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Multiregional tumor drug-uptake imaging by PET and microvascular morphology in end-stage diffuse intrinsic pontine glioma


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ABSTRACT

Introduction
Inadequate tumor uptake of the vascular endothelial growth factor (VEGF) antibody bevacizumab could explain lack of effect in diffuse intrinsic pontine glioma (DIPG).

Methods
By combining data from a positron emission tomography (PET) imaging study using zirconium-89\(^{89}\text{Zr}\)-labeled bevacizumab and an autopsy study, a 1-on-1 analysis of multiregional in vivo and ex vivo \(^{89}\text{Zr}\)-bevacizumab uptake, tumor histology and vascular morphology in a DIPG patient was performed.

Results
In vivo \(^{89}\text{Zr}\)-bevacizumab measurements showed heterogeneity between lesions. Additional ex vivo measurements and immunohistochemistry of cervicomedullary metastasis samples, showed highest uptake in the area with marked microvascular proliferation. In the primary pontine tumor all samples showed similar vascular morphology. Other histological features were similar between samples studied.

Conclusion
In vivo bevacizumab-PET serves to identify heterogeneous uptake between tumor lesions, while subcentimeter intra-lesional heterogeneity could only be identified by ex vivo measurements. Bevacizumab uptake is enhanced by vascular proliferation, although our results suggest it is not the only determinant of intra-lesional uptake heterogeneity.
INTRODUCTION

End-stage DIPG shows glioblastoma histology and overexpression of proangiogenic factors, including VEGF. Whether DIPG patients benefit from anti-VEGF treatment, such as the monoclonal antibody bevacizumab, however, is still unclear. So far, results of trials with bevacizumab have been disappointing. A recent drug imaging study assessing tumor uptake of zirconium-89-labeled bevacizumab in DIPG showed inter and intra-patient heterogeneity, but factors determining uptake are currently unknown. Therefore, this study aims to perform a 1-on-1 analysis of multiregional in vivo and ex vivo 89Zr-bevacizumab uptake, tumor histology and vascular morphology in a DIPG patient.

MATERIALS AND METHODS

A 12-year old female patient presented with a left abducens nerve paralysis. Brain MR-imaging revealed a T1-weighted hypointense, T2-weighted hyperintense lesion infiltrating over 50% of the pons suggestive of DIPG. Biopsy demonstrated high-grade diffuse glioma features, and Sangers sequencing of H3F3A and HIST1H3B revealed a wild-type status. Whole exome sequencing showed no mutations in HIST1H3C or HIST2H3C either. A diagnosis of Histone H3 wild-type DIPG was made. The patient was enrolled in the DIPG study VUmc-01 (Dutch Trial Register: NTR2391) - phase A, and treated with radiotherapy (30x1.8=54Gy) and weekly gemcitabine (175mg/m²/week) for six weeks. Progression-free survival was 3.8 months. After disease progression, the patient participated in a molecular imaging study (NTR3518), including an immuno-PET scan at 145 hours postinjection of 0.1mg/kg bevacizumab labeled with 0.9MBq/kg zirconium-89, as described. Four days afterwards, the patient died (death was unrelated to study participation) and participated in an autopsy study. All three studies were approved by the Institutional Review Board of VU University Medical Center and performed in accordance with the Declaration of Helsinki. For each study, both parents gave written informed consent.

In vivo 89Zr-bevacizumab Uptake Measurements

In vivo 89Zr-bevacizumab uptake was quantified as decay-corrected maximum activity concentration (corr. ACmax in Bq/mL) in manually delineated volumes of interest, i.e. tumor areas with visually enhanced uptake. Activity concentrations were also converted
into Standardized Uptake Values (SUVs), i.e. decay-corrected maximum activity concentration normalized to injected dose/body weight.

**Ex vivo 89Zr-bevacizumab Uptake Measurements**

Brain autopsy was performed 2 hours post-mortem. Multiple 0.5 cm³ tumor and control samples (macroscopically non-affected brain, cerebrospinal fluid (CSF) and blood) were obtained for ex vivo ⁸⁹Zr-radioactivity measurements and histological analysis. Ex vivo radioactivity concentrations were measured with a gamma well counter (Wallac 1480 Wizard, Turku, Finland). The in vivo PET scanner (Philips Ingenuity TF-128 PET-CT-scanner, Philips Healthcare) was cross-calibrated with the well counter. Ex vivo ⁸⁹Zr-radioactivity concentrations were also quantified as corr. ACmax and converted into SUV.

**Immunohistochemistry**

Four-μm-thick formalin-fixed, paraffin-embedded tissue sections were stained with Hematoxylin&Eosin (H&E) and immunostained as previously validated and described against glial fibrillary acidic protein (GFAP; Dako, 1:400); MIB-1 (Dako, 1:40); GLUT-1 (Thermo Scientific, 1:200), smooth muscle actin (SMA, Dako, 1:200), collagen-IV (Dako, 1:50), CD34 (Dako, 1:50) and VEGF (Pharamgen, 1:50). Specific stainings were chosen to determine differences in proliferation grade in the tumor in vascular endothelial cells (MIB-1), to highlight vascular morphology by identifying endothelial cells (CD34), vascular smooth muscle cells and activated pericytes (SMA) and the vascular basal membrane (Coll-IV), to visualize tumor morphology and resident astrocytes (GFAP), to identify blood brain barrier integrity and areas of increased hypoxia (GLUT1), and to determine VEGF expression (VEGF). Immunopositivity was detected with 3,3’-Diaminobenzidine (DAB). Omitting primary antibodies yielded no significant staining. Sections were photographed using a Leica DM6000B microscope.

**RESULTS**

**In vivo ⁸⁹Zr-bevacizumab Uptake**

The T1 post-gadolinium-MR-images obtained eight days pre-mortem showed a 3.6x3.3cm primary pontine tumor, with strong contrast enhancement but no apparent necrosis. Furthermore, extensive meningeal and subependymal metastases in the right ventricular trigone (VTM) and cervicomedullary junction (CMM) (Fig. 1), as well as in the right frontal lobe, left Sylvian fissure, left lateral ventricle posterior horn, and along the spinal cord were observed (Supplemental Figs. 1A-F). At t=145 the most intense ⁸⁹Zr-
bevacizumab uptake-positivity was seen in the CMM (corr. ACmax 7800Bq/mL, SUV 9.9, volume 2.0mL), followed by the primary pontine tumor (5607Bq/mL, SUV 7.1, 13.1mL) and the VTM (2537Bq/mL, SUV 3.2, 1.2mL).

**Ex vivo $^{89}$Zr-bevacizumab Uptake**

Ex vivo radioactivity measurements at t=242 were performed in multiple tumor samples (Fig. 2A), including the VTM, primary tumor, CMM, and a small 0.02gram fragment of dural metastasis that was previously undetected by MRI and PET (Supplemental Fig. 2), and macroscopically normal areas of the pons, cerebellum and dura. Tracer uptake was highest in the dural metastasis (22652Bq/mL, SUV 28.7) and CMM samples, with considerable difference between the cranial (27152Bq/mL, SUV 34.4) and caudal (16184Bq/mL, SUV 20.5) part. Differences in uptake were also observed between the cranial (2949Bq/mL, SUV 3.7) and caudal (11343Bq/mL, SUV 14.4) part of the primary

![Image of Gadolinium-enhanced T1-MR-images obtained 8 days before death and corresponding PET slices obtained 4 days before death (t=145 post-$^{89}$Zr-bevacizumab-injection) showing the primary pontine tumor, and metastases in the right ventricular trigone (VTM) and cervicomedullary junction (CMM). White arrows represent areas of disease.](image-url)
Radioactivity was low in normal tissue samples (<1844Bq/mL, SUV <2.3), CSF (62Bq/mL, SUV 0.1) and blood (716Bq/mL, SUV 0.9).

**Correlation Between In vivo and Ex vivo 89Zr-bevacizumab Uptake**

Fig. 2B shows the ratio between in vivo and ex vivo 89Zr-bevacizumab uptake of the primary tumor, VTM and CMM.

**Correlation Between Ex vivo 89Zr-bevacizumab Uptake and Histology**

After ex vivo radioactivity measurements, multiple samples from the primary tumor and CMM were investigated with (immuno)histochemistry to explore differences in histology as possible explanation for tracer uptake heterogeneity. H&E showed diffuse growth...
Figure 3 | Histology and immunohistochemistry of samples from the cranial and caudal part of the primary pontine tumor and CMM, respectively (A) H&E; (B) MIB-1 (for detection of proliferating cells); (C) astrocyte marker GFAP; (D) CD34 (endothelial cell marker) and Glut-1 (a glucose transporter expressed by brain endothelial cells as part of the blood brain barrier, and often deregulated in areas with hypoxia) (E) SMA (a marker of vascular smooth muscle cells and activated pericytes) and Coll-IV (a component of the vascular basal membrane) (F) VEGF staining. Original magnification of all images x200. Note: the diffuse growth pattern in all samples; extensive necrosis and glomerular microvascular proliferation with multiple MIB-1 positive (endothelial) cells in the microvascular walls in the caudal CMM; partial loss of CD-34 in the caudal pons; similar staining against GFAP, GLUT-1, VEGF in all samples; all (micro)vessels highlighted in the stain against collagen-IV and SMA.
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pattern, high cellularity and variable cytonuclear atypia (Figs. 3A and 3B) in all tumor samples, and confirmed absence of tumor in macroscopically normal tissue. Necrosis and florid glomeruloid microvascular proliferation were only present in the cranial CMM (with highest ex vivo radioactivity) (Fig. 3A). In this sample, MIB-1 staining was positive in up to 50% of tumor cells and in numerous (endothelial) cells in the microvascular walls (Fig. 3B). In the remaining samples microvascular proliferation was less pronounced: here, 30% (in both primary tumor samples) to 50% (in the caudal CMM sample) of tumor cell nuclei were MIB-1-positive, whereas endothelial cells were MIB-1-negative (Fig. 3B). GFAP was expressed in tumor cells in the primary tumor and CMM, as well as in residual non-neoplastic astrocytes (Fig. 3C). Staining of CD34 showed partial loss of microvascular immunoreactivity in the caudal part of the primary tumor. In the other samples, CD34-immunoreactivity was maintained (Fig. 3D). Immunoreactivity for GLUT-1 was similar in all samples (Fig. 3E). Staining of collagen-IV and SMA highlighted the vascular basal lamina and tunica media, respectively, in all samples without obvious differences (Fig. 3E). Staining of VEGF was similar amongst all samples (Fig. 3F).

DISCUSSION

This study directly correlates multiregional $^{89}$Zr-bevacizumab uptake to tumor histology and vascular morphology in a patient with histone H3 wild-type DIPG$^7$. Observed differences between in vivo and ex vivo radioactivity measurements reflect tracer pharmacokinetics, e.g. ongoing tracer clearance from blood over time, in combination with spatially and/or temporally heterogeneously increasing bevacizumab tissue deposition. Moreover, in vivo PET underestimates true activity in small lesions (in this case the CMM and VTM) because of partial volume effects. Due to this effect$^{248}$, the actual radioactivity concentration as assessed by imaging at t=145 post-$^{89}$Zr-bevacizumab injection was likely underestimated by circa 70%. Finally, as opposed to ex vivo measurements, at t=145 intravascular $^{89}$Zr-bevacizumab still contributes to the in vivo PET tumor signal (blood pool SUV~4$^{247}$). Thus, where in vivo PET serves to identify whole body tracer distribution and heterogeneity between lesions, subcentimeter intra-lesional heterogeneity of tracer distribution can only be defined with ex vivo measurements.

Interestingly, H&E and MIB-1 staining showed prominent microvascular proliferation in the cranial part of the CMM, with higher $^{89}$Zr-bevacizumab uptake, versus the caudal part with lower uptake. No other histological differences were found between the two samples, such as pattern of tumor growth, extent of necrosis or different immunoreactivity for endothelial
markers. In the two samples from the pons, intra-lesional uptake heterogeneity was also observed, although here both areas showed similar vascular morphology, tumor growth, and extent of necrosis. These findings suggest that vascular proliferation is an important, yet not the only determinant of intra-lesional heterogeneity in bevacizumab uptake.

The presence of microvascular proliferation and disorganized vessels\textsuperscript{251} in WHO grade IV glioma is considered a consequence of hypoxia-induced overexpression of proangiogenic factors, including VEGF\textsuperscript{30,244}. In the cranial CMM sample, glomeruloid microvascular structures with increased endothelial cell turnover (as determined by MIB-1-immunoreactivity) were prominent. Based on this, VEGF was expected to be overexpressed. VEGF immunoreactivity, however, showed mostly aspecific staining and did not differ between samples. This may be explained by the very short half-life of the VEGF protein (15-20 minutes\textsuperscript{252}) being incompatible with its immunohistochemical detection two hours post-mortem. Glut-1 staining was similar in all samples analyzed, suggesting a partially intact blood-brain barrier (BBB). However, our histological analysis did not provide definitive clues to determine the role of BBB integrity in bevacizumab uptake in this case.

CONCLUSION

Although concerning a single case, we conclude that \textit{in vivo} PET is capable of detecting heterogeneity in bevacizumab uptake between lesions, which correlates well to \textit{ex vivo} measurements. However, PET cannot detect subcentimeter intra-lesional uptake heterogeneity. Furthermore, our results suggest that targeting of bevacizumab is enhanced in areas with vascular proliferation. However, as significant inter-and intra-lesional heterogeneity was also observed in areas that did not show differences in vascular proliferation, other factors present in the DIPG microenvironment likely also play a role. Because the DIPG microenvironment is heterogeneous and dynamic in nature\textsuperscript{245}, patients are likely to fail treatment if timing and patient selection are not optimized. The results of this study underline the potential of immuno-PET studies, especially when combined with biopsy or/and autopsy studies, in the quest for optimal selection and timing of treatment schedules.

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Supplemental Figure 1 | Gadolinium-enhanced T1-MRI, PET and macroscopy (if available) images of (A) the primary pontine tumor and metastases at the (B) cervicomedullary junction, (C) spinal cord, (D) posterior horn of the left lateral ventricle, (E) right frontal lobe and (F) left Sylvian fissure. White arrows represent areas of 89Zr-bevacizumab uptake and/or disease.
Supplemental Figure 2 | Macroscopic picture of the dural metastases (white arrows), which were detected during autopsy but were undetected by MRI and PET. The dotted arrow indicates the primary pontine tumor.