BioGENAI Supplementary Material: NRP2 Validation

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Glioblastoma Resistance to Immunotherapy

Angiogenic processes are regulated and initiated through multiple proteins, particularly neuropilin ligands: vascular endothelial growth factor (VEGF) proteins [1] and semaphorins [2]. These proteins are enhanced by the receptor neuropilin 2 (NRP2). NRP2 acts as a primary receptor for semaphorin-based angiogenesis signaling and a coreceptor for VEGF, allowing it to bind to other pro-tumor proteins. Hence, the effects of NRP2 were further investigated as the inhibition of the protein could potentially downregulate angiogenesis and immunosuppressive behaviors of GBM, allowing targeted therapies to take effect such as PD-L1 inhibition therapy.

To identify GBM-advancing biomarkers, an unsupervised deep learning model was utilized to identify genes correlated to GBM resistance to viral-immunotherapy. Resistance to viral immunotherapy, PVSRIPO, was hypothesized to indicate an immunosuppressive tumor microenvironment (TME). Furthermore, tumor angiogenesis may be correlated with immunosuppression [3]. Angiogenesis involves the promotion of recruitment and proliferation of immunosuppressive cells such as Treg cells, MDSCs, and M2-TAMs, creating a immunosuppressive TME.

Analysis of mRNA sequencing with Deep Learning

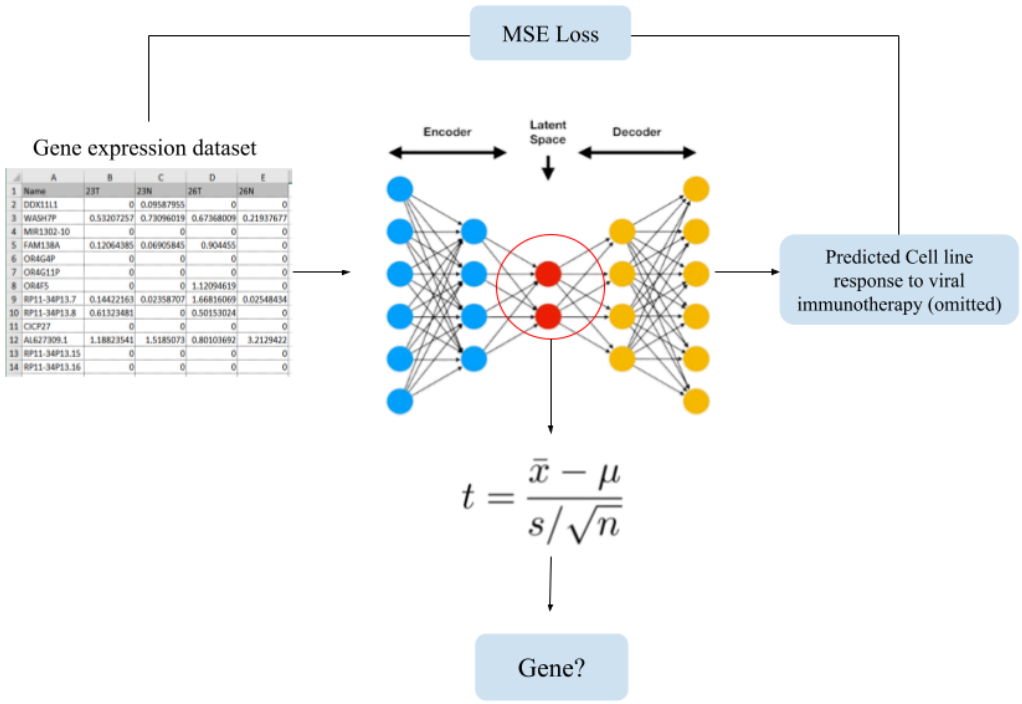
Data was acquired from a study conducted by Thompson et al. through the gene expression omnibus (GEO) [4]. Data consisted of mRNA sequencing of GBM cell lines through the stages of being treated with viral immunotherapies, G207 and PVSRIPO [4]. This included 6 total cell lines. Following experimentation, based on LD50 values, cell lines were either classified as resistant or responsive to the treatment. Finally, the data was labeled as resistant or responsive according to its LD50 results after treatment .

Neural Network Architecture and Training

An unsupervised autoencoder neural network was implemented to capture the genes with highest influence in GBM resistance to viral immunotherapy. The model compresses the data into a latent representation and then learns to reconstruct the gene expressions based on its label as resistant or responsive. In doing so, the model learns extractable patterns and features (gene expressions) that correlate to a certain class. *Fig. 2* depicts the approach to identify significant genes using an autoencoder neural network.

Figure 2

Depicts process of analyzing gene expression data using unsupervised learning



The autoencoder architecture consisted of 6 fully connected layers; 3 encoder, and 3 decoder layers. Additionally, dropout with a rate of 0.2 as well as ReLU activation were used after each layer. Data

was z-score normalized and input to the autoencoder model. The model was trained on Google Colab's A100 GPU for 100 epochs].

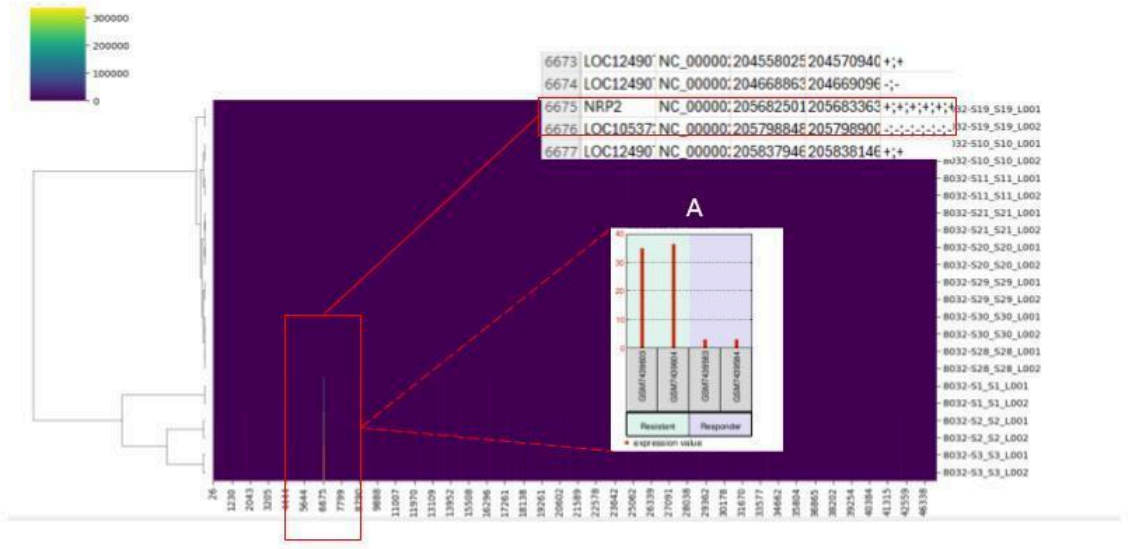
Training and validation loss were monitored for overfitting which did not occur. Following this, the model was saved, including the encoder saved separately. Next, the entirety of the gene expression dataset was passed into the encoder portion of the model. A t-test with a rigorous variance threshold of 0.001 was implemented on the encoder output latent space and plotted using a heatmap, highlighting genes with significance to the specified class of resistance.

Results I

Shown in *Fig. 3,* when the results of the t-test were plotted onto a heatmap, the gene ID 6675 was revealed to be highly prevalent in resistant samples. This gene corresponded to neuropilin 2 (NRP2), coding for the NRP2 protein. Since the model captured patterns which correlated to resistance, potentially not gene expression, the expression of NRP2 was investigated in resistant samples in comparison to responsive cell lines. Depicted in *Fig. 3,* the bar chart shown next to the letter A is the expression of NRP2 in resistant cell line D283 (left) versus responsive cell line D341 (right). Two bars are depicted for each cell line as according to the procedures described in the article accompanying the data, expression values were read in lanes, therefore compelling the need to analyze both lanes for each cell line. As shown, NRP2 is approximately 7 times as expressed in the resistant cell line D283. This was also the case for the other cell lines tested. Therefore, it was clear that NRP2 is expressed much more in GBM cells resistant to viral immunotherapies.

Figure 3

Output from t-test heatmap analysis. According to the corresponding chart, a highly influential gene is NRP2. This is reflected in raw gene analysis data shown by the chart.



Rigorous analysis and literature review of NRP2 was then conducted to determine the meaning of high expression in resistant GBM cells. According to Klagsbrun et al., NRP2 has great significance in tumor angiogenesis [5]. NRP2 acts as a coreceptor for most VEGF isoforms, extensively enhancing its signal. VEGF plays a keystone role in the regulation of tumor angiogenesis, allowing for it to take place altogether. Therefore, the inhibition of NRP2 to act as an amplifier for VEGF theoretically will reduce tumor angiogenesis. This connects to the results of the machine learning experiment previously described before, as according to Voron et al., tumor angiogenic development plays a crucial role in tumor immunosuppression [3]. Additionally, tumor hypoxia, common in fast-growing tumors like GBM, leads to accelerated angiogenesis. Hypoxic stress, caused by lack of oxygen in tumoral tissues, requires excess vasculature, regulated by angiogenesis caused by VEGF. Furthermore, VEGF is responsible for the migration and proliferation of endothelial cells (EC) which line newly developed blood vessels. ECs modulate many varieties of immune cells such as T-cells, causing immune cells attempting to destroy

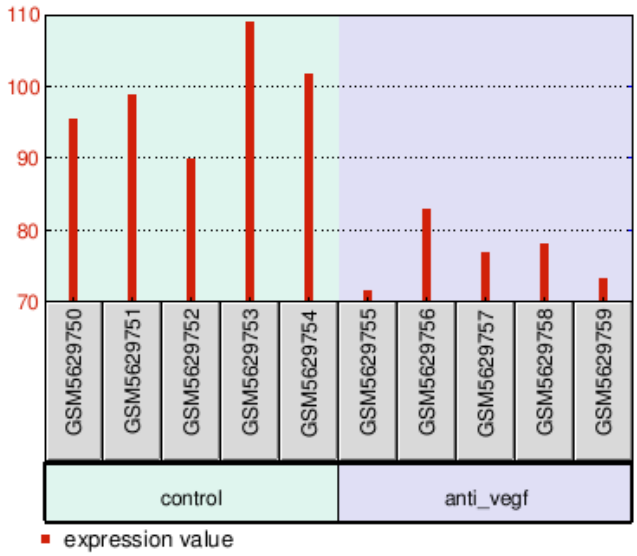
cancer cells to be repelled. Therefore, VEGF regulation does indeed play a role in immunosuppression due to regulation of ECs.

Moreover, the effects of NRP2 inhibition were studied in knockout mice by Takashima et al [9]. In transgenic mice, NRP2 knocked out, regular body angiogenesis was severely impaired, characterized by the lack of vasculature development. Thus, similarly in tumors, NRP2 is instrumental in angiogenesis, leading to the formation of new blood vessels and vascular pathways necessary for tumor survival.

Furthermore, resistance to many treatments has been associated with VEGFC/NRP2 signaling [6, 7]. This signaling pathway mediates tumor autophagy, a process that enhances cancer cell survival to many therapies such as chemotherapy and immunotherapy. This is due to VEGFC/NRP2 autocrine functions, which initiate tumor autophagy. Moreover, the correlation between NRP2 and VEGF expression becomes further apparent in a study by Chrzanowska et al., investigating the immunosuppressive behaviors of VEGF [8]. In their study, a proposed protein, Rap1B, was hypothesized to correlate to VEGF signaling for increased proliferation of endothelial cells. Inhibiting the protein in mice models down regulated VEGF behavior, lowering its expression and overall EC presence. This caused CD8+ T-cells to invade the tumor, allowing for a large-scale anti-tumor immune response. Therefore, VEGF-induced EC proliferation resulted in the body, reflecting its behaviors enhancing immunosuppression inside the TME. Investigating this study further on the GEO, mRNA gene expression was analyzed. Shown in *Fig. 4* are the expression results for NRP2 in regular mice tumor cells (left) and tumor cells with the inhibition of Rap1B (right). Clearly, NRP2 is considerably less expressed in cells with compromised VEGF signaling. Therefore, it was concluded that NRP2 contributes to VEGF immunosuppressive behaviors, downregulating once VEGF was repressed.

Figure 4

Profile graph of NRP2 in regular mice tumor samples ("control") and Rap1B inhibited cells ("anti_vegf")



1. Klagsbrun M, Takashima S, Mamluk R. The Role of Neuropilin in Vascular and Tumor Biology. In: Madame Curie Bioscience Database [Internet]. Austin (TX): Landes Bioscience; 2000-2013. Available from: https://www.ncbi.nlm.nih.gov/books/NBK5991/
2. Folkman J. Tumor angiogenesis. Adv Cancer Res. 1985;43:175-203. doi: 10.1016/s0065-230x(08)60946-x. PMID: 2581424.
3. Rahma OE, Hodi FS. The Intersection between Tumor Angiogenesis and Immune Suppression. Clin Cancer Res. 2019 Sep 15;25(18):5449-5457. doi: 10.1158/1078-0432.CCR-18-1543. Epub 2019 Apr 3. PMID: 30944124.
4. Thompson EM, Kang KD, Stevenson K, Zhang H, Gromeier M, Ashley D, Brown M, Friedman GK. Elucidating cellular response to treatment with viral immunotherapies in pediatric high-grade glioma and medulloblastoma. Transl Oncol. 2024 Feb;40:101875. doi: 10.1016/j.tranon.2024.101875. Epub 2024 Jan 5. PMID: 38183802; PMCID: PMC10809117.

5. Zetter BR. Angiogenesis and tumor metastasis. Annu Rev Med. 1998;49:407-24. doi: 10.1146/annurev.med.49.1.407. PMID: 9509272.
6. Islam R, Mishra J, Bodas S, Bhattacharya S, Batra SK, Dutta S, Datta K. Role of Neuropilin-2-mediated signaling axis in cancer progression and therapy resistance. Cancer Metastasis Rev. 2022 Sep;41(3):771-787. doi: 10.1007/s10555-022-10048-0. Epub 2022 Jul 1. PMID: 35776228; PMCID: PMC9247951.
7. Duffy AM, Bouchier-Hayes DJ, Harmey JH. Vascular Endothelial Growth Factor (VEGF) and Its Role in Non-Endothelial Cells: Autocrine Signalling by VEGF. In: Madame Curie Bioscience Database [Internet]. Austin (TX): Landes Bioscience; 2000-2013. Available from: https://www.ncbi.nlm.nih.gov/books/NBK6482/
8. Sharma GP, Kosuru R, Lakshmikanthan S, Zheng S, Chen Y, Burns R, Xin G, Cui W, Chrzanowska M. Endothelial Rap1B mediates T-cell exclusion to promote tumor growth: a novel mechanism underlying vascular immunosuppression. Angiogenesis. 2023 May;26(2):265-278. doi: 10.1007/s10456-022-09862-5. Epub 2022 Nov 20. PMID: 36403190.