

Retrospective immunohistochemical evaluation of high-grade glioma patients treated with bevacizumab and irinotecan, with or without cetuximab



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Background

- Recent data have shown that bevacizumab (B) and irinotecan (I) as well as cetuximab (C) combined with B and I induce significant responses in recurrent high-grade gliomas (HGG)
- HGG tumors are known to be hypoxic, which among other things stimulate transcription of Vascular Endothelial Growth Factor (VEGF)
- VEGF induces angiogenesis and HGG's are known to be highly vascularized with pronounced tumor vascularity significantly correlated with poor survival
- Tumor vessels are often malformed and occlusions are frequent, and as such intratumoral hypoxic areas will remain. Moreover, tumor vessels are leaky, leading to tumor edema and increased intratumoral pressure, which further increases hypoxia
- Hypoxia leads to, among others, stabilization of the HIF-1 α and HIF-2 α subunits that initiate transcription of pro-angiogenic factors such as the VEGF
- In a recent study by Sathornsumetee *et al.* hypoxia, as measured by expression of the hypoxia inducible transmembrane enzyme, carbonic anhydrase 9 (CA9), was associated with poor survival outcome

Aim

Retrospective immunohistochemical evaluation of biomarkers related to angiogenesis and hypoxia in HGG pts treated with BI and CBI

Methods

- Tumour material**
 - CBI group (N=43)**
 - Recurrent primary Glioblastoma Multiforme (GBM) patients with progression <8 months from standard treatment with radiotherapy and concomitant temozolomide followed by adjuvant temozolomide
 - Treatment: cetuximab, bevacizumab and irinotecan
 - BI group (N=42)**
 - Recurrent HGG pts with progression after receiving standard primary treatment including surgical interventions and usually at least two chemotherapy regimens
 - Treatment: bevacizumab and irinotecan
- Immunohistochemistry**
 - Surgical specimens obtained prior to treatment with either BI or CBI were formalin-fixed and paraffin-embedded
 - Evaluation of the slides was performed independently and under blind conditions by J.E. and B.H.
 - Antibodies:**
 - HIF1: Mouse monoclonal anti-HIF1 α (clone 54) (1:250 dilution) BD Biosciences, NJ, USA
 - HIF2: Mouse monoclonal anti-HIF-2 α (ep190b) (1:300 dilution) Novus Biological, CO, USA
 - VEGF: Mouse monoclonal anti-VEGF (C-1) (1:400 dilution) Santa Cruz Biotechnology, Inc, CA, USA
 - CD34: Mouse monoclonal anti-CD34 (NCL-L-END) (1:200 dilution) Novo Castra, UK
 - CA9: Mouse monoclonal anti-CA9 (M75) (1:200 dilution) from S. Pastorekova, Slovakia (Pastorekova *et al* 1992)
 - GLUT1: Mouse monoclonal anti-GLUT1 (SPM498) (1:200 dilution) Abcam, MA, USA
 - All Sections were counterstained with Mayer's hematoxylin.
- Scoring of immunohistochemistry:**
 - CD34 positive vessels were counted in 3 hotspots at 400x magnification in tumor tissue. The mean value were used in the analysis
 - The remaining slides were scored semiquantitative on a scale from 0 – 100% of positive staining cells in non-necrotic tumour tissue

Statistics:

- Statistical analyses were performed using SPSS (SPSS, IL, USA). Survival was determined from the time of treatment initiation, until time of death or last follow-up. Kaplan-Meier curves were used to graphically describe progression free survival between Complete Response(CR)/Partial Response (PR) and Stable Disease(SD)/Progressive Disease(PD). Log-rank tests were used to compare survival between these subgroups.

Cluster analysis:

- In an attempt to identify molecular markers, which could assist the prediction of response to therapy, cluster analysis was performed using SPSS implementation (TwoStep function) of the balanced iterative reducing and clustering using hierarchies method. Cluster analysis is an unsupervised classification method for data mining, directed toward identifying tumours with a similar molecular "fingerprint". First after the clusters have been identified, they are compared in terms of outcome (response and/or survival). Because the material in this study is limited, the results from cluster analysis should be read with caution but the method could be an possible approach in the future in the search for tumour profiles based on molecular markers.

Conclusions

- The presence of a molecular signature in HGG which could be predictive for OS and response to anti-angiogenic treatment, might be found among angiogenic and/or hypoxic markers using Cluster analysis
- However, due to the limited patient material, these biomarker results need further statistic evaluation and the presented results should only be considered as indicative

Results

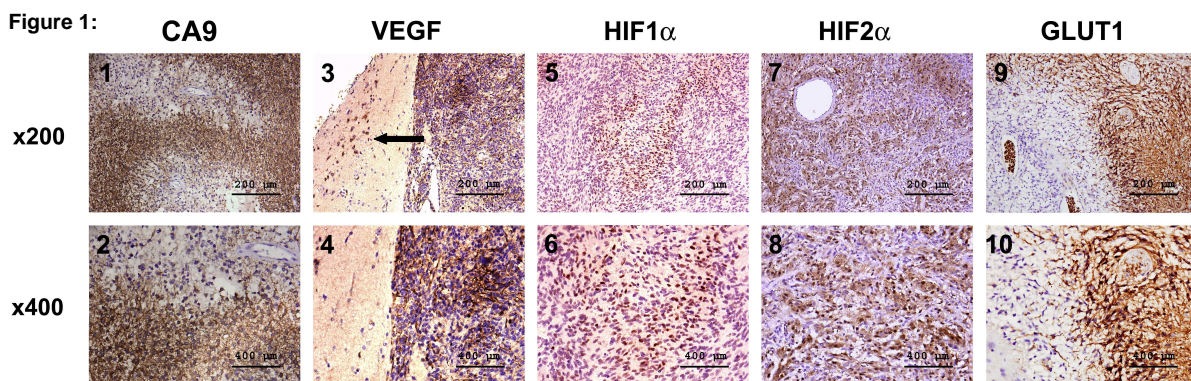


Figure 1: Representative immunostaining detection of angiogenic/hypoxic markers. (1 and 2) Carbonic anhydrase 9 (CAIX) on tumour cell membrane and cytoplasm, adjacent to necrotic area. (3 and 4) Vascular endothelial growth factor (VEGF) in tumour cytoplasm and stroma. Notice that there is no positive staining of normal brain tissue (black arrow). (5 and 6) HIF1 α in tumour cell nuclei. (7 and 8) HIF2 α in nuclei and cytoplasm. (9 and 10) GLUT1 in cytoplasm and endothelial. The images are shown in x200 magnification (1,3,5,7,9) and x400 magnification (2,4,6,8,10). These 5 molecular markers showed a tendency towards clustering, when tested by TwoStep Clustering methods in SPSS, as shown in Figure 3.

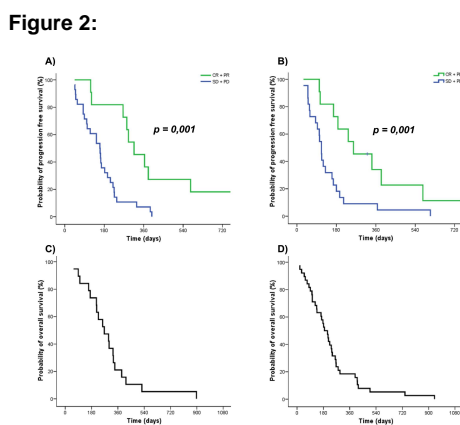


Figure 2: Kaplan Meier estimate showing Time To Progression (TTP) for evaluable pts treated with: A) Bevacizumab and Irinotecan (BI) (N=37) or B) Cetuximab, Bevacizumab and Irinotecan (CBI) (N=32). Green curve showing TTP for the total CR and PR pts whereas the blue curve shows TTP for the total SD and PD pts. The Overall Survival is illustrated for GBM patients receiving BI (C) (N=24) or CBI (D) (N=43) by Kaplan-Meier estimate. GBM pts receiving BI and CBI shows similar response to treatment.

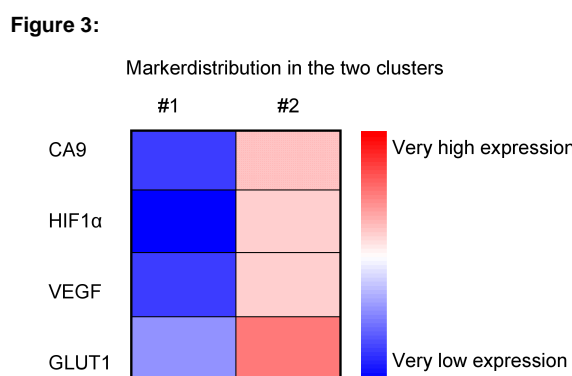


Figure 3: Molecular marker expression profiles in 49 GBM pts receiving BI or CBI treatment with available tumour tissue. Results from the reduced balanced iterative reducing and clustering using hierarchies cluster models. The expression scale values from the different markers were standardized and profiles normalized to the overall average of the clusters. The following molecular markers were tested: CAIX, HIF1 α , HIF2 α , VEGF, GLUT1 and CD34. Blues corresponds to a value <overall average, white corresponds to average value, and red corresponds to a value >average. Chi-square tests, with Bonferroni adjustment, were used to assess significant cluster formation. The two clusters found (32 pts in cluster 1, 17 pts in cluster 2) by this method were used in Figure 4. Due to the limited pts in this material (N=49), caution must be taken when interpreting these results using the cluster method.

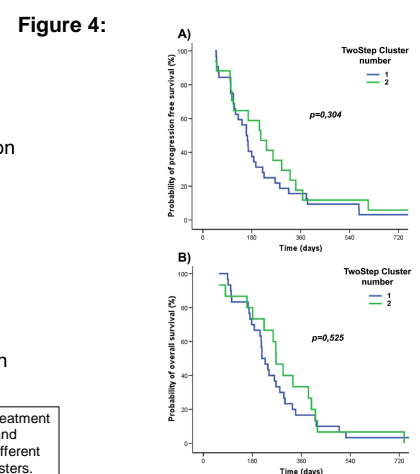


Figure 4: Kaplan Meier plot showing A) PFS and B) OS factored by molecular marker expression profile: cluster 1 (blue), cluster 2 (green). Evaluable GBM pts in receiving BI or CBI are included (N=49). Log rank statistics and the level of significance is shown ($p=0,304$ and $p=0,525$ respectively)