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HOX Gene Signatures Predict Survival Outcomes of Glioblastoma Patients

Dilbar Salman[1] and Yasin Mamatjan[1]

[1] Faculty of Science, Thompson Rivers University, 835 University Dr, Kamloops, V2C 0C8, BC, Canada

Abstract— **Diffuse gliomas represent over 80% of malignant brain tumors ranging from low-grade to aggressive high-grade lesions. Molecular characterization of gliomas has led to the development of a more accurate World Health Organization (WHO) classification system comprising specific glioma subtypes. The adult-type diffuse glioma was classified into three categories: astrocytomas (IDH-mutated), oligodendrogliomas (IDH-mutated and 1p/19q-deleted), and glioblastomas (IDH-wildtype (IDH-wt)) in 2021. The HOX (homologous box) gene is known as the leading gene of cell differentiation and vertebrate growth. HOX genes display important roles by regulating several hallmarks of cancer. HOX genes are virtually absent in healthy adult brains when they are detected in malignant brain tumors, namely gliomas. There is a need for new molecular biomarkers that can accurately predict patient outcomes. Our goal is to characterize glioblastoma and identify the HOX gene signatures of the outcome to understand which HOX-gene biomarkers predict bad survival in glioblastoma, IDH-wt. We used 237 and 310 IDH-wt glioblastoma gene expression (RNAseq) from The Cancer Genome Atlas (TCGA) and Chinese Glioma Genome Atlas (CGGA) respectively. To identify prognostic HOX genes, supervised analysis and COX analysis on grade 3 vs 2 and grade 4 vs 3 were performed separately, then find out the common significant HOX genes between supervised analysis and COX analysis. The selected 8-HOX genes (*HOXA2, HOXA3, HOXC11, HOXC6, HOXC9, HOXD11, HOXD12, HOXD4*) were used for gene expression and survival analysis. Notably, 8-HOX gene signatures in the TCGA dataset split the glioblastoma cohort into two prognostic groups that strongly predict the survival probability of glioblastoma patients ($p < 0.01$). Furthermore, the identified 8 HOX-gene signatures associated with patient survival was validated by using with an independent CGGA dataset ($p < 0.01$). Thus, glioblastoma can further be stratified into clinically relevant categories based on HOX gene expression which shows the importance of HOX genes in the outcome of IDHwt gliomas to identify relevant molecular subtypes for practical tumor classification.**

Keywords— **HOX gene, Glioblastoma, mRNA, biomarker, The Cancer Genome Atlas project (TCGA)**

I. Introduction

Diffuse gliomas can be divided based on the presence or absence of mutation in IDH genes into IDH mutant (IDH-mut) and IDH wildtype (IDH-wt) tumors. IDH mutations often occur in younger patients and the presence of an IDH mutation confers a distinct survival advantage compared to similar IDH-wt tumors [1]. Glioblastoma is widely recognized as the prevalent neoplasm of the central nervous system, constituting roughly 57% of all gliomas and 48% of all primary malignant central nervous system tumors [15]. This subtype is most commonly associated with clinically defined primary or de novo glioblastoma, and it is more prevalent among patients aged 55 years and above [2].

Importantly, the Consortium to Inform Molecular and Practical Approaches to Central Nervous System Tumor Taxonomy (cIMPACT-NOW) update 3 proposes that gliomas with histologic grade 2 and 3, lacking isocitrate dehydrogenase (IDH) mutation and exhibiting epidermal growth factor receptor (EGFR) amplification, whole chromosome 7 gain / whole chromosome 10 loss, or TERT promoter (pTERT) mutations should be classified as glioblastomas, IDH-wt, which are categorized as grade IV by the WHO in 2021. However, some researchers assessed the usefulness of molecular classification based on pTERT status and copy-number alterations (CNAs) in Lower-Grade Gliomas (LGGs, grade 2 and 3) lacking IDH mutation. They believe that the determination of the TERT promoter mutation status is both essential and adequate for the diagnosis of IDH-wt diffuse astrocytic glioma characterized by the molecular features typically observed in glioblastoma [3]. Drivers of IDH-wt tumors are not well understood and molecular changes in IDH-wt tumors render them more resistant to cytotoxic agents [4].

HOX gene expression is associated with poor survival in glioblastoma patients and predicts resistance to temozolomide therapy [5]. Altered HOX gene expression and methylation in glioblastoma are linked to increased resistance to chemotherapy and sustained proliferation of glioma-initiating cells [6, 7]. Despite these discoveries, there remains a high variability in the survival outcome of glioblastoma that is unexplainable by currently known molecular markers for glioblastoma. In this study, we aim to identify mRNA signatures of outcome in glioblastoma focusing on HOX genes using The Cancer Genome Atlas project (TCGA) glioblastoma datasets, and further validate the clinically and biologically relevant subgroups within glioblastoma using Chinese Glioma Genome Atlas (CGGA) glioblastoma datasets.

II. Methodology

Overview: In this study, we aimed to identify HOX gene signatures of outcome in IDH-wt gliomas (glioblastoma) in the TCGA dataset and validate it with an independent cohort from CGGA. The identification of HOX gene patterns correlated with clinical survival and mutational analysis, and we initially identified the HOX gene markers correlated with clinical outcome (survival data) in glioblastoma from the TCGA cohort by COX model-based analysis of prognostic HOX genes that predict worse survival in glioblastoma and further performed supervised analysis between grades 2, 3, and 4 tumors.

Datasets: We used 237 glioblastoma gene expression (RNAseq) datasets from TCGA to select significant HOX genes and downloaded 310 glioblastoma gene expression (RNAseq) from CGGA to perform independent validation. Regarding the glioma classification study, many researchers mentioned that TCGA and CGGA are the main sources of molecular information about gliomas, including clinically annotated transcriptomic and genomic profiles. Although TCGA itself has played a pivotal role in developing the WHO CNS5 classification, its proprietary databases still retain outdated diagnoses, which frequently appear incorrect and misleading according to the WHO CNS5 standards [12, 13]. The prognostic difference did not reach statistical significance within the TCGA dataset. This lack of significance may stem from the abundance of censored data within the TCGA or potential racial influences [13]. To address this issue, we validated our results with an independent glioblastoma cohort from CGGA. Thus, this HOX gene cluster study will provide accurate and reliable results about HOX gene signatures in glioblastoma.

Supervised analysis: For supervised RNAseq data analysis, we used a limma-based modeling approach (Bioconductor) and compared different grades based on one versus the other (grades 4 versus 2, grades 3 vs 2, and grades 4 vs 3). Fold change $(FC) > 2$ is considered to be significant. Then, we reported the adjusted p-value (p.Adj) by selecting $p.Adj < 0.05$ for the final gene signature selection.

COX regression analysis: The COX regression analysis was performed on the TCGA glioblastoma data and assessed associations between HOX gene expression and survival using univariate COX regression. We ranked a list of HOX genes that showed a significant survival difference. Significant Hazard Ratio (HR) means that genes were linked to differences in survival. For HOX genes with $HR > 1$, high expression or up-regulation is associated with short survival, while for genes with $HR < 1$, high expression or up-regulation is associated with long survival. By combining these two approaches (COX model and supervised analysis), we identified prognostic HOX gene biomarkers that can predict glioblastoma patient survival.

Approaches to selecting HOX gene signatures: We identified differentially expressed HOX genes between the high grade compared to the low-grade group (grades 4 vs 2, and grades 3 vs 2). Differentially expressed genes in grades 3 vs 2 showed 32/40 significantly enriched HOX genes upregulated (overexpressed) in grade 3. Interestingly, grades 4 vs 2 showed similar patterns of increased expression of HOX genes (26/40) ($p.Adj < 0.01$, FC = 2) that all these 26 significant HOX genes fully overlap with the significant HOX genes for grades 3 vs 2. This adds much novelty to the approach to the field of molecular pathology since these groups of co-regulated HOX gene expression have similar profiles with positive correlations. They may be functionally related, as overexpression of these HOX genes is associated with poor survival. The only differences came from HOXB clusters: (*HOXB3, HOXB4, HOXB5, HOXB6, HOXB9*) and *HOXC8*, which will guarantee further investigation. Furthermore, we performed COX analysis to select significant HOX genes from 26 HOX genes to predict survival in the TCGA glioblastoma cohort. We selected 8 HOX genes based on COX analysis ($p < 0.01$, $FC > 1.1$). These 8 HOX gene signatures include *HOXA2, HOXA3, HOXC11, HOXC6, HOXC9, HOXD11, HOXD12, HOXD4* that are located at the chr7, chr2 and chr12.

III. Results

To show the survival probability of three types of glioma, we applied Kaplan-Meier diagram using 168 Mutant-codel (IDH mutant 1p/19q co-deletion corresponds to Oligodendroglioma), 258 Mutant-non-codel (IDH mutant 1p/19q non-co-deletion corresponds to Astrocytoma), and 237 IDH wild type (Corresponds to Glioblastoma) patients information from TCGA (Fig. 1A). As shown in Fig. 1A, the IDH-wt patients have the shortest survival time of around one to two years, the Mutant-non-codel patients have a median survival time of approximately five to six years, and Mutant-codel patients have the longest survival time of about 10 years after diagnosis. Furthermore, to compare the HOX gene expression in those three types of gliomas the Boxplot analysis was performed (Fig. 1C). Interestingly, the better survival tumor type corresponds to lower HOX gene expression (Mutant-codel) while the worse survival tumor type has high HOX gene expression (IDH-wt) ($P < 0.0001$) (Fig. 1A, 1C). Normal brain tissue has lowest expression values of HOX genes compared to all subtypes of gliomas (Fig. 1C).

Furthermore, glioblastoma, IDH-wt includes grades 2, 3, and 4 gliomas according to the WHO classification for CNS

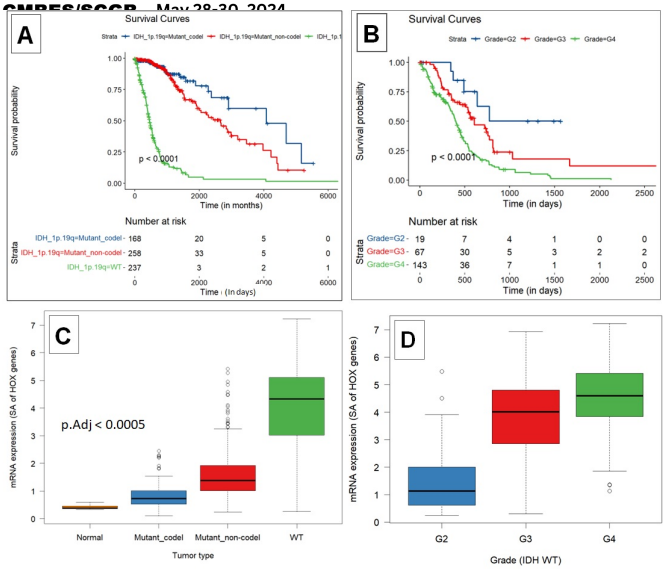

Fig. 1: HOX gene expression in glioma patients based on tumor types and Glioblastoma grades. (A) Kaplan-Meier diagram for overall survival of Mutant-codel (IDH mutant 1p19q co-deletion corresponds to Oligodendroglioma), Mutant-non-codel (IDH mutant 1p19q non-co-deletion corresponds to Astrocytoma), and IDH wild type (corresponds to glioblastoma) brain tumor patients ($p < 0.0001$). (B) Kaplan-Meier diagram showing patient survival probability for grades 2, 3, and 4 of glioblastoma ($p < 0.0001$). (C) Boxplot showing the HOX genes mRNA expression in Normal brain tissue (Orange), IDH Mutant-codel (Blue), IDH Mutant-non-codel (Red), and IDH-wt (Green) samples. HOX gene values were calculated based on the Signed Average of 40 HOX genes ($p.Adj < 0.0005$). (D) Boxplot representing the corresponding mRNA expression of HOX genes in grades 2, 3, and 4 of glioblastoma (IDH-wt) patients.

tumors in 2021 [14]. Here, we have checked the survival rates of those three grades of glioblastoma. The Kaplan-Meier diagram result shows that grades 2, 3, and 4 have long to short survival times (Fig. 1B) and it corresponds to the low to high HOX gene expressions in grades 2, 3, and 4 of glioblastoma (Fig. 1D).

The significant HOX gene signatures *HOXA2, HOXA3, HOXC11, HOXC6, HOXC9, HOXD11, HOXD12, HOXD4* were identified by supervised analysis and COX analysis using 237 glioblastoma gene expression (RNAseq) from TCGA datasets. We explored 8-HOX genes to see if they are prognostic biomarkers in glioblastoma, IDH-wt. The results showed that the TCGA glioblastoma cohort was stratified by the HOX gene where overexpression showed worse survival while separating the 8-HOX genes into two groups (Fig. 2A) ($p = 0.0016$) and three groups (Fig. 2B) by high and low expression ($p = 0.0067$).

Furthermore, an independent validation with CGGA was performed. Given the robustness of the 8-HOX gene signature in determining glioblastoma prognosis, we applied our 8-HOX gene signature to the CGGA glioblastoma dataset for survival stratification. To examine this, we used a total of 310 glioblastoma-based mRNA datasets. Similar to our findings using the CGGA dataset we show that in glioblastoma HOX gene overexpression predicts worse survival differences while significantly separating CGGA glioblastoma cohort into two groups ($p < 0.0001$) by high and low expression (Fig. 2C) and three groups (Fig. 2D) by high, medium, and low expression of the 8-HOX gene expression ($p < 0.0001$).

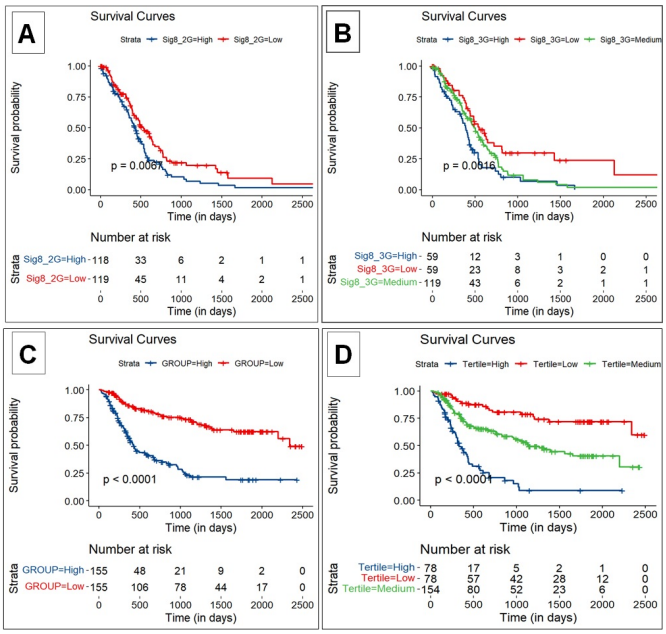

Fig. 2: 8 HOX gene signatures selected based on supervised analysis and COX analysis using TCGA glioblastoma datasets. We used glioblastoma gene expression (RNAseq) from TCGA and CGGA datasets. (A) Kaplan-Meier diagram for overall survival of significant HOX genes highly expressed group and lower expressed group of glioblastoma patients. $p = 0.0067$. (B) Kaplan-Meier diagram for overall survival of glioblastoma patients when the 8-HOX genes have high, medium, and low expression. ($p = 0.0016$). (C) Kaplan-Meier diagram for overall survival of significant 8-HOX genes highly expressed and lower expressed glioblastoma patients. Validated results by using CGGA show ($p < 0.0001$). (D) Kaplan-Meier diagram for overall survival of glioblastoma patients when significant 8-HOX genes have high, medium, and low expression. Validated results by using CGGA showed ($p < 0.0001$).

IV. Discussion and Conclusion

In this study, we characterized a HOX-gene signature of outcome in glioblastoma and performed molecular analysis to identify the mRNA signature of outcome, especially to understand which are HOX-gene biomarkers that predict bad survival in glioblastoma. 40 HOX-gene analysis from the TCGA dataset resulted in 8-HOX gene signatures: (*HOXA2, HOXA3, HOXC11, HOXC6, HOXC9, HOXD11, HOXD12,*

HOXD4) correlating increased gene expression with poor patient outcome seen in IDH-wt tumors further demonstrate the importance of this HOX-gene signature as a biomarker to predict patient outcome in glioblastoma. In addition, we evaluated our 8-HOX gene signature using the independent glioblastoma datasets from CGGA. The glioblastoma cohort was stratified by HOX gene expression level from low to high with 8-HOX gene signatures showing significant survival differences for gene expression analysis. Pathways that have been considerably stimulated in more mature oligodendrogliomas were found to be associated with developmental transcription factors [11]. The excessive expression of *HOXD12* was correlated with both the age and survival rate of patients, whereas the excessive methylation of *HOXD12* was linked to age, tumor grade, and survival in both the TCGA and CGGA datasets [10]. We also saw a pattern with *HOXD12* where *HOXD12* expression highly significantly correlates with poor patient survival in our study.

The differentiation between IDH-mut and IDH-wt gliomas has also become more distinct, as the term "secondary" or IDH-mut glioblastoma has been omitted in favor of the designation diffuse IDH-mut astrocytoma, with a WHO grade of 4. Expression of HOX genes offers significant prognostic insights in IDH-mut gliomas that are not encompassed by existing molecular diagnostics. In this article, we are providing initial results with 40 HOX gene analyzing based on the WHO classification of CNS tumors in 2021, Overall, HOX genes are more highly expressed in IDH-wt glioma than IDH-mut glioma, since IDH-wt gliomas are clinically more aggressive than IDH-mut tumors and high expression HOX genes correspond to worse survival of glioblastoma patients. The patient survival outcome related seven-HOX gene demonstrated considerable disparities in survival rates for both 1p/19q deleted and non-deleted IDH-mut gliomas [8]. Although IDH-wt glioma of grades 2 and 3 have clinical and molecular characteristics similar to primary glioblastoma of grade 4, our HOX gene expression analyses indicate the different expression of HOX genes among different grades 2, 3, and 4 of glioblastoma.

Numerous centers currently choose targeted next-generation sequencing panels, which enable the examination of all pertinent molecular markers of glioblastoma in a single custom assay, however, the clinical applicability of molecular testing via targeted sequencing panels remains restricted beyond the confirmation of diagnosis and prognosis [9]. In certain studies, it has been proposed that the activation of HOX genes may be linked to patient survival in the context of molecular characteristics of glioblastoma [16].

In summary, we observed a high degree of positive correlation between HOX gene expression with worse outcomes in glioblastoma. Using HOX gene analyses, we further show that the 8-HOX gene signature has the potential to improve the classification of glioblastoma. Those results will be the basis of future studies on accurate classification and survival prognosis, HOX gene cluster molecular signaling pathways, and also for effective treatment options for glioblastoma.

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