In Vivo Detection of EGFRvIII in Glioblastoma via Perfusion Magnetic Resonance Imaging Signature Consistent with Deep Peritumoral Infiltration: The $\phi$-Index

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Abstract

Purpose: The epidermal growth factor receptor variant III (EGFRvIII) mutation has been considered a driver mutation and therapeutic target in glioblastoma, the most common and aggressive brain cancer. Currently, detecting EGFRvIII requires postoperative tissue analyses, which are ex vivo and unable to capture the tumor’s spatial heterogeneity. Considering the increasing evidence of in vivo imaging signatures capturing molecular characteristics of cancer, this study aims to detect EGFRvIII in primary glioblastoma noninvasively, using routine clinically acquired imaging.

Experimental Design: We found peritumoral infiltration and vascularization patterns being related to EGFRvIII status. We therefore constructed a quantitative within-patient peritumoral heterogeneity index (PHI/$\phi$-index), by contrasting perfusion patterns of immediate and distant peritumoral edema. Application of $\phi$-index in preoperative perfusion scans of independent discovery ($n = 64$) and validation ($n = 78$) cohorts, revealed the generalizability of this EGFRvIII imaging signature.

Results: Analysis in both cohorts demonstrated that the obtained signature is highly accurate (89.92%), specific (92.35%), and sensitive (83.77%), with significantly distinctive ability ($P = 4.0033 \times 10^{-10}$, AUC = 0.8869). Findings indicated a highly infiltrative-migratory phenotype for EGFRvIII tumors, which displayed similar perfusion patterns throughout peritumoral edema. Contrarily, EGFRvIII tumors displayed perfusion dynamics consistent with peritumorally confined vascularization, suggesting potential benefit from extensive peritumoral resection/radiation.

Conclusions: This EGFRvIII signature is potentially suitable for clinical translation, since obtained from analysis of clinically acquired images. Use of within-patient heterogeneity measures, rather than population-based associations, renders $\phi$-index potentially resistant to inter-scanner variations. Overall, our findings enable noninvasive evaluation of EGFRvIII for patient selection for targeted therapy, stratification into clinical trials, personalized treatment planning, and potentially treatment-response evaluation. Clin Cancer Res; 23(16); 4724–34. ©2017 AACR.

Introduction

Glioblastoma (GBM) is the most common and aggressive primary malignant adult brain tumor, with an average survival of 14 months (1) following standard treatment with surgical resection and chemoradiation, and 4 months otherwise. Despite treatment advances over the past 20 years, the lack of substantial improvement in the overall survival rates, in part, relates to the underlying molecular and cellular heterogeneity of GBM (2–5). Primarily this heterogeneous genetic landscape of GBMs and the resulting different treatment responses gave rise to recognizing the beneficial effect of personalized medicine, leading to the current investigation and testing of new treatment options targeting specific molecular characteristics (6).

The epidermal growth factor receptor (EGFR) is a regulator of normal cellular growth in epithelial origin tissues (7), which has been well-validated as a target for cancer therapy, as abnormalities in its expression have been associated with reduced response to aggressive therapy (8, 9). Overexpression of the cell-surface EGFR leads to dysregulation in its signaling, which is a main contributor to the formation of many epithelial malignancies in humans (10). In such cases, which is the majority of patients with GBM (11), there is typically an associated gene amplification (12) or mutation (13) in EGFR (9). The most common extracellular EGFR mutation in GBM is the variant III (EGFRvIII), alternatively named de2-7EGFR or ΔEGFR, which is an important factor in driving tumor progression and defining prognosis (14). Half of
EGFR-amplified tumors harbor the EGFRvIII mutation, which is a gene rearrangement due to in-frame deletion of exons 2–7 from this receptor tyrosine kinase (15). This deletion consequently causes constitutive signaling in the absence of ligand binding (16). In contrast to wild-type EGFR, which can be found in normal tissue, EGFRvIII is expressed only in tumor cells (17), can be found nearly in 33% of GBM patients (14, 18), and its overexpression worsens the prognosis (11, 14). EGFRvIII is also associated with activation of numerous oncoprogenic processes leading to aggressive tumor growth and proliferation (13), hence evidence of the mutant’s presence can have an impact on treatment decisions, as well as on evaluating treatment response. For these reasons, vaccination against EGFRvIII is a potentially promising immunotherapy (17), and EGFRvIII represents a potentially viable therapeutic target for GBM patients (19, 20) that has been the target of several investigational drug trials and pilot studies (6, 21–24).

Although determination of EGFRvIII status is vital for targeted therapeutics in GBM, invasive studies are required for current tissue-based approaches, which include immunohistochemistry and next-generation sequencing (NGS; refs. 25, 26). The process of such approaches is primarily hindered by the spatial (4, 5) and temporal heterogeneity (6, 27–29) of molecular alterations within the GBM tumor that give rise to sampling error. Furthermore, the invasive nature of repeated biopsies makes it nearly impossible to evaluate the dynamic equilibrium of mutations and molecular characteristics that occur during the course of treatment, hence adapt the treatment accordingly. Patient stratification and selection for treatment is limited for the same reason. In other cases, the biopsy (or resection) of the tumor might not always be possible, such as in deep-seated tumors, in which there is no sufficient sample size for histopathologic analysis. Finally, molecular testing may be unavailable in certain clinical settings due to cost or equipment availability.

Thus, the aim of this study is to determine the EGFRvIII status based solely on quantitative magnetic resonance imaging (MRI) phenotypes. To our knowledge, this study is the first to establish a robust, reproducible, noninvasive and easy to evaluate imaging signature of the EGFRvIII expression in GBM. We hypothesized that the EGFRvIII+ tumors, which are thought to have aggressive, migratory, and infiltrative phenotypes, would present imaging signatures consistent with deep infiltration throughout the peritumoral edematous tissue. In addition, we hypothesized that assessment of the tumor cell infiltration heterogeneity (30) in the peritumoral edema, may be discriminatory of the EGFRvIII status and hence lead to a distinctive imaging biomarker. Taking into consideration that edema is a result of infiltrating tumor cells, as well as a biological response to the angiogenic and vascular permeability factors released by the spatially adjacent tumor cells (31), dynamic susceptibility contrast (DSC) MRI was used to indirectly measure changes in perfusion as they relate to EGFRvIII.

Materials and Methods
Study design
Preoperative multiparametric MRI data from a combined (discovery and replication) cohort of 142 patients (80 males, 62 females) with de novo (i.e., primary) GBM were included. The inclusion criteria for those patients comprised: (i) pathologic diagnosis of de novo GBM confirmed by our board-certified neuropathologist (M. Martinez-Lage), (ii) NGS-based EGFRvIII expression, and (iii) availability of contrast-enhanced T1-weighted (T1-C), T2-weighted fluid-attenuated inversion recovery (T2-FLAIR), and DSC MRI volumes. Tissue specimens of these patients were obtained by surgical resection and tested for the status of EGFRvIII at the Hospital of the University of Pennsylvania (HUP, Philadelphia, PA) between July 2006 and July 2015. Mean and median age was 59.82 and 60.95 years, respectively (range: 18.65–86.95 years). EGFRvIII was negative in 100 patients (57 males, 43 females; 70.42%) and positive in 42 patients (23 males, 19 females; 29.58%), a proportion consistent with reported rates (29) of molecular alterations with respect to de novo GBM and positive EGFRvIII in GBM patients (31). Gender and age did not differ significantly between the GBM and EGFRvIII+ patients. The protocol was approved by the Institutional Review Board at HUP, and informed consent was obtained from all subjects. No randomization method was used for allocating samples to experimental groups.

Equipment and imaging data
The multiparametric MRI data used in this study were acquired in the axial plane using a 3-Tesla Siemens Magnetom Trio A Tim clinical MRI system, according to a standardized acquisition protocol followed at HUP. For the DSC-MRI scan, the total intravenously injected dose of contrast material was divided by an initial intravenously loading dose to help minimize errors due to potential contrast leakage out of intravascular space, and a second bolus intravenous injection after a 5-minute delay. The DSC acquisition was performed during the second injection. The exact contrast material dosage amount was dependent on patient weight, i.e., 0.3 ml/kg. The dosing ratio of the initial over the second dose was 1/3 prior to September 2013 (107 patients), and 1/1 afterwards (35 patients). The contrast material used early in the study (105 patients) was gadodiamide (Omniscan, GE Healthcare) while later in the study (37 patients) was gadobenate dimeglumine (MultiHance, Bracco SpA). The T2-FLAIR volumes were acquired between the initial intravenous injection and the DSC acquisition. The dimensions of the T2-FLAIR images were 192 × 256 × 60 pixels, with spatial resolution of 0.938 × 0.938 ×
Image preprocessing

The provided MRI volumes were smoothed using a low-level image processing method, namely Smallest Univariate Segment Assimilating Nucleus, to reduce high-frequency intensity variations (i.e., noise) in regions of uniform intensity profile while preserving the underlying structure (32). The intensity nonuniformity caused by the inhomogeneity of the magnetic field during image acquisition were removed using a nonparametric, nonuniform intensity normalization algorithm (33). The volumes of all the modalities for each patient were coregistered to the T1-CE anatomic template using a 6-degrees-of-freedom affine registration, and then skull-stripped (34).

Regions of interest and perfusion temporal dynamics

To assess the tumor cell infiltration heterogeneity within the peritumoral edema (i.e., the peritumoral area described by hyperintensity on the T2-FLAIR volumes; ref. 30), two regions of interest (ROIs) were annotated for each subject by an expert (Fig. 1), blinded to EGFRvIII status. These two ROIs were used to sample tissue located on the two boundaries of edema near to and far from the tumor, respectively, and hence to evaluate the heterogeneity or spatial gradient of perfusion signals. The T1-CE and T2-FLAIR volumes were used to define the ROIs near to and far from the tumor, respectively. Speciﬁcally, the T1-CE volume was used to initially define the ROI adjacent to the enhancing part of the tumor, described by hyperintense signal on T1-CE, and the T2-FLAIR volume was then used to revise this ROI in terms of all its voxel values being within the peritumoral edematous tissue, described by hyperintense signal on the T2-FLAIR volume. The T2-FLAIR volume was also used to deﬁne the ROI at the farthest from the tumor but still within the edematous tissue, i.e., the enhancing FLAIR abnormality signal. These ROIs are described by lines drawn in multiple slices of each image (T1-CE and T2-FLAIR) for each subject, whereas the visual example of Fig. 1 shows only a single slice. The perfusion temporal dynamics for each of these ROIs were obtained from the DSC-MRI volume (Fig. 2A). Specifically, the perfusion of each voxel during 45 time-points was used to form a feature vector of 45 dimensions. Principal component analysis (PCA) was then used to summarize the perfusion signal of each ROI, as in ref. 31. Speciﬁcally, the property of PCA to represent data as an ellipsoidal population in a lower dimensional space, while retaining most of its variance, was exploited on these feature vectors. As shown in Fig. 2B, each of these feature vectors
can be represented as a single point in a three-dimensional space. The voxels of each ROI with similar dynamic behavior would form almost elliptical clusters of points (ellipsoids) in this three-dimensional space.

It should be noted that while drawing these ROIs, (i) the voxels of both ROIs are always within the edema, (ii) not in proximity to the ventricles, (iii) representative of infiltration into white matter and not into gray matter, (iv) the distant ROI is at the farthest possible distance from the enhancing part of the tumor while still within edema, and (v) no vessels are involved within any of the defined ROIs, as denoted in the T1-CE volume.

**Measurement of heterogeneity**

The Bhattacharyya coefficient (35) is used as a measure of heterogeneity within the peritumoral edema [peritumoral heterogeneity index (PHI/\(\phi\)-index)], by measuring the separability...
between the summarized ellipsoids of these ROIs for each patient. The Bhattacharyya distance is considered the most reliable for the purpose of measuring the distance of a point observation from a distribution of observations along each principal axis, namely after conducting PCA (36).

Robustness analysis
Although calculation of the ϕ-index is mostly automated, it currently requires expert drawing of the immediate and distant peritumoral ROIs (Fig. 1). To test the robustness and reproducibility of the index with respect to this expert input, the intra- and interrater agreements were evaluated, using the intraclass correlation coefficient (ICC). Specifically, 40 patients of the combined cohort were randomly selected and new ROIs were defined by (i) the same operator but on a different instance (3 months later); (ii) another operator. The new set of ROIs was drawn in a much faster and less detailed manner, to test the reproducibility of PHI in a more typical clinical setting. The definition of the ROIs was conducted by a medical doctor (H. Akbari) and a computer scientist (S. Bakas) working in medical image analysis for 8 and 10 years, respectively. This implies that any operator with medical image understanding can conduct this analysis and retrieve PHI, as long as (s)he follows the aforementioned rules, without the background of a neuroradiologist being required.

Code availability
We developed the PHI-Estimator, to enable others easily conduct this analysis and retrieve our PHI. The code source and executable installer are available on: www.med.upenn.edu/shia/phiestimator.html.

Results
Brief description of the experiment
To assess the peritumoral heterogeneity, two ROIs were defined within the peritumoral edema: one ROI adjacent to the enhancing part of the tumor, typically depicted by high T1-Ce signal intensity, and the other at the farthest from the tumor but still within the edema (Fig. 1). The average size for the near and far ROIs (across all subjects) is 72.2 and 172.3 voxels, respectively, which can be considered sufficient in order to account for potentially noisy voxels included in the ROIs. A recently published method (31) utilizing PCA was used to summarize the perfusion temporal dynamics of each ROI into a group of few principal components capturing more than 95% of the signal’s variance. The Bhattacharyya coefficient (35) was then used to measure the separability between the summarized perfusion measurements of the two ROIs for each patient, and evaluated as a biomarker of \( \text{EGFRVI}\). We refer to this separability measurement as PHI/ϕ-index. Values of the ϕ-index close to 0 indicate similar perfusion dynamics between the two ROIs, consistent with deeply and aggressively infiltrating and vascularized tumors (37). Such values could also indicate normal phenotype throughout the edema, which is rather unlikely in these aggressively infiltrating tumors. Conversely, values of the ϕ-index close to 1 indicate substantially different perfusion characteristics between the two ROIs, consistent with a less migratory phenotype of more localized peritumoral infiltration and vascularization, in which tumor-like perfusion characteristics are relatively confined to the vicinity of the tumor.

PHI yields significantly distinct distributions for \( \text{EGFRVIII}^+ \) and \( \text{EGFRVIII}^- \) patients
The ϕ-index was initially estimated for a discovery cohort of 64 patients (22 \( \text{EGFRVIII}^+ \)) with de novo GBM and displayed significantly distinct distributions between \( \text{EGFRVIII}^+ \) and \( \text{EGFRVIII}^- \) patients (\( P = 1.5725 \times 10^{-7} \), AUC = 0.9459), with median ϕ values of 0.3097 and 0.0961 and interquartile range (IQR) of (0.1855–0.4808) and (0.0509–0.1095), respectively (Fig. 3A).

Subsequently, an independent replication cohort of 78 patients (20 \( \text{EGFRVIII}^+ \)) was analyzed in the same way, and the ϕ-index distributions for the \( \text{EGFRVIII}^+ \) and \( \text{EGFRVIII}^- \) tumors returned equivalently distinct results (\( P = 2.8164 \times 10^{-4} \), AUC = 0.8336), with median values of 0.2586 (IQR: 0.1659–0.3938) and 0.0952 (IQR: 0.0411–0.1348), respectively (Fig. 3B). The best threshold (accuracy = 0.9219, specificity = 0.9762, sensitivity = 0.8182) in the ϕ-index for the discovery cohort was 0.1372, which when applied to the replication cohort returned an accuracy of 0.8590 (specificity = 0.8793, sensitivity = 0.8) confirming its generalizability.

Furthermore, the two cohorts were combined into one larger cohort, of 142 patients (42 \( \text{EGFRVIII}^+ \)), and the distinctiveness of the distributions of the ϕ-index for \( \text{EGFRVIII}^+ \) and \( \text{EGFRVIII}^- \) tumors was even more significant (\( P = 4.0033 \times 10^{-10} \), AUC = 0.8869), with median values of 0.2806 (IQR: 0.1759–0.4088) and 0.0961 (IQR: 0.0505–0.1309), respectively. Comparison of the median values, as well as the first and the third quartiles, between the two distributions reveals the ability to distinguish between them based solely on the PHI (Fig. 3A). To statistically evaluate the significance of the results obtained for the combined cohort, a two-tailed paired \( t \)-test was used to compare between the two distributions (Fig. 3C). This statistical analysis returned a \( P \) value = 4.0033 \times 10^{-10}, which confirmed at the 5% significance level.
level that the patients in the pool of \textit{EGFRvIII} and \textit{EGFRvIII}², come from populations with unequal means, with the confidence interval (CI) on the difference of the means being (0.1526–0.2795). A receiver operating characteristic (ROC) analysis was also used in the combined cohort to illustrate the performance of PHI on an individual patient basis (Fig. 4L). The ROC curve was created by plotting the sensitivity against the false positive rate (i.e., 1 – specificity) at various thresholds of PHI. The threshold set on 0.1377 returned the best accuracy (88.73%), with sensitivity and specificity equal to 80.95% and 92%, respectively (AUC = 0.8869; standard error = 0.0351; 95% CI, 0.8180–0.9558).

To demonstrate that the results of perfusion heterogeneity between the two ROIs for each subject within the two \textit{EGFRvIII} groups were not confounded by a potentially larger extent of edema in one of the two groups, we assessed the distance between the two ROIs against the $j$-index for each patient (Supplementary Fig. S1) and noted that there is no correlation between them (correlation coefficient: 0.0519, P-value = 0.5394). Furthermore, by assessing the distribution of distances between the two ROIs for each \textit{EGFRvIII} group, we show that the extent of the edema between these two groups has no significant difference (P-value = 0.6728; Supplementary Fig. S2). This shows that the obtained results of PHI variation between \textit{EGFRvIII}² and \textit{EGFRvIII} tumors is a true finding and not an effect of different amount of edema observed between the two groups. A single slice of an example subjects of each \textit{EGFRvIII} group are shown in Supplementary Fig. S3, to show the similar extent of the edema.

Unbiased estimates of performance through nested cross-validation

A nested 10-fold cross-validation was also performed over the combined cohort using a model configuration of three sets: the...
training set, for deriving the predictive model; the validation set, for selecting the optimal threshold for the $\phi$-index; and the test set, for testing the generalization of predictions on new/unseen data, thereby avoiding optimistically biased estimates of performance. The cross-validated accuracy, sensitivity, and specificity were estimated equal to 89.92%, 83.77%, and 92.35%, respectively, and the optimal threshold of the $\phi$-index was found to be 0.1377 in consistency with the one found in the ROC analysis.

Repeatability and reproducibility of PHI
The median $\phi$ values for the intrarater subset were 0.2761 (IQR: 0.1572–0.4046) and 0.065 (IQR: 0.0389–0.1303) for EGFRvIII$^{-}$ and EGFRvIII$^{+}$ patients, respectively ($P = 2.8529 \times 10^{-5}$; AUC = 0.8846), whereas the median $\phi$ values for the interrater subset were 0.2273 (IQR: 0.1512–0.3426) and 0.1112 (IQR: 0.0579–0.1294; $P = 0.003$; AUC = 0.8242). The ICC was 0.825 among the same rater and 0.775 among different raters.

Discriminative value of other MRI modalities
Additional MRI modalities were assessed to investigate their discriminative ability, compared to that of the DSC-MRI. These comprised: native T1-weighted (T1), T1-CE, T2, T2-FLAIR, DTI (TR), DTI(FA), DTI(RAD), and DTI(AX). The number of patients was reduced to 140 due to data availability. It is observed that all additional modalities had notably poorer discrimination ability (Fig. 4A–H) and the distributions of PHI for each of them were not distinct between the different EGFRvIII genotypes. Furthermore, a support-vector-machine was used for a multivariate analysis of a complete joint/multifaceted model, where only the DTI(TR) ($P = 0.0053$) and T1 ($P = 0.0054$) were found to be significant (38), additive to the DSC. ROC analysis of these two joint models, namely DSC-TR and DSC-TR-T1 showed very small improvement over DSC-MRI alone (Fig. 4I–K), with AUC$_{DSC}$ = 0.8857, AUC$_{DSC-TR}$ = 0.8985, and AUC$_{DSC-TR-T1}$ = 0.9019.

Discussion
This study is the first to establish a robust, reproducible, noninvasive, and easy to evaluate imaging signature of EGFRvIII in de novo GBM, based on quantitative analysis of peritumoral regions and not on assessment of intratumoral regions that the current general knowledge and understanding of the EGFRvIII status in GBMs is currently based. The results demonstrate that assessment of the heterogeneity of perfusion temporal dynamics throughout the peritumoral edema on in vivo MRI data predicts the EGFRvIII mutation status, hence reveals an accurate (89.92%), sensitive (83.77%), and specific (92.35%) imaging biomarker of the mutation, which can be used clinically for personalized treatment decisions and response evaluations. Our findings support the strengths of an emerging approach that we have termed computational molecular imaging, which refers to molecular target determination by virtue of their distinct imaging phenotypic patterns without the need to deliver specialized molecular probes to the tissue. Importantly, results are obtained using commonly acquired clinical MRI scans as part of standard clinical practice, thereby increasing the likelihood of their translation to the clinic.

EGFRvIII identification in GBM patients using radiographic analysis alone holds significant clinical relevance in terms of personalized medicine. Traditional identification of genomic mutations, such as EGFRvIII by tissue-based techniques, requires invasive surgical resection or biopsy, and is obtained from a single tissue specimen, whereas we report a non-invasive, purely image-based approach for pre-operative evaluation of this molecular target. Because glioma cells bearing the mutant are not uniformly distributed throughout a tumor, sampling error may occur with tissue-based approaches. Conversely, imaging captures the tumor’s spatial heterogeneity more completely, minimizes bias potentially occurring by evaluating a limited portion of tumor, and can provide data on the regional EGFRvIII expression. Such global assessment of the mutant could be used as a more accurate guide to patient selection for clinical trials. Furthermore, once the mutation is identified, EGFRvIII-targeted therapies can be selected. In addition to selecting initial treatment, there may be significant value in detection of EGFRvIII at additional time-points following treatment initiation, as it has been shown that expression of the mutant may be lost at the time of progression (29) following standard chemoradiation in approximately half of patients (28). There is also a high probability (>80%) of losing EGFRvIII expression following EGFRvIII peptide vaccination (27). Consistent with this finding, antigen editing with quantitative loss of EGFRvIII is also observed, after infusion of genetically modified chimeric antigen receptor (CAR) T cells targeting EGFRvIII in recurrent GBM patients (21). By using standard clinical imaging sequences, a longitudinal evaluation of EGFRvIII in patients both after treatment and with recurrent tumors, represents a feasible approach to detect changes in EGFRvIII expression. Unlike repeated biopsies, such monitoring can be performed repeatedly without risk and with decreased cost over time. Thus, an imaging-based approach for EGFRvIII identification can aid in all phases of care of the GBM patient from diagnosis to targeted therapy to response surveillance.

Although most of the attention in characterizing tumors has been placed on the tumor bulk, the peritumoral edema, typically depicted by high T2-FLAIR signal intensity, holds much additional data. Despite the fact that more than 90% of recurrences occur in edema (39) due to the highly infiltrative nature of GBM, there is limited research focused on the assessment of this region and its microenvironment (2, 40). Edema results from infiltrating tumor cells and the biological response to the angiogenic and vascular permeability factors released by the spatially adjacent tumor cells (31). Although the peritumoral edema remains mostly unresolved and is generally not aggressively treated, by virtue of hosting the tumor’s “propagating font” it is critically important for diagnostic and therapeutic purposes.

Large GBM tumors typically outgrow their blood supply, which results in ischemia, secretion of angiogenic factors, such as vascular endothelial growth factor (VEGF), and cytokines that eventually lead to neovascularization, increased permeability, and edema (41, 42). These new vessels, when compared with the existing healthy blood vessels, have an increasingly tortuous and branched structure, as well as higher permeability, which typically affect the brain circulation. Such alterations in the brain circulation are captured by DSC-MRI, which is based on the decay of T2 signal during the first pass of a paramagnetic contrast medium through the capillary bed. Therefore, DSC-MRI enables the generation of a perfusion curve by assessing the dynamic changes in the signal intensity of the peritumoral region of a GBM through time. Analysis of the complete perfusion signal through PCA enables microvascular imaging and provides a visual correlation of blood flow, blood volume, and vessel permeability (31).

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Variations in the perfusion signal between the immediate and distant peritumoral ROIs relate to phenotypic characteristics conferred by the presence of EGFRvIII. Based on the \( \phi \)-index, we found that EGFRvIII+ tumors had very similar, and relatively normal immediate and distant peritumoral perfusion patterns, in contrast to the EGFRvIII– tumors (Fig. 2). This finding is consistent with relatively more locally infiltrating EGFRvIII+ tumors, accompanied by localized immediate peritumoral vascularization. Note that "more locally infiltrating" does not refer to a generally more infiltrative tumor. Conversely, deeply infiltrating and migrating EGFRvIII+ tumors displayed a more uniform peritumoral perfusion phenotype, consistent with less intense peritumoral vascularization facilitated by the migratory characteristics of EGFRvIII+ tumors that likely allows them to gain access to blood supply farther from the bulk of the tumor. Differences in the perfusion signal (Fig. 2) enabled us to derive an accurate, sensitive, and specific imaging biomarker based on DSC-MRI. Specifically, the distribution of the \( \phi \)-index values (Fig. 3C) across the EGFRvIII+ population has a much larger range of values (0.0340–0.8944) and IQR (0.1759–0.4088) when compared to the distribution across the EGFRvIII– patients (range: 0.0080–0.5039, IQR: 0.0505–1.1309). This discrepancy might reflect the underlying expression heterogeneity (2–5), which is prevalent in GBM, with the EGFRvIII+ patients potentially expressing the mutant form in areas that were not sampled for tissue analysis, and tumors that were found to be EGFRvIII+ being more likely to have developed the full phenotype of the mutant. It is well-documented that oncogenic EGFRvIII confers a more motile and invasive phenotype to neural stem cells (43) and GBM cells (37). Furthermore, the narrow range of the \( \phi \)-index distribution across the EGFRvIII+ patients suggests high specificity in terms of identifying a new EGFRvIII+ patient, which can be achieved without significant loss of sensitivity.

DSC-MRI alone was the focus of this study, even though an advanced imaging modality that is not always available. However, mounting evidence for the importance of this modality (18, 31, 40) has rapidly increased its adoption in standard clinical settings. Nevertheless, assessment of additional MRI modalities to investigate if a joint/multifaceted model of the peritumoral heterogeneity could lead to an improved biomarker of EGFRvIII showed that only DTI(TR) and T1 were significant, in addition to DSC-MRI (Fig. 4I–K). However, considering the improvement offered by including these modalities, we still think there is little value in adding other MRI modalities to DSC, unless those additional sequences are acquired anyway for other reasons. Notably, we found that the differences in PHI between the EGFRvIII+ and EGFRvIII– patients were minimal for DTI(TR) (Fig. 5). This would be consistent with similar cell density between the two defined ROIs and for both EGFRvIII genotypes. The actual difference between the EGFRvIII+ and the EGFRvIII– patients lays in the gradient of vascularization throughout the edema that looks to be almost identical for the EGFRvIII+ patients, as opposed to the EGFRvIII– patients, who show a larger drop in the perfusion of the immediate peritumoral ROI, i.e., more confined infiltration and vascularization (Figs. 2 and 6). This implies that the EGFRvIII+ patients might benefit from a slightly more extended resection and focused radiation, in order to include the immediate peritumoral edema.
Our study has several aspects that distinguish it from prior related studies (18, 40, 44–47), which were either demonstrating population-wide associations, thereby not focusing on establishing an individual-patient biomarker, or not validating their results in an independent replication cohort, which is critical for a clinically useful biomarker. Firstly, and most importantly, the results obtained in our study are based on individual-patient in vivo measures and show high accuracy in addition to providing pathophysiological insights, hence increasing the likelihood of the ϕ-index being clinically applicable. Secondly, instead of limiting the use of perfusion imaging in retrieving isolated hemodynamic features (e.g., leakage corrected relative cerebral blood volume; Supplementary Fig. S4), we use the complete perfusion signal via PCA, which allows for more comprehensive analysis as it encapsulates the complete hemodynamic information. Thirdly, instead of reporting only results on a discovery set, we use two independent cohorts for the purpose of identification (initial discovery set) of the proposed ϕ-index and confirmation (independent replication cohort) of its discriminatory generalizability in unseen data. These two cohorts could be noted as retrospective and prospective, since the images of the replication (i.e., prospective) cohort were obtained after the index was identified in the discovery (i.e., retrospective) cohort, and $\text{EGFR}^\text{vIII}$ status for the replication cohort was obtained after the ϕ-index was estimated for all its patients. Furthermore, we combined these two cohorts under a nested cross-validation scheme, to quantitatively validate the generalization performance of PHI and its threshold, whilst providing unbiased performance estimates. The advantage of cross-validation lies upon the observation that high accuracy score obtained for the training set, might have been obtained through “overfitting” to the training data. The accuracy score obtained for the training set is likely to be higher than the accuracy score obtained by applying the method to new examples, not seen in the training set. Thus, the reported cross-validated performance score and its corresponding ϕ-index threshold may be considered unbiased. Additionally, none of these previous studies investigate for the reproducibility of their findings, whereas in our case both inter- and intra-rater agreements are evaluated in almost one third of the included data.

This study evaluated the expression status of $\text{EGFR}^\text{vIII}$ alone, as a binary present/absent value, and did not account for other mutations or amplifications in EGFR that might alter the perfusion signal. However, the frequency of $\text{EGFR}^\text{vIII}$ was similar to the rates reported in the literature (11). It is known that EGFR amplification may increase between the initial diagnosis and recurrence (28), and that loss of $\text{EGFR}^\text{vIII}$ may be due to EGFR amplification and individual cells harboring varying levels of $\text{EGFR}^\text{vIII}$ (48) or even regulated by the tumor (49). Thus, it would be informative to include the EGFR amplification values in a future analysis, which could further explain the widespread of PHI values for the $\text{EGFR}^\text{vIII}$- tumors. We currently consider patients labeled as $\text{EGFR}^\text{vIII}$-, but with low ϕ-index values, as patients that may potentially express the mutant in areas that were not sampled for tissue analysis, resulting in inappropriate classification. Future prospective studies could be conducted for retrieving the mutant status on specific spatially distinct radiologically guided localized biopsies, as described in other studies (4, 50). Then, the proposed ϕ-index would be used for evaluating the mutant on these specific known locations, facilitating the creation of a parametric map of $\text{EGFR}^\text{vIII}$ expression. Last but not least, a larger cohort should be considered for analysis, consisting of patients scanned using different equipment, with the intention of validating the robustness of the proposed marker to acquisition differences.

The ability to noninvasively determine the status of $\text{EGFR}^\text{vIII}$ in GBM patients, only by assessing DSC-MRI scans, can assist in obtaining the mutant status faster and more easily. Application of PCA in the raw DSC-MRI signal reveals informative features that represent distinctive imaging phenotypes correlating to $\text{EGFR}^\text{vIII}$ in GBM. This $\text{EGFR}^\text{vIII}$ imaging signature is constructed in a manner that should be robust to MRI scanner variations, by virtue of evaluating within-patient heterogeneity measures, rather than relying on population-wide associations (45–47). The obtained cross-validated results demonstrate that discrimination of the $\text{EGFR}^\text{vIII}$ status, which is critical for personalized treatment decisions and response evaluation, can be achieved based solely on assessing the peritumoral heterogeneity on in vivo perfusion imaging data, whilst potentially obviating costly and not widely available tissue-based genetic testing. The cross-validation scheme over the available patient data provided unbiased performance estimates and quantitatively validated the generalization performance of the ϕ-index and its classification threshold. The proposed ϕ-index contributes to personalized medicine by allowing the identification of an important molecular target on an individual patient basis, using widely available clinical imaging protocols. These characteristics enable the identification of individual patients that could benefit from selective treatments in a more efficient and less invasive way than by current options, with the intention of improving patient prospects while minimizing the risk of side effects.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
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