding areas of primary pancreatic adenocarcinoma xenograft 5061 grown in scid mice is displayed.
The HIF-1α immunoreactivity is displayed on the left side and the corresponding tumor area with GLUT-1
staining is displayed on the right side. The HIF-1α immunoreactivity is moderate to strong throughout the whole
vital tumor. No GLUT-1 staining can be observed.

In 5061 primary pancreatic adenocarcinoma xenograft an even, moderate to strong HIF-1α immunoreactivity throughout the nuclei of the whole lobule-like organized tumor can be detected. The strongest nuclear staining was observed at the border of the tumor to the normal tumor stroma surrounding the tumor. HIF-1α staining is regular and does not show intensity changes next to or far away from blood vessels.

The sections stained for GLUT-1 do not show staining in cell membranes and thus no correlation between GLUT-1 and HIF-1α staining can be detected in primary pancreatic adenocarcinoma of the 5061 cell line. No necrosis can be observed in 5061 primary tumor xenograft.

4.8 5072 primary pancreatic adenocarcinoma xenograft
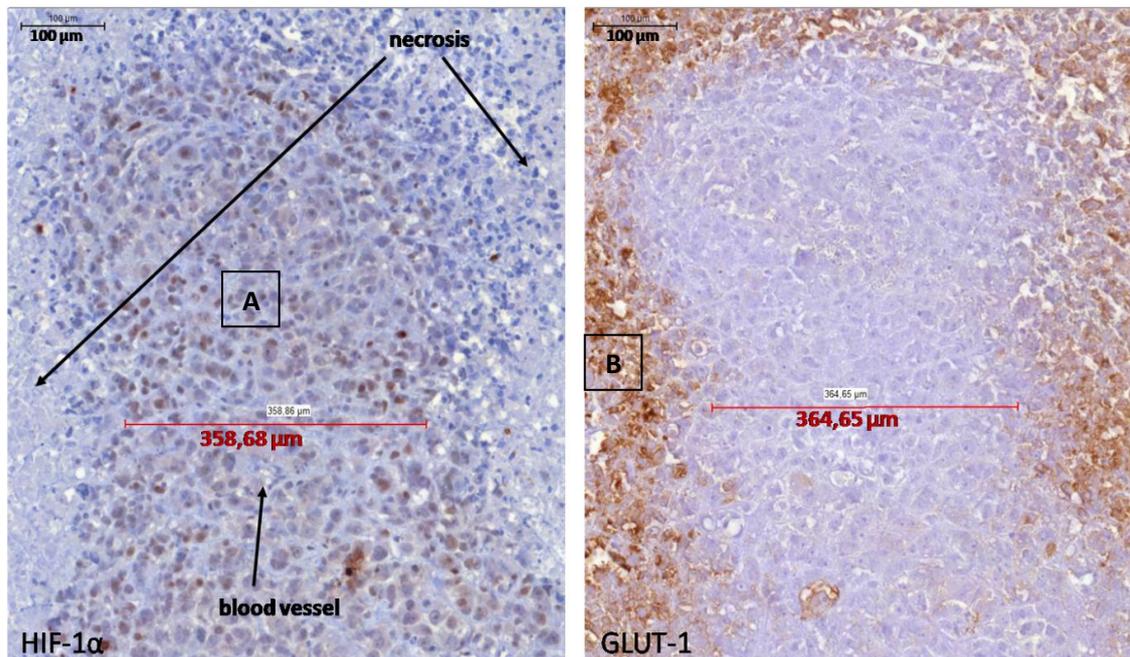
Fig. 15 5072 primary pancreatic adenocarcinoma
Corresponding areas of primary pancreatic adenocarcinoma 5072 xenograft is displayed. HIF-1α immunoreactivity is displayed on the left side and GLUT-1 immunoreactivity on the right side. (A) Moderate HIF-1α immunoreactivity can be observed in all nuclei of vital tumor cells. (B) GLUT-1 immunohistochemistry of cell membranes is reduced to a margin via necrosis and in necrosis itself.

In this primary pancreatic adenocarcinoma 5072 cell line HIF-1α immunoreactivity is detected in the nuclei throughout whole vital tumor tissue. The HIF-1α staining intensity increases with distance to blood vessels and at the tumor border to necrosis. The lobular structure is not very obvious.

The GLUT-1 immunoreactivity of cell membranes is moderate to intensive located in a marginal zone between vital tumor cells and areas of necrosis. The staining reaches highest intensity in necrotic areas and at the border areas between vital tumor cells and necrosis only overlapping with a very few HIF-1α positive cell layers. Areas around blood-vessels do not show any staining.

In primary pancreatic adenocarcinoma of the 5072 cell line a correlation between HIF-1α immunoreactivity and GLUT-1 immunoreactivity cannot be detected.

4.9 PC3 primary prostate adenocarcinoma xenograft
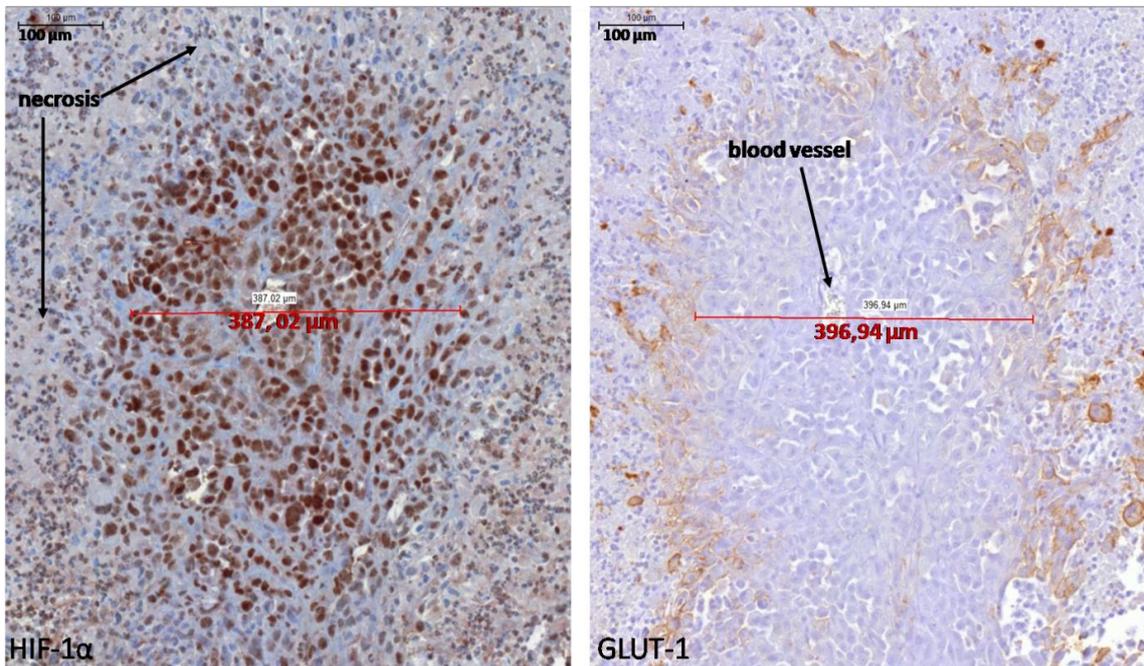
Fig. 16 PC3 primary prostate adenocarcinoma xenograft
PC3 Primary prostate adenocarcinoma xenograft grown in scid mice. The HIF-1α immunoreactivity is displayed on the left side and the GLUT-1 immunoreactivity is displayed on the right side. (A) HIF-1α immunoreactivity with moderate to strong intensity throughout the whole vital tumor. (B) GLUT-1 staining of cell membranes at the margin to necrosis and in necrosis next to vital tumor tissue.

In PC3 primary prostate adenocarcinoma xenograft the HIF-1α immunoreactivity can be detected throughout the nuclei of vital tumor cells. The staining intensity is moderate to strong increasing with growing vessel distance of the cells.

GLUT-1 immunoreactivity of cell membranes can be found in necrotic areas and in a small marginal area of vital cells adjacent to necrosis overlapping with a few HIF-1α positive cell layers.

In PC3 primary prostate adenocarcinoma no correlation between HIF-1α and GLUT-1 immunoreactivity can be observed.

4.10 T47D primary breast adenocarcinoma xenograft
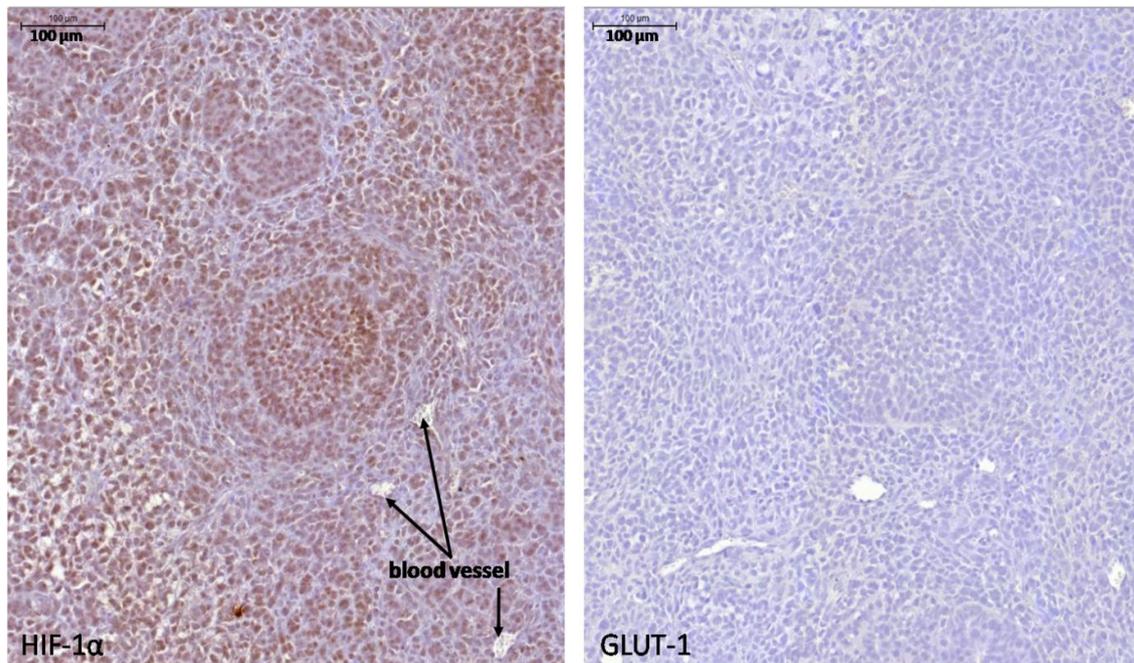
Fig. 17   T47D primary breast adenocarcinoma xenograft
Corresponding areas of T47D primary breast carcinoma xenograft grown in a scid mouse is displayed. HIF-1α immunohistochemistry is displayed on the left side and corresponding area with GLUT-1 immunohistochemistry on the right side. HIF-1α immunoreactivity throughout the whole tumor with moderate to strong intensity. No GLUT-1 immunoreactivity.

In T47D primary adenocarcinoma xenograft a strong HIF-1α immunoreactivity in all nuclei throughout the whole tumor independent from their position to blood vessels can be observed. On the border to tumor stroma an increase in staining intensity of HIF-1α can be observed.

The GLUT-1 immunoreactivity of cell membranes can only be found unspecifically and sporadically in some necrotic areas inside the solid tumor. Only a few small necrotic spots can be found in this tumor.

In primary breast carcinomas of T47D cell line grown in scid mice no correlation between immunohistochemistry of HIF-1α and GLUT-1 can be observed.

4.11 MCF 7 primary breast adenocarcinoma xenograft
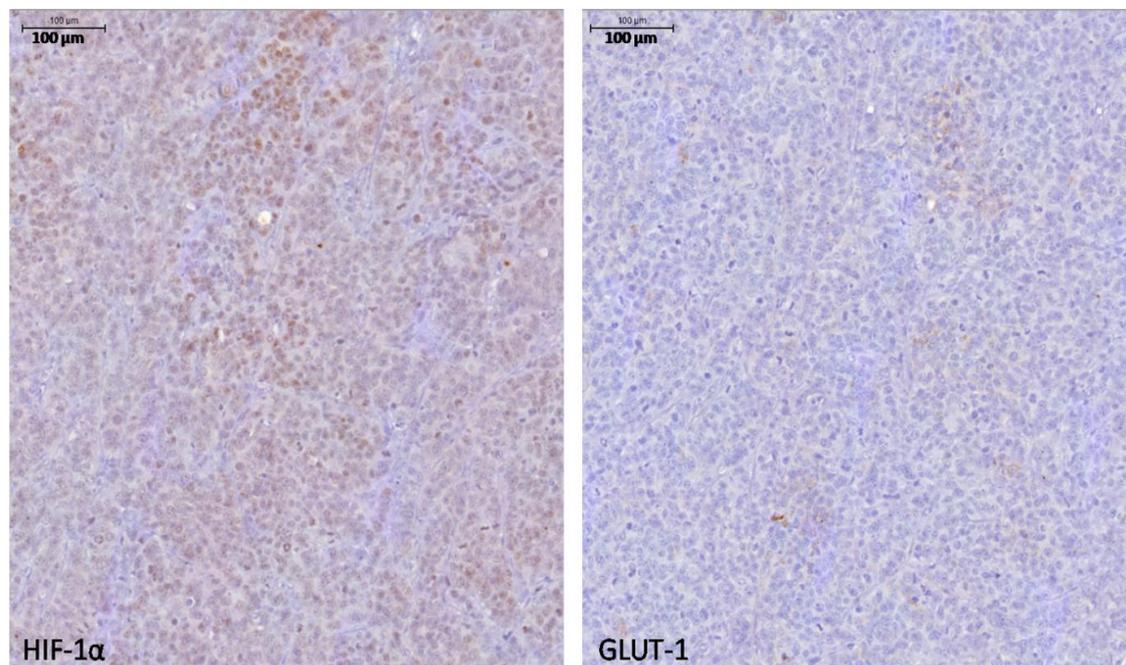
Fig. 18   MCF7 primary breast adenocarcinoma xenograft
Corresponding areas of MCF7 breast carcinoma xenograft grown in a scid mouse are displayed. HIF-1α immunoreactivity on the left and GLUT-1 immunoreactivity on the right. HIF-1α immunoreactivity can be observed throughout the whole tumor. GLUT-1 staining can only be found in some central areas connected to tumor stroma.

In primary MCF7 breast adenocarcinoma xenograft grown in scid mice a moderate to weak HIF-1α immunoreactivity can be observed throughout all nuclei of the whole tumor. The staining is more intense in the marginal area to the normal tumor stroma. A few nuclei positioned directly adjacent to small blood vessels do not show HIF-1α staining. In some parts of the tumor connective tissue septae divide the tumor tissue into lobules.

GLUT-1 immunoreactivity of cell membranes can only be found in a few, small and central necrotic areas and in a few marginal areas connected to tumor stroma. The staining of cell membranes is moderate to strong.

HIF-1α and GLUT-1 do not show a correlating staining pattern.

4.12 OH1 primary small-cell lung carcinoma xenograft
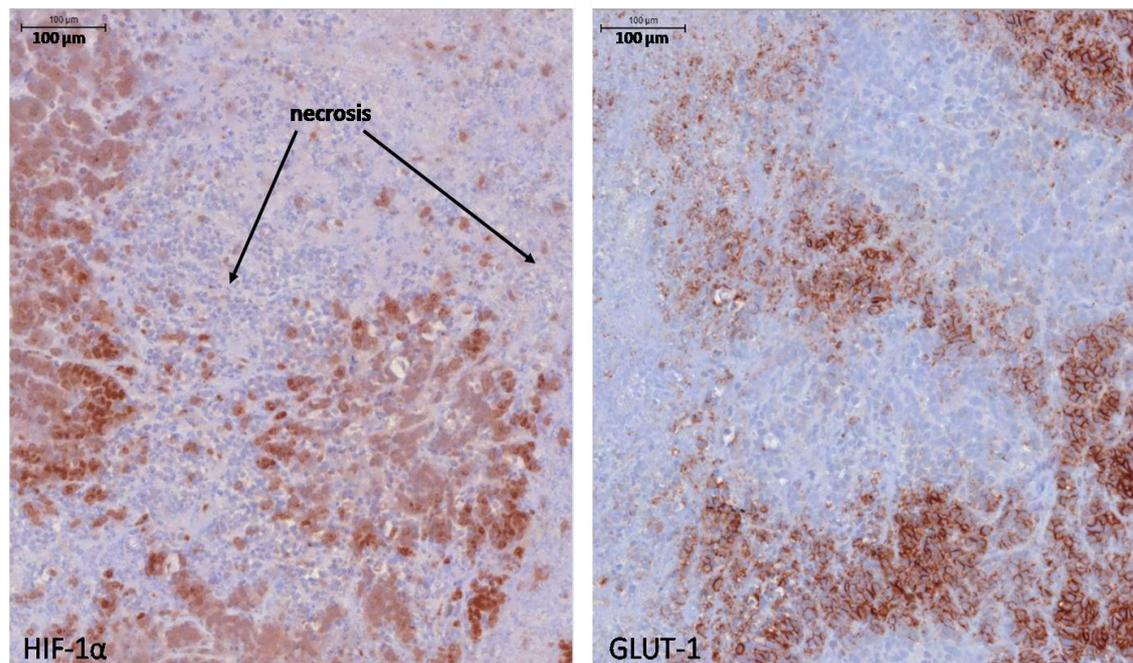
Fig. 19   OH1 primary small cell lung carcinoma xenograft
Corresponding areas of OH1 small lung cell carcinoma xenograft are displayed. The HIF-1α immunoreactivity is displayed on the left side and the corresponding area of GLUT-1 immunoreactivity on the right side. HIF-1α immunoreactivity can be found in whole vital tumor cell nuclei. GLUT-1 stained membranes can be found in necrosis and margin areas to necrosis.

In primary OH1 small-cell lung carcinoma xenograft a moderate HIF-1 staining throughout the nuclei of the whole vital cell population can be observed. At the borders to necrotic areas and in some areas inside vital tumor tissue and in marginal area to tumor stroma a more intense staining can be observed. Inside necrotic areas islands of vital tumor tissue which show an increasing staining intensity proportional to vessel distance can be found. Necrotic areas can be found throughout the whole tumor mostly in central locations without HIF-1α staining.

GLUT-1 staining in small-cell lung carcinoma is most intense in necrotic areas and bordering areas of the tumor next to tumor stroma.

 In this entity the patterns of both immunoreactivities do not match.

5. Discussion

This investigation was undertaken to analyze the expression of HIF-1α and GLUT-1 as markers of hypoxia in human tumor cells xenografted in immunodeficient mice. As the suitability of these two proteins as a marker of hypoxia is discussed in the literature, an experimental system to analyze these two markers would be desireable for future investigations.

HIF-1α is the central mediator in cell-reaction to hypoxia and regulatory processes seem to play a fundamental role in tumor behavior with regard to malignancy and therapeutic response. This transcription factor seems to mediate the tumor survival under hypoxia and regulates the way of glucose uptake and utilization[34]. This is the reason why HIF-1α itself and its target genes seem to be suitable markers to assess tumor hypoxia. This hypothesis is also supported by *in vitro* experiments that show direct correlation between O_2 availability and expression of HIF-1α and its target genes including GLUT-1.[35]

The advantage of having an endogenous hypoxia marker would be that no hypoxia sensitive agents used as hypoxia markers would have to be applied before biopsy as it is necessary for current exogenous hypoxia markers. In addition, a retrospective evaluation of hypoxia would also be possible. Because of these advantages, an internal hypoxia marker would be very desirable.

In addition to HIF-1α, target genes of this transcriptional factor have been suggested as an internal hypoxia markers. One such target gene is the glucose transporter GLUT-1, which has been considered to be a reliable hypoxia marker.[36] However, no correlation between HIF-1α and GLUT-1 expression has been found in our tumor series. Therefore GLUT-1 is not suitable as hypoxia marker as it has also been proposed by other authors.[37]

Comparing the different tumor entities and staining results analogies and differences become obvious. In all samples an over expression for HIF-1α but not automatically for GLUT-1 was found. Some tumors did not even show relevant GLUT-1 staining such as LOX, 5061 and T47D although HIF-1α immunhistochemistry showed an increased HIF-1α expression.

The strongest GLUT-1 staining can be found in greatest distance to blood vessels and in tumor stroma, but most intensive staining is to be found in a marginal zone next to necrosis or surrounding tumor stroma. Some entities do not even show significant GLUT-1 staining. Therefore two different pattern of immunohistochemistry of HIF-1α and GLUT-1 can be devised. In one pattern (pattern I) a significant HIF-1α staining but no significant GLUT-1 staining can be found. The other pattern (pattern II) shows immunoreactivity for both HIF-1α and GLUT-1 (see figure 20 and figure 5).
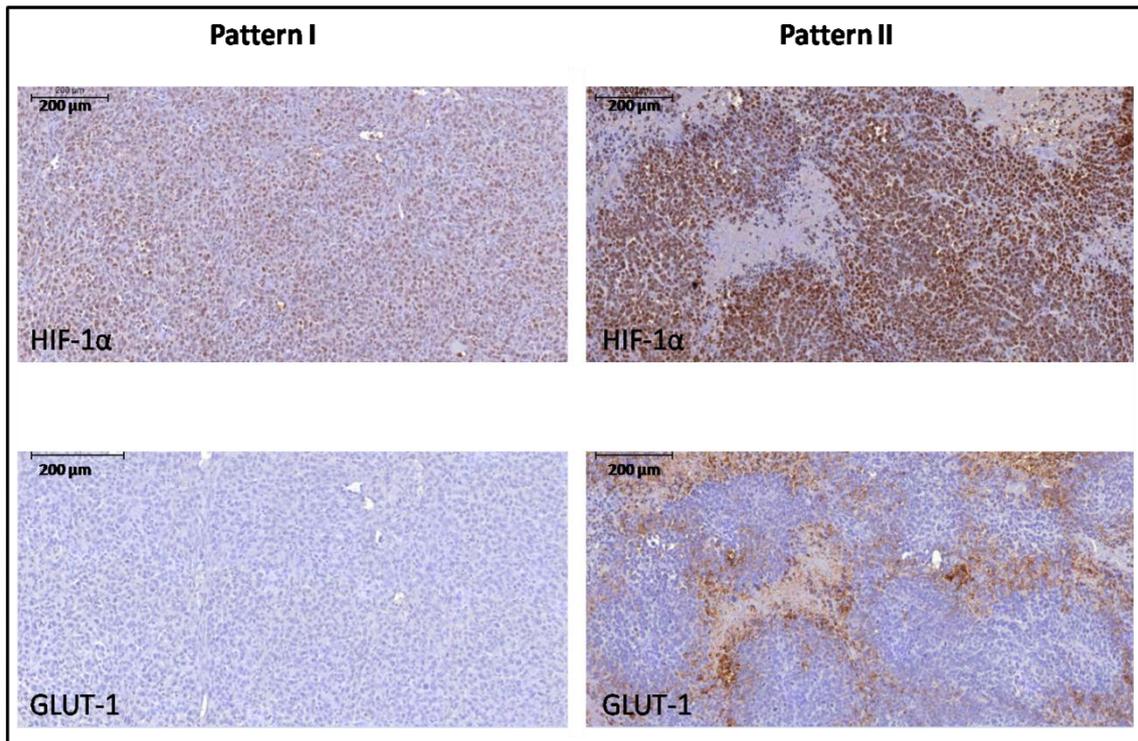
FIG. 20 Staining pattern of HIF-1α and GLUT-1 immunoreactivity of human tumor cell lines xenografted into immunodeficient animals. Exemplary HIF-1α immunoreactivity in the upper row and GLUT-1 immunoreactivity in the second row. On the left (Pattern I) 2-dimensional HIF-1α immunoreactivity can be observed while corresponding tumor tissue does not show GLUT-1 immunoreactivity. On the right (Pattern II) HIF-1α and GLUT-1 immunoreactivity can be observed while there is no significant correspondence. Pattern I staining behavior is observed in LOX, 5061, T47D and MCF7 primary tumor xenografts while Pattern II staining behavior is observed in SKNSH, KELLY, MV3, MEWO, 5072, OH1 and PC3 primary tumor xenografts in our study.

It is notable that HIF-1α staining does not show a uniform pattern throughout our investigated series of tumor entities. Again, two different pattern can be observed (see figure 21). One pattern characterized by the HIF-1α expression pattern being directly linked to the distance to a supplying vessel (Type A). The staining intensity increases proportional to the blood vessel distance. The other pattern observed is a consistent staining throughout the whole tumor without or with only little influence to expression intensity in relation to supplying blood vessels (Type B).
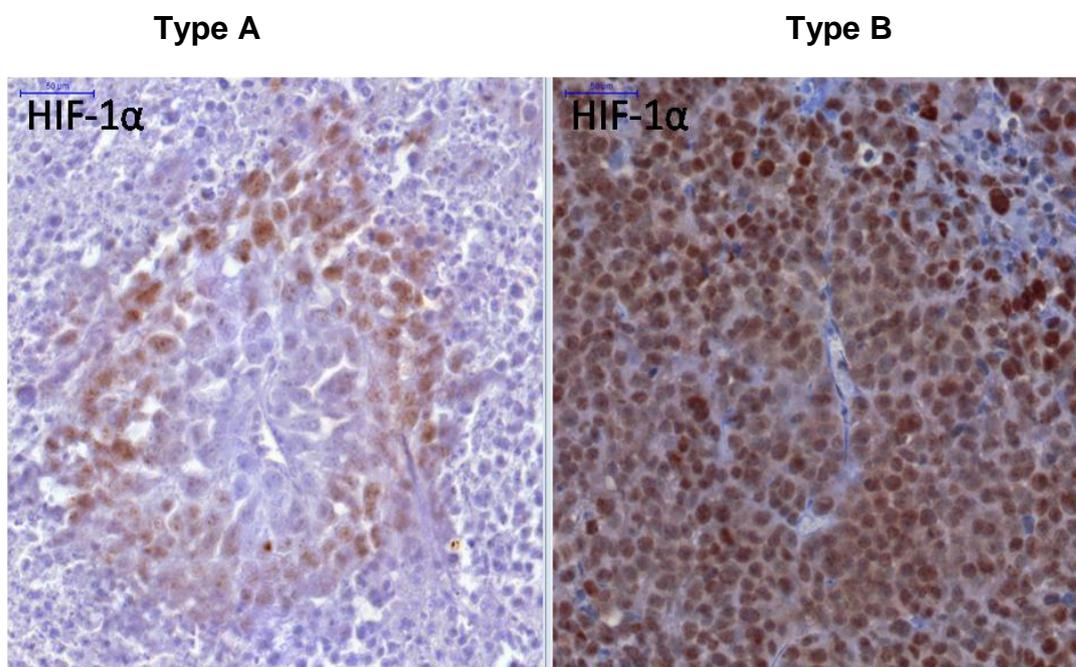
Fig. 21 This figure shows the two different expression patterns of HIF-1α. On the left we see a neuroblastoma (SKNSH) with increasing staining correspondent to vessel distance. The right picture shows a melanoma (FEMX) and demonstrates the second staining pattern with 2-dimensional staining throughout whole vital tumor tissue.

Type A	Type B
SKNSH	T47D
MEWO	LOX
KELLY	5061
5072	OH1
PC3	FEMX
MV3	MCF7

Fig. 22 Listing of different HIF-1α expression pattern. On the left entities with increasing expression intensity corresponding to blood vessel distance. On the right entities with 2-dimensional staining throughout whole vital tumor tissue.

Differences in the immunoreactivity of HIF-1α and GLUT-1 are not unexpected as HIF-1α and GLUT-1 are not equal in the latency of their expression in response to hypoxia. The HIF-1α expression is very fast in its reaction to hypoxic conditions. Already two minutes after the exposure of a cell culture to hypoxia under *in vitro* conditions the HIF-1α molecule becomes detectable in the nucleus. The accumulation increases rapidly until 30 minutes post exposure and reaches a maximum level after about 60 minutes.[38] The degradation is similarly fast with a half-life of only 5 minutes under normoxic conditions[39]. This very fast reaction on changing oxygen levels makes HIF-1α suitable to detect acute hypoxic conditions. In contrast to acute hypoxic conditions, tumor hypoxia is a chronic one as indicated by the presence of necrosis. In principle through the killing of the mice and the tumor preparation acute hypoxia can be caused in the process of tumor preparation. This fact needs further investigation.

In contrast the expression behavior of GLUT-1 differs from that of HIF-1. The maximum mRNA and thus protein levels are not reached until 16 hours after exposition to hypoxia. The degradation of GLUT1 is also significantly longer with a half-life of about 19 hours.[40,41] So GLUT-1 expression is in contrast to HIF-1α a delayed marker of hypoxia. In addition it needs much longer time in decrease after reoxygenation. These different response mechanisms to hypoxia make it in principle difficult to compare these hypoxia markers as HIF-1α visualizes primarily acute changes in oxygen levels while GLUT-1 indicate a more chronic hypoxia. This could be the reason for the observed staining pattern I (LOX, 5061, T47D, MCF7) in which we observe HIF-1α immunoreactivity but no GLUT-1 presence. In pattern I entities we also cannot find distinctive necrotic areas as ultimate indicator for chronic hypoxia.

There is no final proof of the hypothesis that HIF-1α does correctly sign hypoxia in tumor cells *in vivo*. There are other ways of stabilizing and accumulating HIF-1α under normoxic conditions such as loss of pVHL, influence of growth factors such as insulin-like growth factors I and II, epidermal growth factor, fibroblast growth factor II and tumor necrosis factor-α. [42] As mentioned already inactivation of pVHL or p53 or stabilization through RAS-oncogenes could be a reason for the over expression of HIF-1α in normoxia. Another described way for the stabilization of HIF-1α under normoxic conditions are mutations of TCA

cycle tumor suppressors such as succinat-dehydrogenase (SDH) an fumarat-hydratase (FH) which lead to a decrease of α-ketoglutarate or an increase of succinate which both lead to inhibited degradation of HIF-1α through PHDs.[43] Obviously there are other influencing factors for the stabilization of HIF-1α than hypoxia in tumor cells. The same problem exists for the expression of GLUT-1. Except hypoxia the GLUT-1 level seems also to be influenced by glucose disposability.[44,45]

Another important aspect that has to be discussed is the stabilization of HIF-1α under circumstances of inflammation. In many of our screened tumors we see inflammatory signs. Knowles et al.[46] and Jung et al.[47] describe an increased stabilization of HIF-1α under normoxic conditions through a destabilization of the labile iron pool and thus a decreased activity of PHDs by a lack of cofactor (Fe^{2+}) availability and by inflammatory mediator IL-1β.[,]

Other discussed forms of stabilizing HIF-1α under non-hypoxic inflammatory circumstances are NO-dependent inhibition of PHD activity and PHD2 inhibition by influence of transforming growth factor β.[48] It is not improbable that further mechanisms despite hypoxia will be discovered influencing the expression of HIF-1α in tumor cells.

Different results can be found in the literature with regard to endogenous hypoxia markers. Some authors find a weak correlation between direct pO_2 measurement and the expression of GLUT-1.**[49]** The experiments were carried out on malignant tumors of the uterine cervix. The use of GLUT-1 in this case has been validated by a secondary study comparing GLUT-1 appearance to the dissemination of exogenous hypoxia marker pimonidazol.[50, 51]

But this finding is not undisputed as other studies did not support these results. In a study carried out by Mayer et al. on locally advanced cervix cancer the result was contrary to the previous cited study.[52] The study by Mayer et al. also had the aim to investigate if there exists a correlation between direct pO_2 levels and appearance of endogenous hypoxia marker GLUT-1 by additionally using direct pO_2 measurement methods. However, no correlation between direct pO_2 measurement and endogenous marker GLUT-1 was found.[53] These findings are supported by other studies carried out on the same tumor entity by Sakata et al.[54] comparing the expression of endogenous hypoxia markers such as GLUT-

1, vascular endothelial growth factor, involucrin and osteopontin to direct pO_2 measurement via polarographic oxygen electrodes. No significant correlation between direct pO_2 detection and expression of endogenous hypoxia markers was found in the study. This controversial discussion shows that the use of GLUT-1 as endogenous hypoxia marker or even HIF-1α is not validated by direct pO_2 measurements.

As GLUT-1 is a transcriptional target of HIF-1 *in vitro* and correlates with its expression and pO_2 level it would be necessary to see a correlation *in vivo* to assume that GLUT-1 could be used as endogenous hypoxia marker. Without this correlation it cannot be suggested that GLUT-1 or even HIF-1α seems to be an indicator of a certain level of tumor hypoxia in general. It is not excluded that the expression of one of these proteins could be used as a predictor for prognosis and probably tumor behavior as there seem to be many other influencing factors but a use as hypoxia marker seems to be doubtful. Taking our findings into account we therefore we agree with Mayer[55] and other authors that the reliability of endogenous markers to predict hypoxia is not evidenced up to now. The comparison of both staining results showed that there is no correlation between HIF-1 and GLUT-1 distribution throughout the different tumor entities. The GLUT-1 staining is mainly found in cell membranes of viable cells on the border of the viable tumor cells to necrotic areas and in necrotic areas itself. It is notable that there is not a similar GLUT-1 expression in different tumors of the same entity such as in case of melanoma cell lines in our study. In opposite to melanoma cell lines MEWO, MV3 and FEMX there is no significant GLUT-1 immunohistochemistry detectable in tumor of LOX primary melanoma xenograft in our investigation. This is a point that needs further investigation. The HIF-1α staining varies from tumor entity to tumor entity and partly also in primary tumors of the same cell-line grown in different animals. HIF-1α immunoreactivity varies from that of GLUT-1, therefore GLUT-1 cannot be considered as reliable endogenous hypoxia marker if HIF-1α expression is considered to be the primary indicator of hypoxia. It has to be considered that GLUT-1 expression *in vitro* is not necessarily linked to the actual level of oxygen in solid tumors and other influencing co-factors such as inflammation need to be explored in this context.

6. Summary

For the treatment of solid malignant tumors measuring tumor hypoxia would be clinically desirable as hypoxia influences the resistance of a solid malignant tumor with regard to chemo- and radiotherapy.

The transcriptional factor HIF-1α plays a fundamental role in adaption of tissue to hypoxic conditions. It plays a crucial role for the survival of tissue under these conditions and coordinates the adaption to these circumstances by regulation of cell proliferation, angiogenesis, metabolic changes and many other factors as well. GLUT-1 a target gene of the transcriptional factor HIF-1α is discussed as endogenous hypoxia marker but its suitability is disputed up to now.

We investigated different tumor entities for a correlation in expression of HIF-1α and GLUT-1 expression in order to investigate if there is a correlation of HIF-1α and GLUT-1 expression.

A series of archived primary human xenograft tumors grown in immunodeficient mice were analyzed for the correlation of the expression of HIF-1α and GLUT-1 by using immunohistochemistry.

The comparison of both revealed no correlation in HIF-1α and GLUT-1 expression throughout different tumor entities. HIF-1α expression showed two different distribution pattern, pattern I and II. Pattern II shows an increase in staining intensity of HIF-1α in dependence to blood vessel distance and GLUT-1 expression in a marginal zone at the border to necrosis and in necrosis itself. In pattern I a nearly constant HIF-1α expression throughout the whole vital tumor cell nuclei without an influence of supplying blood vessels and without any significant GLUT-1 expression can be observed.

Another difference exists in the expression of HIF-Iα. In type A the HIF-1α expression intensity is increasing correspondent to blood vessel distance and in type B consistent staining intensity can be observed throughout all vital tumor cells without any or only small influence by blood vessels.

As the distribution of HIF-1α and GLUT-1 does not match *in vivo* there is no clear connection between the central mediator in adaption to hypoxia HIF-1α and its target gene GLUT-1. Hence GLUT-1 cannot serve as an as endogenous hypoxia marker.

7. References

1   Semenza GL (2003) Targeting HIF-1 for cancer therapy. Nat Rev Cancer Oct;3(10):721-32

2   Ke Q, Costa M. (2006) Hypoxia-Inducible Factor-1 (HIF-1). Mol Pharmacol 70: 1469-1480; 2006

3   Arvelo F, Cotte C (2009) Hypoxia in cancer malignity. Review. Invest Clin. Dec;50(4):529-46

4   Airley R, Loncaster J, Davidson S, Bromley M, Roberts S, Patterson A, Hunter R, Stratfort I, West C (2001) Glucose Transporter Glut-1 Expression Correlates with Tumor Hypoxia   and Predicts Metastasis-free Survival in Advanced Carcinoma of the Cervix. *Clin Cancer Res* 2001;7:928-934.

5   Coer A, Legan M, Stiblar-Martincic D, Cemazar M, Sersa G (2007) Comparison of two hypoxic markers: pimonidazol and glucose transporter 1 (Glut-1); 11th Mediterranean   Conference on Medical and Biomedical Engineering and   Computing 2007, pp. 465- 468

6   Mayer A, Höckel M, Vaupel P (2008) Endogenous Hypoxia Markers: Case not Proven!; Adv Exp Med Biol (2008) Volume: 614, Pages: 127-136

7   Höckel M, Vaupel P (2001) Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 93(4): 266-76

8   Hall EJ, Giaccia AJ (2005) Radiobiology for the Radiologist. Lippincott Williams and Wilkins; sixth Edition, chapter six; 85-105

9   Kumar P (2000) Impact of anemia in patients with head and neck cancer. Oncologist. 2000;5 Suppl 2: 13-8

10   Harrison L, Blackwell K (2004) Hypoxia and Anemia: Factors in Decreased Sensitivity to Radiation Therapy and Chemotherapy? Oncologist 2004; 9 Suppl 5:31-41

11   Meyer LHG (2004) Die Messung des Sauerstoffpartialdrucks am Unterkieferknochen mit der polarographischen Feinnadelsonde - eine neue Methode; med. Dissertation Halle

12   Franko AJ, Chapman JD (1982) Binding of 14C-misonidazole to hypoxic cells in V79 spheroids; Br J Cancer. 1982 May;45(5):694-9

13   Nordsmark M, Loncaster J, Aquino-Parsons C, Chou SC, Ladekari M, Harvesteen H, Lindegaard JC, Davidson SE, Varia M, West C, Hunter R, Overgaard J, Raleigh JA (2003); Measurements of hypoxia using pimonidazole and polarographic oxygen-sensitive electrodes in human cervix carcinomas; Radiother Oncol 2003 Apr; 67(1): 35-44,

14   Bentzen L, Keiding S, Nordsmark M, Falborg L, Hansen SB, Keller J, Nielsen OS, Overgaard J (2003) Tumor oxygenation assessed by ^{18}F-fluoromisonidazole PET and polarographic needle electrodes in human soft tissue tumors. Radiol Oncol 2003 Jun;67(3):339-44

15   Delorme S, Knopp MV (1998) Non-invasive vascular imaging: assessing tumor vascularity. Eur Radiol 1998;8(4);517-27

16   Bentzen L, Keiding S, Nordsmark M, Falborg L, Hansen SB, Keller J, Nielsen OS, Overgaard J (2003) Tumor oxygenation assessed by ^{18}F-fluoromisonidazole PET and polarographic needle electrodes in human soft tissue tumors.Radiol Oncol 2003 Jun;67(3).339-44

17 Semenza GL, Wang GL (1992) 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 Dec;12(12):5447-5454

18 Epstein AC, Gleadle JM, McNeil LA, Hewitson KS, O`Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, Ratcliffe PJ (2001) C. Elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell. 2001 Oct 5;107(1):43-54.

19 Smith TG, Robbins PA, Ratcliffe PJ (2008) The human side of hypoxia-inducible factor; Br J Haematol. 2008 May; 141(3): 325–334.

20 Maynard MA, Ohh M (2004) Von Hippel-Lindau tumor suppressor protein and hypoxia- inducible factor in kidney cancer. Am J Nephrol 2004;24(1):1-13

21 Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG Jr (2001)   HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing;   Science. 2001 Apr 20;292(5516):464-8

22 Hewitson KS, McNeil LA, Riordan MV, Tian YM, Bullock AN, Elkins JM, Oldham NJ, Bhattachaya S, Gleadle JM, Ratcliffe PJ, Pugh CW, Schofield CJ (2002) Hypoxia-inducible factor (HIF) asparagin hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. J Biol Chem 2002 Jul 19;277(29)

23 Jewell UR, Kvietikova I, Scheid A, Bauer C, Wenger RH, Gassmann M (2001) Induction of HIF-1alpha in response to hypoxia is instantaneous; FASEB J. 2001 May;15(7):1312-4

24 Maynard MA, Ohh M (2004) Von Hippel-Lindau tumor suppressor protein and hypoxia- inducible factor in kidney cancer. Am J Nephrol 2004;24(1):1-13

25 Luo JC, Shibuya M (2001) A variant of nuclear localization signal of bipartite-type is required for the nuclear translocation of hypoxia inducible factors (1alpha, 2alpha and 3alpha).Oncogene 2001 Mar 22; 20(12):1435–1444

26 Semenza GL (2001) Hypoxia inducible factor 1: oxygen homeostasis and disease pathophysiology. Trends Mol Med 2001 Aug;7(8):345-350;

27 Semenza GL (2002)   Signal transduction to hypoxia-inducible factor 1; Biochem Pharmacol 2002 Sep;64(5-6):993-998

28 Lim JH, Lee ES, You HJ, Lee JW, Chun YS (2004)   Ras-dependent induction of HIF-1alpha via the Raf/MEK/ERK pathway: a novel mechanism of Ras-mediated tumor promotion. Oncogene 2004 Dec 16; 23(58);9427–9431

29 Bussink J, Kaanders JH, van der Kogel AJ (2003) Tumor hypoxia at the micro-regional level: clinical relevance and predictive value of exogenous and endogenous hypoxic cell markers; Radiother Oncol. 2003 Apr;67(1):3-15

30 Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, Semenza GL (1998)

Cellular and developemental control of O_2 homeostasis by hypoxia inducible factor 1alpha. Genes Dev. 1998 Jan 15;12(2):149-162

[31] Ouiddir A, Planes C, Fernandes I, VanHesse A, Clerici C (1999) Hypoxia upregulates activity and expression of the glucose transporter GLUT1 in alveolar epithelial cells. Am. J. Respir Cell Mol. Biol. 1999 Dec;.21(6):710-8

[32] Airley RE, Loncaster J, Raleigh JA, Davidson SE, Hunter RD, Stratfort IJ (2003) Glut-1 and CA IX as intrinsic markers of hypoxia in carcinoma of the cervix: relationship to pimonidazole binding. Int J Cancer 2003 Mar 10;104(1):85-91

[33] Lim JH, Lee ES, You HJ, Lee JW, Chun YS (2004) Ras-dependent induction of HIF-1alpha via the Raf/MEK/ERK pathway: a novel mechanism of Ras-mediated tumor promotion. Oncogene 2004 Dec 16; 23(58);9427–9431

[34] Ziello JE, Jovin IS, Huang Y (2007) Hypoxia-Inducible Factor (HIF)-1 regulatory pathway and its potentional for therapeutic intervention in malignancy and ischemia; Yale J Biol.Med. 2007 June; 80(2):51-60

[35] Mayer A, Höckel M, Vaupel P (2008) Endogenous Hypoxia Markers: Case not Proven!; Adv Exp Med Biol (2008) Volume: 614, Pages: 127-136

[36] Airley RE, Loncaster J, Raleigh JA, Davidson SE, Hunter RD, Stratfort IJ (2003) Glut-1 and CA IX as intrinsic markers of hypoxia in carcinoma of the cervix: relationship to pimonidazole binding.Int J Cancer 2003 Mar 10;104(1):85-91

37 Mayer A, Höckel M, Vaupel P (2008) Endogenous Hypoxia Markers: Case not Proven!; Adv Exp Med Biol (2008) Volume: 614, Pages: 127-136

38 Jewell UR, Kvietikova I, Scheid A, Bauer C, Wenger RH, Gassmann M (2001) Induction of HIF-1alpha in response to hypoxia is instantaneous; FASEB J. 2001 May;15(7):1312-1314

39 Jewell UR, Kvietikova I, Scheid A, Bauer C, Wenger RH, Gassmann M (2001) Induction of HIF-1alpha in response to hypoxia is instantaneous; FASEB J. 2001 May;15(7):1312-4

40 Sargeant RJ, Paguet MR (1993) Effect of insulin on the rates of synthesis and degradation of GLUT1 and GLUT4 glucose transporters in 3T3-L1 adipocytes. Biochem J. 1993 March 15; 290 (Pt 3): 913-919

41 Bruckner BA, Ammini CV, Otal MP, Raizda MK, Stacpoole PW (1999) Regulation of brain Glucose transporter by glucose and oxygen deprivation; Metabolism 1999 Apr; 48(4):422-31

42 Powis G, Kirkpatrick L (2004) Hypoxia inducible factor-1α as cancer drug target; Mol Cancer Ther May 2004 3; 647-654

43 Selak MA, Armour SM, MacKenzie ED, Boulahbel H, Watson KD, Pan Y, Simon MC, Thompson CB, Gottlieb E (2005) Succinat links TCA cycle dysfunction to oncogenesis by inhibiting HIF-α prolyl hydroxylase; Cancer Cell 2005 Jan;7(1):77-85

44 Boado RJ, Pardridge WM (2002) Glucose deprivation and hypoxia increase the expression of the GLUT1 glucose transporter via a specific mRNA cis-acting regulatory element. J Neurochem 2002 Feb;80(3):552-4

45 Kitzmann HH, McMahon RJ, Williams MG, Frost SC (1993) Effect of Glucose Deprivation on GLUT1 Expression in 3T3-Ll Adipocytes; J Biol Chem 1993 Jan;268:1320-1325

46 Knowles HJ, Mole DR, Ratcliffe PJ, Harris AL (2006) Normoxic stabilization of Hypoxia-Inducible Factor-1alpha by modulation of the labile iron pool in differentiating U937 macrophages: effect of natural resistance-associated macrophage protein 1, Cancer Res 2006 Mar 1;66(5):2600-7

47 Yung YJ, Isaacs JS, Lee S, Trepel J, Neckers L (2003) IL-β-mediated up-regulation of HIF-1alpha via NFkappaB/COX-2 pathway indentifies HIF-1 as critical link between inflammation and oncogenesis. FASEB J 2003 Nov;17(14):2115-7

48 Frede S, Berchner-Pfannschmidt U, Fandrey J (2007) Regulation of hypoxia-inducible Factors During Inflammation. Methods Enzymol. 2007;405-419

49 Airley R, Loncaster J, Dacidson S, Bromley M, Roberts S, Patterson A, Hunter R, Stratfort I, West C (2001) Glucose Transporter Glut-1 Expression Correlates with Tumor Hypoxia and Predicts Metastasis-free Survival in Advanced Carcinoma of the Cervix. *Clin Cancer Res* 2001;7:928-934

50 Airley RE, Loncaster J, Raleigh JA, Davidson SE, Hunter RD, Stratfort IJ (2003) Glut-1 and CA IX as intrinsic markers of hypoxia in carcinoma of the cervix: relationship to pimonidazole binding.Int J Cancer 2003 Mar 10;104(1):85-91

51 Coer A, Legan M, Stiblar-Martincic D, Cemazar M, Sersa G (2007) Comparison of two hypoxic markers: pimonidazol and glucose transporter 1 (Glut-1); 11th Mediterranean Conference on Medical and Biomedical

Engineering and Computing 2007, pp. 465-   468

52   Mayer A, Höckel M, Wree A, Vaupel P (2005) Microregional expression of
     glucose transporter-1 and oxygenation status: lack of correlation in locally
     advanced cervical   cancers. *Clin Cancer Res* 2005 Apr 1;11(7):2768-73

53   Mayer A, Höckel M, Wree A, Vaupel P (2005) Microregional expression of
     glucose transporter-1 and oxygenation status: lack of correlation in locally
     advanced cervical   cancers. *Clin Cancer Res* 2005 Apr 1;11(7):2768-73

54   Sakata K, Someya M, Nagakura H, Nakata K, Oouchi A, Hareyama M,
     Satoh M (2006) A   clinical study of hypoxia using endogenous hypoxic
     markers and polarographic   oxygen   electrodes;   Strahlenther   Onkol.
     2006 Sep;182(9):511-7

55   Mayer A, Höckel M, Vaupel P (2008) Endogenous Hypoxia Markers: Case
     not Proven!; Adv Exp Med Biol (2008) Volume: 614, Pages: 127-136

8. List of Abbreviations

HIF-1α	Hypoxia-inducible factor 1 alpha
CA IX / XII	Carbonic anhydrase 9 / 12
VEGF	Vascular Endothelial Growth Factor
GLUT-1	Glucose Transporter 1
EPO	Erythropoietin
mm Hg	millimeter column of mercury
PIM	Pimonidazole
PET	Positron Emission Tomography
MRI	Magnetic Resonance Imaging
DCR	Dynamic Contrast Enhancement
PHD	Prolyl-Hydroxylase-Domain
pVHL	Von Hippel-Lindau tumor suppressor protein
FIH	Factor Inhibiting HIF-1
NLS	Nucleus Localizer Sequences
HRE	Hypoxia Response Elements
Da	Dalton (atomic mass unit)
IL-1β	Interleukin-1β
EGF	Endothelial growth factor
IGF	Insulin-like growth factor
DNA	Deoxyrebonucleid Acid
PBST	Phosphate Buffered Saline Tween

9. Acknowledgements

10. Curriculum Vitae

"entfällt aus datenschutzrechtlichen Gründen"

Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Unterschrift:_____