

## Expression of HIF-1 $\alpha$ and iNOS in Astrocytic Gliomas: A Clinicopathological Study

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**Abstract.** *Background:* Hypoxia-inducible-factor-1 (HIF-1) is present at high levels in human tumors and plays a crucial role in tumor promotion by up-regulating several target genes. HIF-1 stimulates the production of NO through the induction of inducible NO synthase (iNOS). *Patients and Methods:* Sixty-three human astrocytic gliomas were analyzed by immunohistochemistry for HIF-1 $\alpha$  and iNOS using formalin-fixed paraffin-embedded material. In 39 cases, the results of immunohistochemistry were correlated with the clinical outcomes. *Results:* HIF-1 $\alpha$  was detected only in astrocytic gliomas grades III and IV, both in the nucleus and in the cytoplasm. The iNOS expression was increased in astrocytic gliomas grades I, II and III and was statistically significantly decreased in astrocytic gliomas grade IV. iNOS was localized round the capillary vessels as well. Statistical analysis showed that the HIF-1 $\alpha$  and iNOS expressions did not correlate with patient survival. *Conclusion:* We believe that HIF-1 $\alpha$  and iNOS expressions merit further investigations in order to understand the biology of astrocytic gliomas. More data are needed from prospective studies.

Gliomas are the most common primary tumours in the brain and are divided into four clinical grades based on their histology and prognosis. Patients with grade IV gliomas (glioblastoma multiforme) have a mean survival of about 1 year, whereas patients with grade III gliomas (anaplastic gliomas) survive for 2-3 years and those with grade II gliomas can survive 10-15 years. Grade I gliomas (pilocytic astrocytomas) are curable by surgery (1). Two different pathways through which high-grade gliomas arise have been proposed. The first pathway includes the progression from a low-grade glioma, which differentiates over time to an

anaplastic phenotype and, finally, to a glioblastoma. The other pathway is the *de novo* appearance of high-grade gliomas or glioblastomas (2).

There are insufficient data regarding the contribution of hypoxia to the progression of tumours from low-grade to high-grade phenotypes. Recent data has shown that hypoxia and low pH increase mutation rates, decrease DNA repair and alter gene expression in cells *in vitro* (2). Tumour cells that are hypoxic may produce the transcription factor HIF-1 (hypoxia-inducible factor 1), that functions as a master regulator of oxygen homeostasis (3). HIF-1 is a heterodimer composed of an inducibly-expressed HIF-1 $\alpha$  subunit and a constitutively-expressed HIF-1 $\beta$  subunit. The  $\alpha$  subunit is regulated by oxygen levels while, under hypoxic conditions subunits  $\alpha$ - and  $\beta$ - are dimerized enabling a transcriptional response involving the coactivator p300. More than 40 genes are regulated by HIF-1 and encode proteins that play roles in critical developmental and physiological processes including angiogenesis, erythropoiesis, glucose transport, glycolysis, iron transport and cell proliferation/survival (4). The activation of these genes requires the binding of HIF-1 to specific sequences termed hypoxia response elements (HRE), which are located in the promoters/enhancers of the hypoxia-inducible genes (3). One of the target genes of HIF-1 is iNOS (inducible NO synthase). iNOS is inducible, Ca<sup>2+</sup>-independent and produces large amounts of NO for prolonged periods of time in response to stimulation by cytokines, lipopolysaccharide, etc. iNOS expression has been observed in the central nervous system in microglia and in oligodendroglomas (5).

In this retrospective study, immunohistochemistry was used to examine the expressions of HIF-1 and iNOS in astrocytic gliomas and the results were correlated with the clinical outcomes of these patients.

### Patients and Methods

**Patients.** Sixty-three tissue specimens from patients, who had been diagnosed with gliomas between 1993-2002, were obtained from the archives of the Pathology Department of the University Hospital

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**Key Words:** HIF-1 $\alpha$ , iNOS, astrocytic gliomas.

**Table I.** Patient characteristics.

Number of patients	63
Male	39
Female	24
Age	Range 24-72 (median age 54)
Histopathology	
Grade I	8
Grade II	6
Grade III	7
Grade IV	42

of Patras, Greece. The patient demographic details are shown in Table I. Tumour classification was done according to the criteria of WHO. By the time that this study was undertaken, 36 patients were found to have died of their disease after a mean survival of 19 months (range 2-68).

All patients had undergone craniotomy and excision biopsies, with radiotherapy being the only adjuvant treatment. The specimens had all been taken before radiotherapy was given, fixed immediately after removal in 10% formalin and processed to paraffin blocks.

**Immunohistochemistry.** Representative serial sections for immunohistochemistry were cut at 4-5 µm. The following antibodies were used: monoclonal anti-HIF-1α 1:500 (clone OZ12, Neomarkers, Fremont, USA) and polyclonal anti-iNOS 1:1000 (Chemicon). Before the primary antibody was applied, the sections had been treated in a microwave oven at 750 W for three cycles of 5 min each in 10 mmol/l sodium citrate buffer pH 6.0. Then, the standard streptavidin-biotin complex immunoperoxidase technique was used with the help of the Dako catalyzed signal amplification system (Dako), as previously described (6). Two independent observers analyzed the specimens.

**Statistical analysis.** All statistical calculations were performed using the SPSS for Windows and the analysis of one-way ANOVA. Differences were considered statistically significant when the *p*-value was ≤0.05.

## Results

HIF-1α was detected only in astrocytic gliomas grades III and IV, but it was not detected in low-grade astrocytomas, as shown in Table II. Immunoreaction of HIF-1α was observed round the necrotic area of the tumour. Also, HIF-1α reactivity was observed both in the nucleus and cytoplasm (Figure 1). Although, HIF-1α was detected only in anaplastic astrocytomas and glioblastoma multiforme, no correlation between the expression of HIF-1α and patient survival (Figure 2) was observed.

Increased iNOS immunoreaction was observed in astrocytic gliomas grades I and II, whereas its expression was statistically significantly decreased in astrocytic gliomas

**Table II.** HIF-1α detection in human glioblastoma.

Grade	HIF-1α detection
I	Not detected
II	Not detected
III	2 cases detected
IV	15 cases detected

grades III and IV (Figure 3). iNOS was expressed from cells that were localized round the capillary vessels (Figure 4). Similar to HIF-1α expression, iNOS expression did not correlate with patient survival (Figure 5).

## Discussion

Glioblastomas are the most common type of primary brain tumours and, in contrast to lower grade astrocytic gliomas, show prominent new blood vessel formation, which is an important independent indicator of poor prognosis (6). The biological steps of angiogenesis are complex and, normally, tightly regulated by the opposing influences of pro- and anti-angiogenic factors (7, 8). The accelerated rate of vascular proliferation in glioblastomas suggests that tight regulation of angiogenesis is altered to favour neoplastic growth (9). One of the main triggers for tumoral angiogenesis is believed to be the physiological response to hypoxia. This is evident in glioblastomas, in which the close temporal and spatial relationship between microvascular hyperplasia and necrosis can be explained best by an angiogenic response to low oxygen levels in nearby necrotic zones (10). Indeed, tumour cells palisading around necrosis express high levels of hypoxia-inducible regulators of angiogenesis such as VEGF (11).

Hypoxia induces a host of biological responses, including altered gene expression through HIF-1. HIF-1, present at high levels in human tumours, plays crucial roles in tumour promotion by up-regulating its target genes, which are involved in anaerobic energy metabolism, angiogenesis, cell survival, cell invasion and drug resistance. Therefore, it is apparent that the inhibition of HIF-1 activity may be a strategy for treating cancer (12, 13). In addition, the subunit HIF-1α may be considered to be a potential marker of tumour hypoxia due to its regulation by the intracellular oxygen concentration (14).

There are studies which showed that the expression of HIF-1α is increased in glioblastomas (6), in line with our findings, and also showed that there is a correlation between the expression of HIF-1α and patients survival (15), in contrast to our results. This difference may be caused by the small number of patients that were analyzed. Moreover, immunoreaction of HIF-1α was found both in the nucleus and

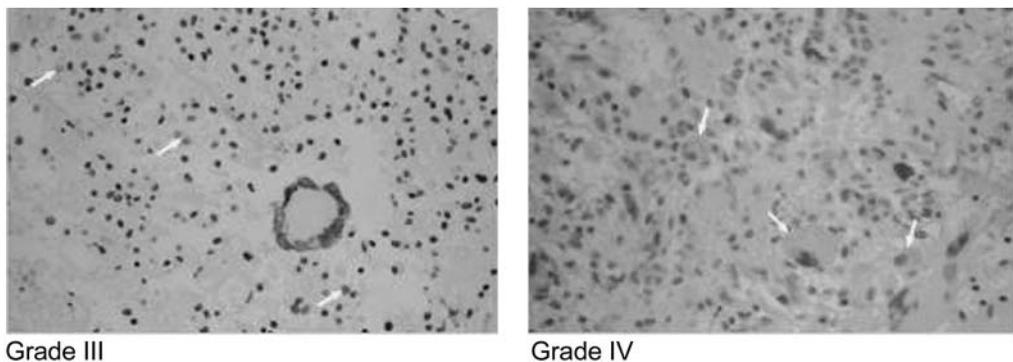


Figure 1. Immunohistochemistry for hypoxia-inducible factor  $\alpha$  (HIF-1 $\alpha$ ). Representative sections of astrocytomas grade III and grade IV. HIF-1 $\alpha$  was detected both in the nucleus and cytoplasm of neoplastic cells. HIF-1 $\alpha$  was not detected in astrocytomas grades I and II. The tissue was counter-stained with haematoxylin.

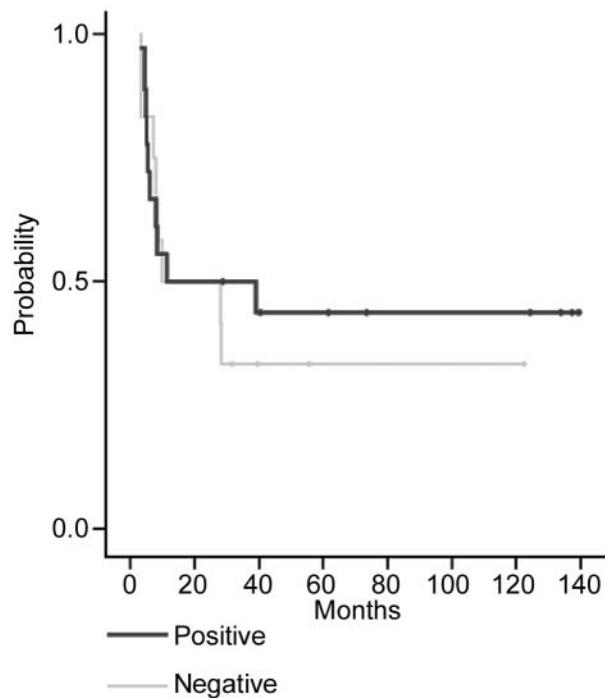


Figure 2. Effect of HIF-1 $\alpha$  expression on overall survival (OS) ( $p=0.69$ ).

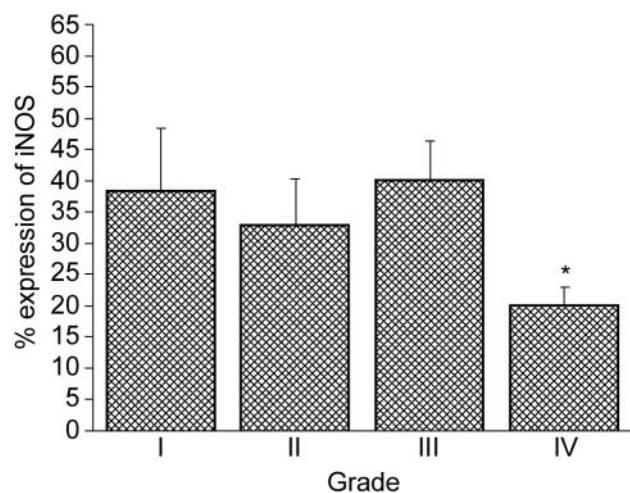


Figure 3. iNOS staining was quantified by two experienced pathologists in sections with samples of astrocytomas grades I, II, III and IV. iNOS expression was statistically significantly decreased in glioblastoma multiforme. \* $p<0.05$

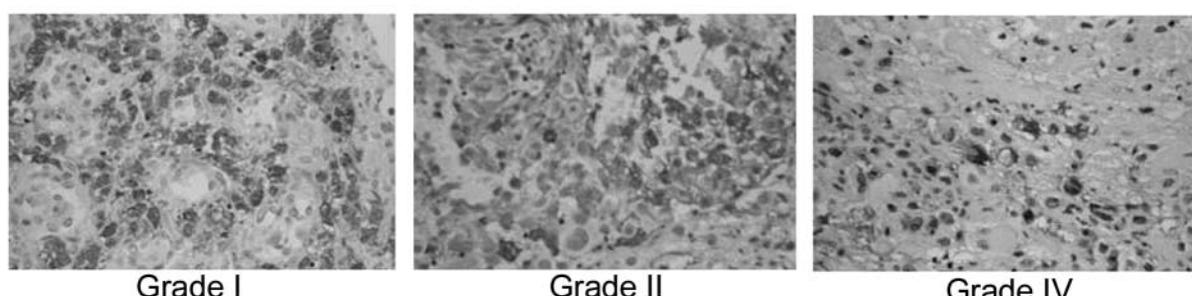


Figure 4. Immunohistochemistry for inducible-nitric oxide synthase (iNOS). Representative sections of astrocytomas grades I, II and IV. iNOS was detected round the capillaries. The amount of iNOS protein was decreased in glioblastoma multiforme. The tissue was counter-stained with haematoxylin.

cytoplasm, although HIF-1 $\alpha$  is a nuclear transcription factor. The location of HIF-1 $\alpha$  in the cytoplasm may be explained by the fact that it has isoforms that do not enter the nucleus and inhibit HIF-1 $\alpha$  dimerization. These isoforms stay in the cytoplasm, and their exact role is unknown to date (16).

An increased level of constitutive and inducible NOS expression and/or activity has been observed in a variety of human tumours including brain tumours (17, 18). Previous studies showed increased expression of cNOS in astrocytic tumour cells, while the highest levels of expression were found in high-grade gliomas (19, 20). In the same studies, iNOS was less frequently detected and expressed at a lower level, predominantly in tumour endothelial cells, by immunohistochemical and Western blot analyses. However, iNOS mRNA expression was higher in human gliomas compared with meningiomas and normal brain tissue. Intense expression of iNOS within gliomas has also been found in some non-endothelial cells, which are presumed to be perivascular and tumour-infiltrating lymphocytes or macrophages (21, 22). These data contradict our findings, in which iNOS expression was higher in astrocytic gliomas grades I and II and decreased in glioblastomas. This could be explained by the fact that the levels of protein may be lower, but mRNA levels may be higher in glioblastomas than in astrocytic gliomas grades I and II. Perhaps, there is a tumour mechanism that interrupts the expression of iNOS protein in order to decrease the NO production. It is known that both endogenous tumoral NO and NO produced by host endothelial cells may block tumour cell adhesion and reduce their metastatic potential (23, 24). In the brain, NO produced by the cerebral vasculature and microglia may suppress the spread of cancer to the brain (25).

Our data suggest rather complicated roles for HIF-1 $\alpha$  and iNOS in the growth and invasion of astrocytic gliomas. Particularly, iNOS may have a dual action, *i.e.* promoting or inhibiting angiogenesis. Further investigation is necessary to elucidate their mechanism of action in the development of astrocytic gliomas and the use of HIF-1 $\alpha$  and iNOS as prognostic factors and anticancer agents.

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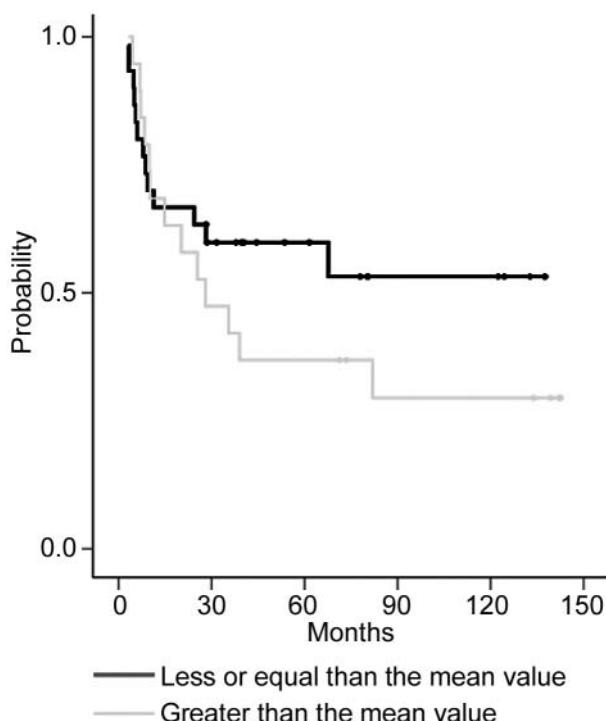


Figure 5. Effect of iNOS expression on overall survival (OS) ( $p=0.28$ ).

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