Distribution of TAM relative to blood vessels in prostate carcinoma: correlation with localization of HIF-1α and HIF-2α

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Introduction **Prostate cancer is the most common form of cancer in men. It is the second leading cause of cancer death in men in the UK and USA after lung cancer. In a variety of tumors, including breast, ovarian, colorectal, and gastric carcinomas, the neoplastic cell population is often outnumbered by CD68-positive tumor-associated macrophages (TAM). TAM are a form of macrophages with an immature phenotype, that are characterized by low expression of carboxypeptidase M and tumor necrosis factor-α and high expression of interleukin (IL)-1 and IL-6, thereby reducing their tumoricidial activities. It also appears that exposure to hypoxia in poorly vascularized areas of tumors may stimulate TAM to release cytokines and enzymes that drive tumor growth, invasion, angiogenesis, and metastasis. Although prostate carcinomas contain significant areas of hypoxia and large numbers of TAM, to date, neither the distribution of TAM relative to blood vessels (and thus hypoxia) nor their expression of hypoxia-inducible factors (HIFs) 1 and 2 has been examined in such tumors.**

Study design **Forty patients with prostate adenocarcinoma were included this study. The sections were double immunostained for the vascular endothelial marker factor VIII, the pan-macrophage marker CD68, and HIF-1α and HIF-2α. The three most vascular areas (vascular 'hotspots') were assessed for mean microvessel density using a 25-point Chalkley array graticule at ×250 magnification (field area 0.189 mm^2). Thereafter, they were assessed for macrophage density. Macrophage and microvessel densities were similarly assessed in the three areas of highest macrophage density (macrophage 'hotspots'). The same method was applied for the analysis of HIF-1α and HIF-2α proteins as well as CD68-positive macrophages.**

Results **This study shows that macrophages are prominent in prostate carcinoma and accumulate in hypoxic areas of tissues as there is a significant and reciprocal relationship between angiogenesis and macrophage infiltration in prostate cancer. HIF-1α and HIF-2α were shown to be expressed in small subsets of macrophages, with a higher expression of HIFs in avascular (35 and 40%) areas when compared with vascular areas (18 and 21%).**

Conclusion **The study gives a valuable indication that TAMs can be used to deliver hypoxically-activated therapeutic DNA constructs to prostate tumors. However, further studies are required to detect the expression of other hypoxia-induced transcription factors in macrophages, which might play a role in activating gene expression in hypoxic tumor areas. *Egypt J Pathol* 32:217–226 © 2012 Egyptian Journal of Pathology.**

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Introduction

Prostate cancer is the most common form of cancer of the male genitourinary tract. It is the second leading cause of cancer deaths in men in the UK and USA after lung cancer. The increase in the number of diagnoses is probably not because of increased incidence but is rather a reflection of the widespread use of the prostate-specific antigen assay in diagnosis. The latter leads to early detection of the tumor and hence radical therapy Dutta Roy *et al.* (2005).

Despite the fact that prostate cancer is now a major healthcare concern and that significant advancements have been made in diagnosis and staging of this disease, radical prostatectomy, radiotherapy, and hormonal therapy are the main conventional treatments for prostate cancer. However, several gene-based approaches have been devised to treat prostate cancer either by direct control of cancer cell proliferation, activation of an immune response against the tumor, inhibition of tumor angiogenesis, or by introduction of other antitumor genes Coffelt *et al.* (2009). Macrophages transfected with hypoxically-activated DNA constructs and then allowed to migrate into hypoxic sites in a tumor mass *in vitro* will activate and express the therapeutic gene (Griffiths *et al.*, 2000). It is not known whether this approach has utility in the treatment of other tumor types such as prostate cancer.

In a variety of tumors, including breast, ovarian, prostate, colorectal, and gastric carcinomas, the neoplastic cell population is often outnumbered by CD68-positive tumor-associated macrophages (TAM; Kelly *et al.*, 1988;

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Negus *et al.*, 1997; Shimura *et al.*, 2000; Nakayama *et al.*, 2002; Ohno *et al.*, 2003). These cells are largely recruited as monocytes from the blood stream through the release of monocyte chemoattractants such as CSF-1 and MCP-1 from both tumor and stromal cells (Ueno *et al.*, 2000). Once they extravasate into the tumor mass, they differentiate into TAM, a form of macrophages with an immature phenotype, that are characterized by low expression of carboxypeptidase M and tumor necrosis factor-α and high expression of interleukin (IL)-1 and IL-6, thereby reducing their tumoricidial activities (Gottfried *et al.*, 2003). This may be partly because of their exposure to tumor-derived anti-inflammatory molecules such as IL-4, IL-10, and transforming growth factor (TGF)-β that block the cytotoxic functions of TAM (Elgert *et al.*, 1998). This suggests that the tumor microenvironment 're-educates' macrophages to perform protumor functions (Guruvayoorappan, 2008). Therefore, it is not surprising that the high number of TAM in breast carcinomas (Leek *et al.*, 1996) and pulmonary adenocarcinomas (Takanami *et al.*, 1999) has been correlated with poor prognosis (i.e. worse overall and relapse-free survival rate). It also appears that exposure to hypoxia in poorly vascularized areas of tumors may stimulate TAM to release cytokines and enzymes that drive tumor growth, invasion, angiogenesis, and metastasis (Leek *et al.*, 1996; Lewis and Pollard, 2006). Although prostate carcinomas contain significant areas of hypoxia (Carnell *et al.*, 2006) and large numbers of TAM, to date, neither has the distribution of TAM relative to blood vessels (and thus hypoxia) nor has their expression of HIFs 1 and 2 been examined in such tumors.

Materials and methods

Case selection

Approval for the study was obtained from the local research ethics committee. A total of 40 prostate adenocarcinoma specimens were obtained from the histopathology archives of the Royal Hallamshire Hospital, Sheffield. Surgical procedures were performed during the period from 1998 to 2000 and patients were selected at random.

In this study, all specimens used were obtained from patients undergoing transurethral resection of the prostate (TURP) for bladder outflow obstruction with no hormonal therapy (incidental tumors), unlike the radical prostatectomies in which the patient received hormonal therapy and the specimen was poorly fixed, which affect the immunostaining. Moreover, TURP was used instead of needle core biopsy because more tissue was available for the study.

For each specimen, sections from each tissue block were stained with hematoxylin and eosin to confirm the original diagnosis. The block containing the most tumors was selected for each specimen, and two serial sections were taken from this block for immunohistochemical staining. Four serial sections of 4 μm thickness were then taken from each block. The first and the third sections were double immunostained for the vascular endothelial marker factor VIII (FVIII) and the pan-macrophage marker CD68. The second and fourth sections were immunostained for hypoxia-inducible transcription factors HIF-1α and HIF-2α, respectively. Negative and positive controls were included to ensure accuracy of the staining.

Immunohistochemistry

Specimens were routinely fixed in 10% neutral buffered formalin and embedded into paraffin wax after routine processing.

CD68/FVIII

Four-micrometer-thick sections were cut from the tissue blocks and mounted onto premium slides, deparaffinized, and rehydrated by passing through graded alcohols. Endogenous peroxidase activity in the sections was inactivated using 3% H_2O_2 for 20 min. The sections were then incubated in a 0.01% solution of protease at 40°C for 40 min. Nonspecific antibody binding was then blocked in a serum-free protein block for 15 min, followed by incubation with a monoclonal antibody specific to human CD68 (Dako Ltd, Ely, Cambridgeshire, UK) at a dilution of 1 : 100 in PBS and a monoclonal antibody specific to human FVIII at a dilution of 1 : 100. The sections were incubated overnight at 4°C and processed by the standard ABC immunostaining procedure using an ABC kit (Vector Laboratories Ltd, Orton Southgate, Peterborough, UK) according to the manufacturer's instructions. To verify the specificity of immunoreactions, some sections were incubated with an IgG control instead of the primary antibody. Immunoreaction products were visualized using diaminobenzidine tetrahydrochloride solution (Vector Laboratories), according to the manufacturer's instructions, and sections were then lightly counterstained with Gill's hematoxylin.

HIF-1α

The monoclonal HIF-1α antibody (Abcam, Cambridge, Cambridgeshire, UK) was raised against amino acids 432–528 of HIF-1α and was specific to HIF-1α as specified by the manufacture, whereas the monoclonal HIF-2α antibody (EP190b, kindly supplied by Professors K. Gatter and A. Harris, Oxford University) was raised against amino acid 535–631 of human HIF-2α and has been shown previously not to cross react with HIF-1α (Talks *et al.*, 2000).

Sections were stained using the Catalyzed Signal Amplification system (Dako), which is based on streptavidin–biotin–horseradish peroxidase complex formation, as previously described by Zhong *et al.* (1999). The 4-μm-thick serial sections were mounted onto premium slides, deparaffinized, and rehydrated by passage through a series of graded alcohols. The slides were treated with a target retrieval solution of sodium citrate (Dako) at 95°C for 40 min according to the manufacturer's instructions. A monoclonal antibody against HIF-1α (Abcam) was used at a dilution of 1 : 1000 for 2 h at room temperature. Negative controls were included in each run in which the antibody was omitted and the same IgG isotype of the antibody was applied. HeLa cells and macrophages were hypoxically incubated to express high levels of HIF-1α

and HIF-2α. Cells were clotted in plasma fibrinogen, embedded in paraffin wax, and used as positive controls. Sections were lightly counterstained with Gill's hematoxylin.

HIF-2α

Sections were stained using the ABC kit (Vector Laboratories). The 4-μm-thick sections were mounted onto premium slides, dried in an oven at 60°C for 15 min, and were then immediately deparaffinized and rehydrated by passage through graded alcohol solutions. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol for 20 min. The slides were then washed in tris-bufffered saline for 5 min before incubation with 1 mmol/l EDTA buffer (pH 8.0) for 6 h at 75°C. Triton X 100 (0.2%) in PBS was applied to the slides for 10 min, which were then rinsed in PBS. The sections were incubated overnight with undiluted primary human monoclonal antibodies against HIF-2α (EP190b). The immunoreactions were visualized using diaminobenzidine tetrahydrochloride solution, and the nuclei were lightly counterstained with Gill's hematoxylin.

Assessment of mean vascular density and macrophage index

The sections of adenocarcinoma stained with CD68/FVIII were scanned at low power (× 10– × 25) to identify the areas of greatest intratumoral macrophage and vascular densities, the so called 'hotspots'. The mean densities of macrophages (MϕD) and blood vessels [mean vascular density (MVD)] within macrophage and vascular hotspots were quantitatively determined according to the Chalkley point array method described by Leek *et al.* (1996). The mean microvessel density and mean macrophage density in the section were assessed microscopically in the three most vascular areas and the three areas with most dense macrophage infiltration using a 25-point Chalkley array eyepiece graticule (× 250 magnification; field area 0.413 mm^2). The graticule was rotated within the eyepiece until the maximum number of graticule dots overlaid the immunohistochemically labeled vessels and their lumens or macrophages. The mean MϕI and MVD were generated from the graticule values taken from the hotspots.

Statistical analysis

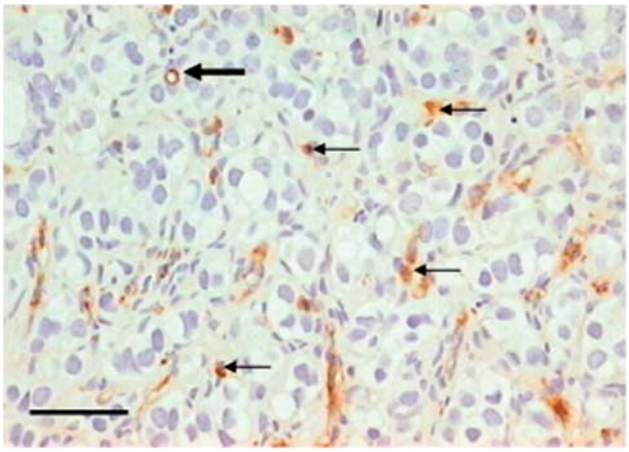
Nonparametric statistical analyses of all data were carried out using the statistical software package SSPS version 12.0 (SPSS Inc., Chicago, Illinois, USA). Correlations between MϕD and MVD among hotspots were calculated using the Wilcoxon rank sum test (Z). The same test was applied to HIF-1α and HIF-2α staining. A P-value less than 0.05 was considered statistically significant.

Results

Macrophage localization within carcinoma of the prostate

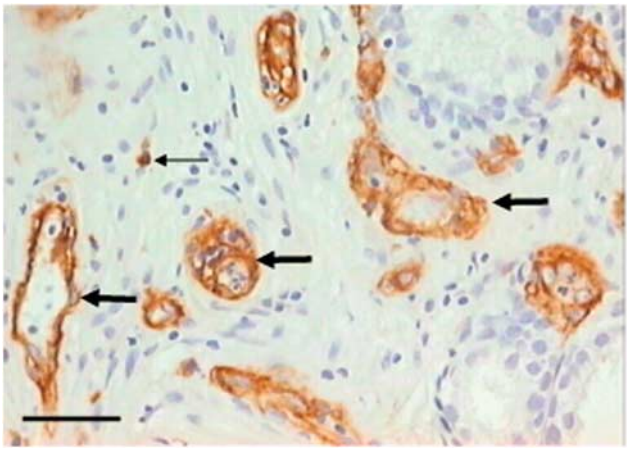
Data obtained from double-stained (i.e. for CD68 and FVIII) prostate carcinomas showed a reciprocal relationship between the MVD and the mean MϕD within vascular and macrophage hotspots in such tumors (Figs 1 and 2). The mean MϕD was significantly ($P < 0.001$) lower in vascular hotspots than in macrophage hotspots, whereas the MVD was significantly ($P < 0.001$) lower in macrophage hotspots than in vascular hotspots (Figs 3 and 4). These data demonstrate that there is a spatial difference in the location of blood vessels and macrophages within prostate carcinomas, with highly vascularized areas containing a low density of macrophages and macrophage-dense areas containing a low density of blood vessels.

Fig. 1



A macrophage 'hotspot' within an avascular area of an adenocarcinoma of the prostate. This has been double stained with the vascular endothelial cell marker factor VIII and the pan-macrophage marker CD68 (both in brown). Thin arrows highlight individual macrophages and thick arrows the occasional blood vessel. Scale bar = 50 μm (ABC immunostain, × 200).

Fig. 2

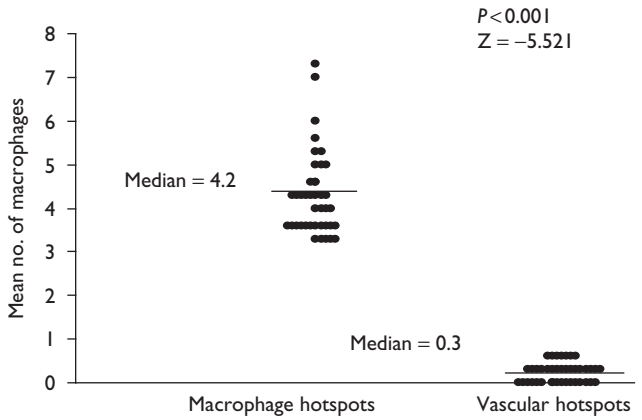


A typical vascular 'hotspot' in an adenocarcinoma of the prostate (double-stained with the vascular endothelial cell marker factor VIII and the macrophage marker CD68; both in brown). In this case, the thin arrows are the blood vessels and the thick arrows the occasional macrophage. Scale bar = 50 μm (ABC immunostain, × 100).

HIF-1α expression in prostate cancer

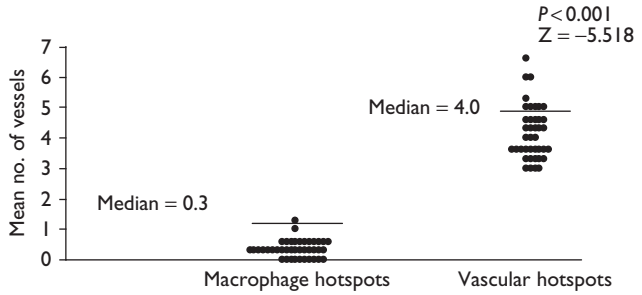
HeLa cells and macrophages were used as controls for the immunostaining procedure and showed HIF-1α and

Fig. 3



Macrophage density (MϕD) in areas of maximal macrophage density and maximum vascular density in 40 specimens of adenocarcinoma of the prostate. Macrophage counts were higher in macrophage hotspots and lower in vascular hotspots ($P<0.001$).

Fig. 4



Microvessel density in areas of maximal macrophage density and maximum vascular density in 40 specimens of carcinoma of the prostate. Vessel counts were significantly ($P<0.001$) higher in vascular hotspots than in macrophage hotspots.

HIF-2α accumulation following exposure to hypoxia but not normoxia (Figs 5 and 6).

Five of the 40 prostate adenocarcinoma (i.e. 12.5%) specimens showed no HIF-1α immunoreactivity in either the malignant cell population or the stromal compartment. In the remaining (i.e. 87.5%) tumors, immunoreactive HIF-1α was detected in the cytoplasm and/or nuclei of both malignant (Fig. 7) and stromal cells such as macrophages (Fig. 8). Furthermore, HIF-1α immunoreactivity was highly focal, appearing in some areas of tumors but not in others.

HIF-1α expression by TAM in macrophage hotspots

The presence of immunoreactive HIF-1α in CD68 + TAM was examined in serially stained sections of 40 prostate carcinoma specimens. Five tumors showed no immunostaining for HIF-1α and in the remaining 35 tumors, ~35% of cells stained positively for both CD68 and HIF-1α in the macrophage hotspots (Fig. 8a and b). Mean MϕD was significantly ($P < 0.001$) higher than the mean macrophage HIF-1α scores in the same areas of tumors (Fig. 9). This indicates that some macrophages in macrophage avascular hot spots do not express detectable levels of HIF-1α.

HIF-1α expression by macrophages in vascular hot spots

Examination of matched serial sections immunolabeled for CD68 and HIF-1α in vascular hot spots (Fig. 10) revealed that there was a significantly ($P < 0.001$) lower density of HIF-1α positive macrophages in these areas compared with CD68-positive cells. Approximately 18% of macrophages were immunoreactive for HIF-1α in these areas.

HIF-2α expression in prostate cancer

Three of the 40 prostate adenocarcinoma specimens examined (i.e. 8%) showed no detectable HIF-2α staining in either the malignant or the stromal compartment of the prostate tumors. In the remaining (i.e. 92%) tumors, HIF-2α immunoreactivity was seen to be highly focal, with immunostaining in both the cytoplasm and/or nuclei of tumor cells (Fig. 11) and TAM (Fig. 12).

HIF-2α expression by TAM in macrophage hot spots

Cells immunolabeled for both HIF-2α and CD68 in serial sections of prostate tumors showed that there was a significantly ($P < 0.001$) higher number of macrophages stained with CD68 alone than with CD68 and HIF-2α in avascular areas (Figs 12a and b and 13). Approximately 40% of CD68 + TAM were immunoreactive for HIF-2 in these areas.

HIF-2α expression by macrophages in vascular hotspots

Examination of serial sections immunolabeled for both CD68 and HIF-2α (Fig. 14) in vascular hotspots revealed that there was also a significantly ($P < 0.001$) higher number of cells stained with CD68 alone than with CD68 and HIF-2α in vascular areas. Approximately 21% of CD68 + TAM were immunoreactive for HIF-2α in these areas.

HIF-1α and HIF-2α expression in vascular and macrophage hotspots

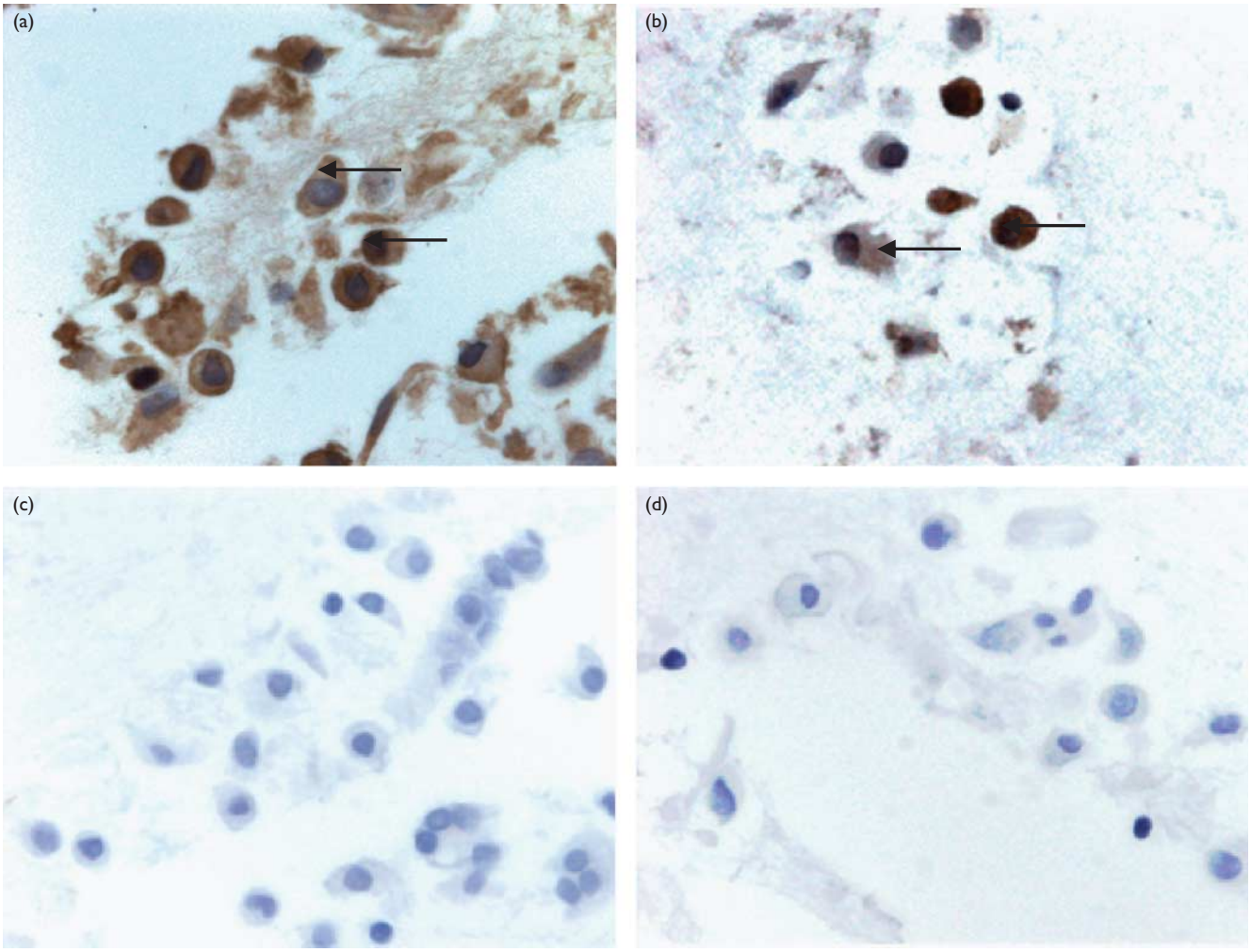
On observing HIF-1α and HIF-2α expression in both vascular and macrophage hot spots, we found that TAM expressed HIFs in both areas but significantly ($P < 0.001$) more in the macrophage hotspots compared with the vascular hotspots (Figs 15 and 16).

Discussion

This study shows that there is a significant and reciprocal relationship between angiogenesis and macrophage infiltration in prostate cancer. HIF-1α and HIF-2α were expressed in small subsets of macrophages, with more HIFs expression in avascular (35 and 40%) areas compared with vascular areas (18 and 21%).

The observation that the areas of high macrophage accumulation were situated away from vascular hotspots implies migration of macrophages away from vessels (i.e. their main sites of entry) into areas of poor vascularity. The factors that attract macrophages into avascular

Fig. 5



Immunolocalization of hypoxia-inducible factor (HIF)-1α (a) and HIF-2α (b) in monocyte-derived macrophages exposed to hypoxia (0.1% O_2; a, b) for 16 h *in vitro*. No immunostaining was seen when cells were exposed to normoxia (20.9% O_2; c, d). Immunoreactive HIF-1α and HIF-2α (brown) were seen in both the cytoplasm and the nuclei (arrows) of these cells following hypoxic induction. Blue staining is the hematoxylin staining of the nuclei of cells. (ABC immunostain, × 400).

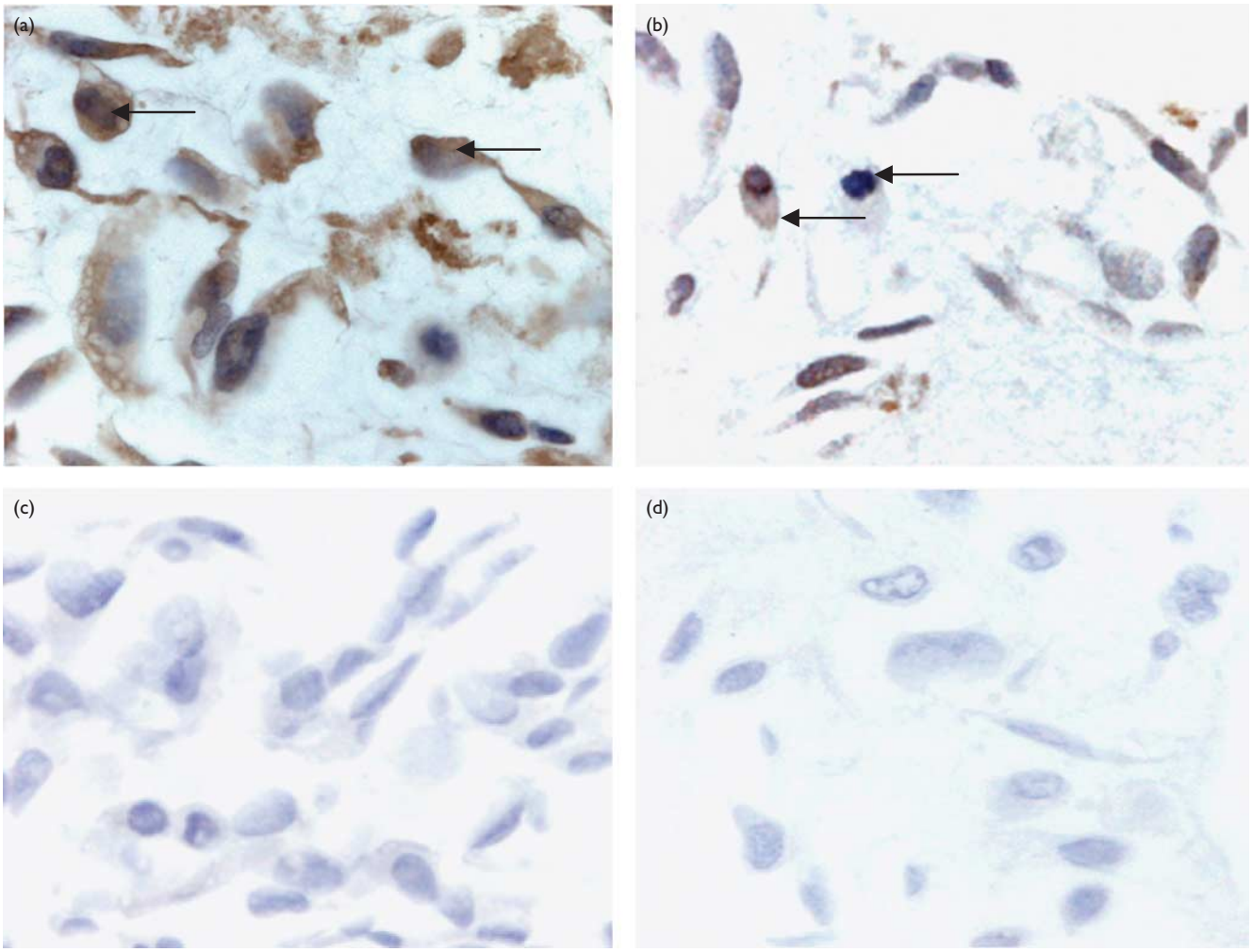
(and possibly hypoxic) areas are not clear but may include the hypoxic induction of release of macrophage chemoattractants by tumor and stromal cells such as VEGF, EMAP-II and ET-2 (Grimshaw *et al.*, 2002). Moreover, the vascular endothelium might produce unidentified factors that repel macrophages away from vessels. Necrosis in areas of chronic ischemia in tumors (i.e. where tumor cells experience hypoxic stress and/or undergo cell death) may also attract TAM.

TAM may also become trapped in hypoxic areas of tumors by the hypoxic release of macrophage migration inhibitory factor by tumor and stromal cells (White *et al.*, 2004). This cytokine, together with the direct inhibitory effect of hypoxia on macrophage migration (Turner *et al.*, 1999), may prevent the movement of TAM out of the hypoxic areas in tumors.

The finding that the expression of both HIF-1α and HIF-2α is highly focal in prostate carcinoma, with both factors accumulating in distinct areas of tumor cells, accords well with other studies on a wide range of human malignancies, including prostate carcinomas (Zhong *et al.*, 1999; Talks *et al.*, 2000; Burke *et al.*, 2002). HIF-1α and HIF-2α are transcription factors known to accumulate in the nuclei of hypoxic cells; it is interesting to note that both HIF-1α and HIF-2α were detected mainly in the cytoplasm of these cells, with occasional nuclear staining. A possible explanation for this might be conformational changes in HIF-1α within the nucleus, thereby reducing its immunoreactivity. Alternatively, ubiquitination may occur, causing them to be rapidly shuttled out of the nucleus and to accumulate mainly in the cytoplasm (Berra *et al.*, 2001), or the protein may have an abnormal conformation, preventing its entry into the nucleus.

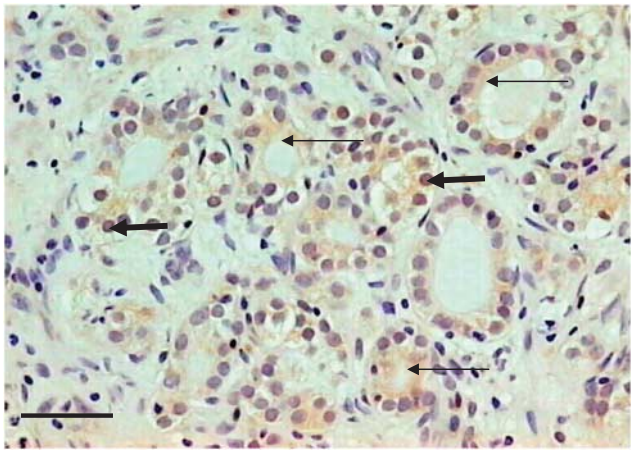
In the present study, only small subsets of macrophages were positive for either HIF in both macrophage or vascular hotspots. It has been suggested that TAM retain a relatively immature macrophage phenotype that is

Fig. 6



Immunolocalization of hypoxia-inducible factor (HIF)-1α (a) and HIF-2α (b) in HeLa cells. Cells were subjected to either normoxia (20.9% O_2; c, d) or hypoxia (0.1% O_2; a, b) for 16 h *in vitro*. Immunoreactive HIF-1α and HIF-2α were seen in both the cytoplasm and the nuclei (arrows) of these cells following hypoxic induction (ABC immunostain, × 400).

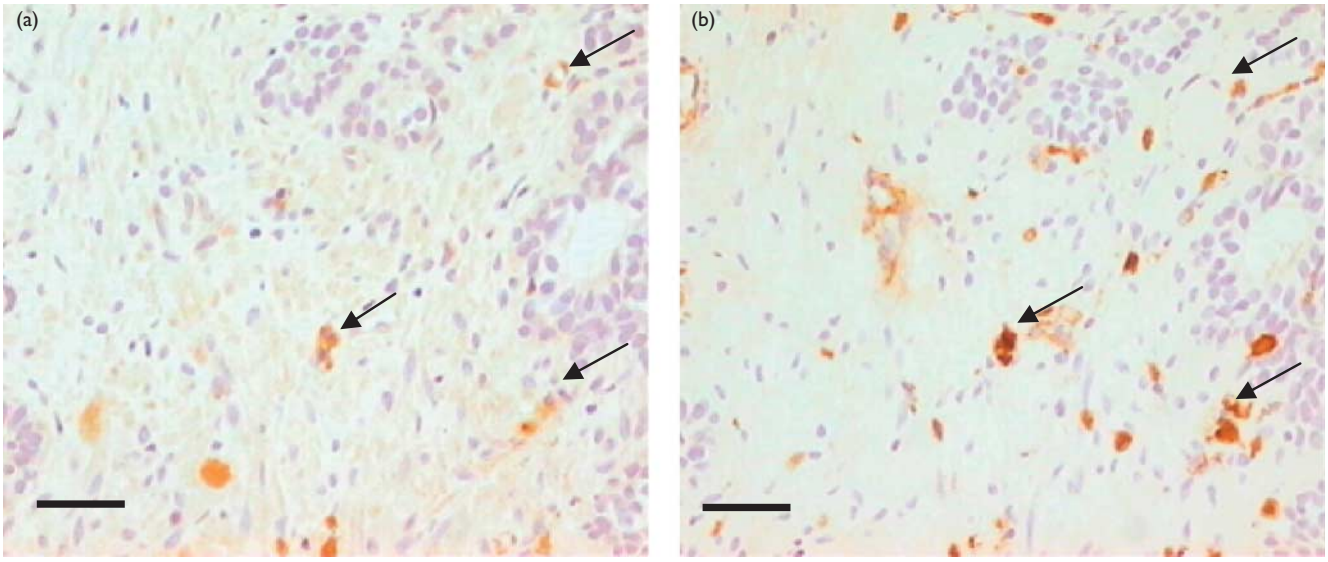
Fig. 7



Expression of immunoreactive hypoxia-inducible factor (HIF)-1α by tumor cells in prostate carcinoma. These cells were identified as tumor cells on the basis of their cell morphology. HIF-1α is present in both the cytoplasm (thin arrows) and the nuclei (thick arrows) of these cells. Scale bar is 50 μm (ABC immunostain, × 200).

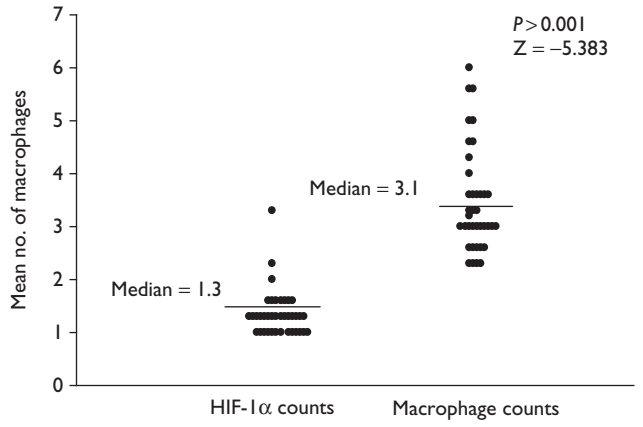
characterized by low expression of differentiation-associated macrophage antigens such as carboxypeptidase M and CD51 (Mantovani *et al.*, 2002). CD68 could be expressed at low levels such as levels at which cpM and CD51 are expressed; therefore, the CD68 antibody might not detect all the macrophages within the prostate tumor examined. However, more TAM were immunoreactive for HIF-1α and HIF-2α in avascular (i.e. presumably hypoxic) macrophage hotspots. These findings accord with those of another study by Koga *et al.* (2004), who stained 69 specimens of invasive bladder carcinoma for both TAM and HIF-2α in serial sections. HIF-2α expression was restricted to small subsets of TAM and this correlated with poor prognosis in such cancers. Although Burke *et al.* (2002) previously showed that HIF-1α is expressed by TAM in prostate tumors, the current findings are contrast with those of Talks *et al.* (2000), who showed HIF-2α to be more widely detected than HIF-1α in TAM in various forms of human tumors, including prostate carcinoma. However, as only five prostate carcinomas were included in their study, no realistic

Fig. 8



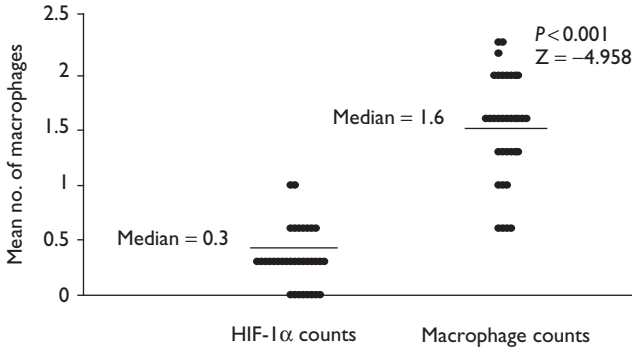
Detection of immunoreactive hypoxia-inducible factor (HIF)-1α in CD68-positive macrophages in prostate carcinoma. Sequential, 4-μm thick sections of wax-embedded tumors were immunostained for HIF-1α (a) and the macrophage marker CD68 (b). HIF-1α is present in a subset of macrophages. Scale bar=50 μm (ABC immunostain, × 100.

Fig. 9



Hypoxia-inducible factor (HIF)-1α expression by tumor-associated macrophages (TAM) in macrophage hotspots in 35 prostate carcinoma. HIF-1α immunostaining was seen in ~35% of macrophages in these areas.

Fig. 10



Hypoxia-inducible factor (HIF)-1α expression by TAM in vascular hotspots of 35 prostate carcinoma. Approximately 18% of macrophages were immunoreactive for HIF-1α in these areas.

comparison can be made with the results of the current study.

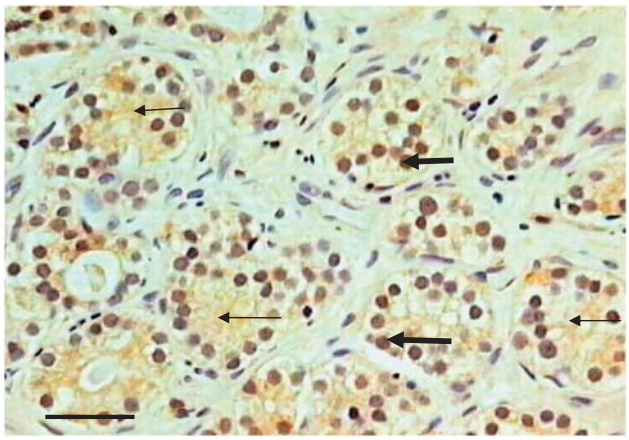
Burke *et al.* (2002) have shown that hypoxic macrophages accumulate more HIF-1α than HIF-2α, suggesting that HIF-1α may be the major hypoxia-inducible transcription factor in hypoxic TAM. This corresponds well with previous studies showing that HIF-1α-deficient mouse embryonic stem cells and embryonic fibroblasts are unxable to exhibit their normal pattern of gene upregulation in hypoxia, suggesting an essential role for HIF-1α (Park *et al.*, 2003). Another study has shown HIF-2α expression by TAM to positively correlate with angiogenesis in breast (Leek *et al.*, 2002) and bladder (Koga *et al.*, 2004) carcinoma. However, as no such analysis for HIF-1α was included in these studies, no direct comparisons could be made between HIF-1α and HIF-2α. Talks *et al.* (2000) showed that both HIFs were more highly expressed near necrotic areas in various human tumors, but this was not seen in prostate carcinomas as they exhibit extremely low levels of necrosis.

Both HIF-1α and HIF-2α are markedly and rapidly induced by hypoxia in human macrophages *in vitro* (Burke *et al.*, 2002). Moreover, hypoxia is common in prostate carcinomas (White *et al.*, 2004). These findings suggest that expression of HIFs by TAM is likely to be due to their exposure to hypoxia within the tumor microenvironment. However, HIF expression can also be

stimulated by cytokines such as tumor necrosis factor-α and IL-1 (Frede *et al*., 2005). The transcriptional activity of HIF-1α can be enhanced by trophic stimuli such as insulin, insulin like growth factors (IGF-1, IGF-2), prostaglandin E_2 (Liu *et al*., 2002), and androgens that upregulate HIF-1α but not HIF-2α expression in prostate cancer (Mabjeesh *et al*., 2003). Furthermore, macrophages release the antibacterial agent PR-39 (Li *et al*., 1997), a 39 amino acid peptide that also inhibits the ubiquitin–proteasome-dependent degradation of HIF-1α protein, resulting in accelerated formation of vascular structures *in vitro* and increased myocardial vasculature in mice (Li *et al*., 2000). It is not known whether any or all of these factors are expressed at higher levels in areas of macrophage accumulation or HIF expression in prostate carcinomas; therefore, these factors could modulate HIF expression in these cells in a hypoxia-independent manner. This could also explain the observation that HIF-1α and HIF-2α were expressed in the vasularized areas of such tumors.

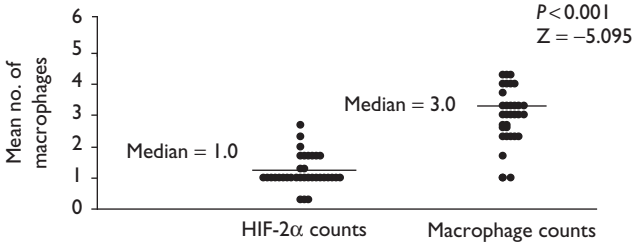
With respect to the concerns over ex-vivo degradation of HIF, although all tissues obtained using TURP are small and rapidly fixed by diffusion, unlike the radical prostatectomy specimen, the time between surgical removal of each tumor biopsy and placing it in formalin

Fig. 11



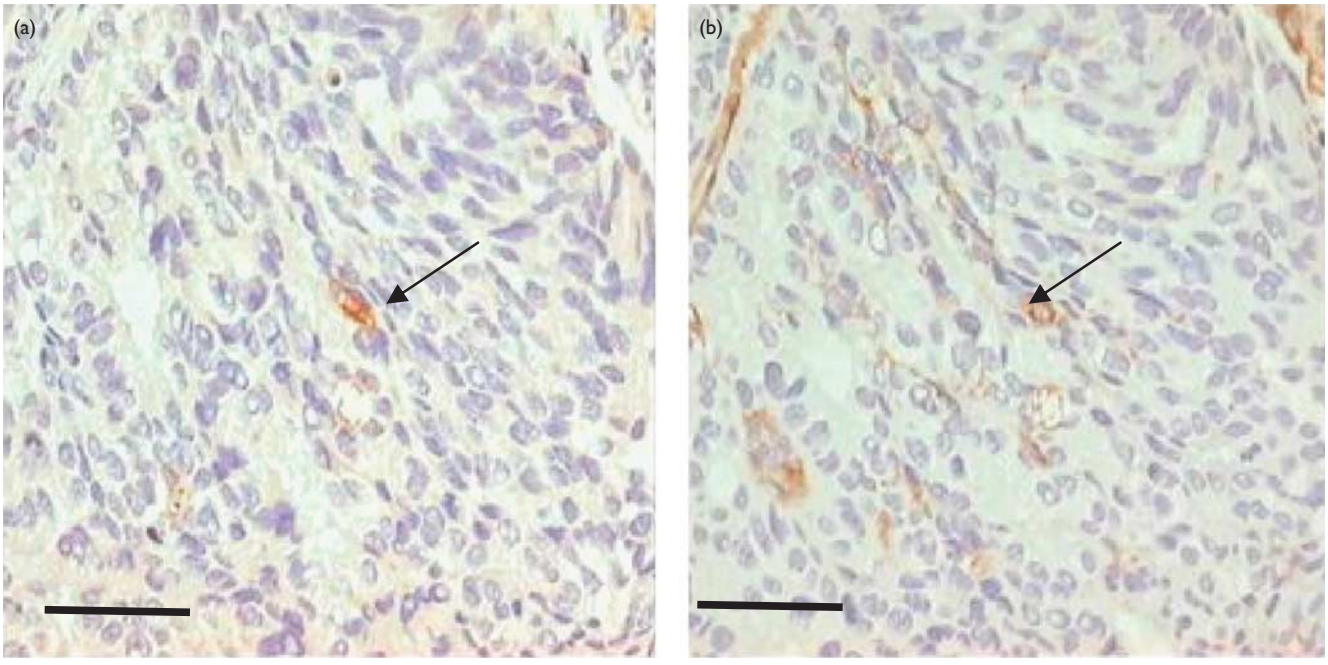
Immunodetection of hypoxia-inducible factor (HIF)-2α in tumor cells in a prostate carcinoma. HIF-1α is present in both the cytoplasm (thin arrows) and the nuclei (thick arrows) of these cells. Scale bar = 50 μm (ABC immunostain, × 200).

Fig. 13



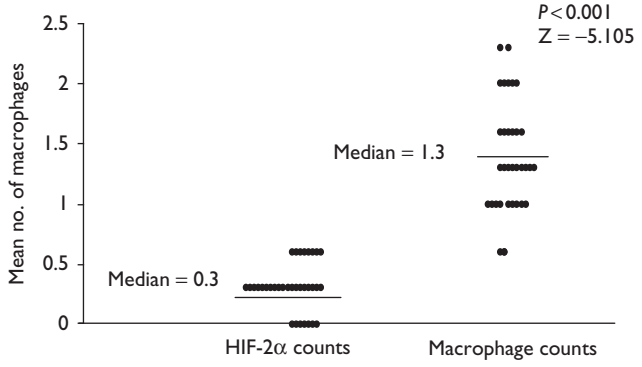
Hypoxia-inducible factor (HIF)-2α expression by tumor-associated macrophages (TAM) in macrophage hotspots in prostate carcinoma. In total, 40% of macrophages were immunoreactive for HIF-2α; 35% of TAM were HIF-1α positive in similar areas of these tumors.

Fig. 12



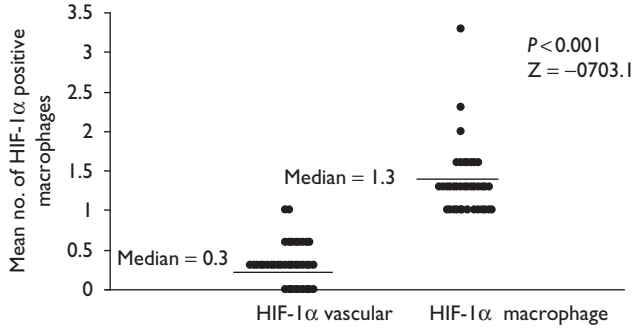
Expression of immunoreactive hypoxia-inducible factor (HIF)-2α (a) by CD68-positive (b) macrophages in an avascular area of a prostate carcinoma. HIF-2α was detectable in 40% of tumor-associated macrophages in these areas. Scale bar = 50 μm (ABC immunostain, × 200).

Fig. 14



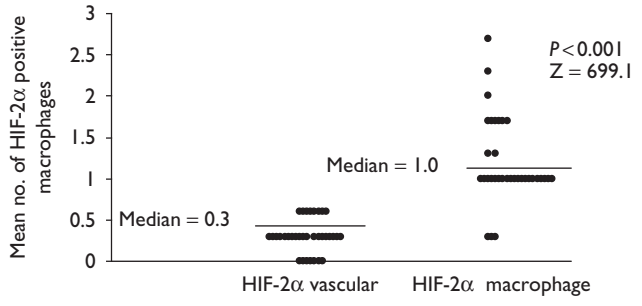
Hypoxia-inducible factor (HIF)-2α expression by tumor-associated macrophages (TAM) in vascular hotspots. There were 21% of macrophages immunoreactive for HIF-2α in prostate cancer; 18% of TAM were HIF-1α positive in similar areas of the tumor.

Fig. 15



Immunoreactive hypoxia-inducible factor (HIF)-1α in macrophages located in vascular versus macrophage hotspots in 35 prostate carcinoma specimens. A greater density of HIF-1α-positive tumor-associated macrophages were seen in the avascular macrophage hotspots than in vessel hotspots.

Fig. 16



Immunoreactive hypoxia-inducible factor (HIF)-2α in tumor-associated macrophages (TAM) in vascular versus macrophage hotspots in 37 prostate carcinoma specimens. A greater density of HIF-2α-positive TAM were seen in avascular macrophage hotspots than in vessel hotspots.

fixative is not known and this may affect the HIF expression within the tumor. Hoedemaeker *et al.* (1998) demonstrated that immersion fixation of radical prostectomy specimens may lead to a discrepancy in preservation between the peripheral and the central part of the specimen.

The findings of this study suggest that not all TAM in hypoxic areas of prostate carcinomas express detectable levels of HIF-1α or HIF-2α. Therefore, the expression of other well-known hypoxia-induced transcription factors by macrophages – including HIF-3α, ATF-4, Egr-1, NFκB, C/EBPβ, and Ets-1 (all of which have been shown to be upregulated by hypoxia in various tumor in cell lines; Oikawa *et al.*, 2001; Blais *et al.*, 2004; Zampetaki *et al.*, 2004) – might play a role in activating gene expression in TAM in hypoxic tumor areas.

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Conflicts of interest

There are no conflicts of interest.

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