Biomolecules and Biomedicine
ISSN: 2831-0896 (Print) | ISSN: 2831-090X (Online)

Journal Impact Factor® (2023): 3.1
CiteScore® (2023): 7.4
www.biomolbiomed.com | www.blog.bjbms.org

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Zottel et al: *TNFSF14* & *CD44* overexpression in GBM immunity

TNFSF14 and *CD44* are overexpressed in glioblastoma and associated with immunosuppressive microenvironment

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DOI: https://doi.org/10.17305/bb.2025.11791

ABSTRACT

Glioblastoma (GBM) is one of the deadliest cancers, and the survival rate has remained low for decades. The aim of the study was the construction of the programmed death-ligand 1 (PD-L1) network, identification of its interactors and over-represented pathways, and analysis of the association between the identified genes and the immunosuppressive microenvironment of GBM. The PD-L1 network was constructed using Cytoscape and Search Tool for the Retrieval of Interacting Genes/Proteins (STRING). Over-representation analysis was performed on WebGestalt using Kyoto Encyclopedia of Genes and Genomes (KEGG), Protein ANalysis THrough Evolutionary Relationships (Panther), and Reactome Pathway Database (Reactome). Gene expression levels were examined in silico using three large datasets (The Cancer Genome Atlas (TCGA), Chinese Glioma Genome Atlas (CGGA), and Rembrandt), as well as with qPCR. The association between PD-L1 gene expression and immune cell infiltration was analyzed using the Tumor Immune Estimation Resource (TIMER 2.0) online tool. Cluster of differentiation 44 (*CD44*) and tumor necrosis factor superfamily member 14 (*TNFSF14*) were found to be significantly overexpressed in GBM compared to lower grade glioma (LGG) and normal brain tissue. Their overexpression was associated with worse overall survival and demonstrated a strong ability to differentiate between GBM and reference brain tissue. Notably, *CD44* and *TNFSF14* were linked to the mesenchymal subtype of GBM and positively correlated with the presence of regulatory T cells, resting NK cells, and PD-L1 expression. Our findings highlight the overexpression of *CD44* and *TNFSF14* in GBM and their potential involvement in creating an immunosuppressive microenvironment. Unraveling the PD-L1 interaction network and its associated pathways offers the potential not only to identify novel biomarkers for GBM prognosis but also to pinpoint alternative therapeutic targets that could be more effective in overcoming the immunosuppressive hurdles inherent in GBM treatment.

Keywords: Glioblastoma; GBM; cluster of differentiation 44; *CD44*; tumor necrosis factor superfamily member 14; *TNFSF14*; immune-checkpoints; tumor microenvironment

INTRODUCTION

With an incidence of 3.1 per 100 000 inhabitants (1) glioblastoma (GBM) is the most common primary brain tumor accounting for 45.6 % brain tumors. The confirmed risk factor are exposure to high dose ionizing radiation,age, race (white) and sex (male) (2, 3). Also, some genetic factors contribute to glioblastoma, such as Li-Fraumeni syndrome and neurofibramotisis (3). The treatment of GBM has not changed since 2005 and it follows Stupp protocol that includes surgical removal of tumor, radiotherapy and chemotherapy with temozolomide (4). Even though some novel treatments have entered clinical practice, such as tumor-treating field, lomustine and regorafenib (5-7), GBM still has exceptionally low prognosis, with a median survival of 15 months. It is characterized by high proliferation rate, core necrosis, microvascular proliferation, and tumor infiltration (8). There are a few reasons why therapy fails, one of which is the presence of the blood-brain barrier (BBB) that prevents penetration of most drugs (9). GBM is also a highly heterogenous tumor, where besides differentiated cells, GBM cancer stem cells are present. These are highly malignant cells, that can form a new tumour, which leads to tumor recurrence (10, 11). Furthermore, the tumor microenvironment (TME) of GBM is a complex network of various cell types, including differentiated cancer and stem cells, dendritic cells, pro-tumor macrophages, and more, creating an immunosuppressive milieu that fosters tumor progression (12). Notably, immune cells within the TME, such as regulatory T cells (Tregs) and NK cells, play significant roles in GBM malignancy, either by promoting immunosuppression or by attempting to cause an anti-tumor response, which is often inhibited by the glioma cells. Tregs contribute to immunosuppression by downregulating cytokine IL-2 and facilitating the conversion of other T cells into Tregs (13, 14). Conversely, NK cells are integral to the anti-tumor immune response, inducing apoptosis in target cells through granzyme B and perforin action (15). However, glioma cells can deploy various strategies to diminish NK cell efficacy (16). Tumor-associated macrophages (TAMs) and resident microglia, which can constitute up to 30% of the tumor mass, fluctuate between M1 (immunopermissive) and M2 (immunosuppressive) phenotypes, with a predominance of M2 macrophages correlating with a worse prognosis due to their role in promoting tumor angiogenesis and resistance to anti-VEGF therapies (17).

Although immunotherapy has shown promise in treating certain cancers, its efficacy in GBM has been largely disappointing, primarily due to the tumor's highly immunosuppressive

microenvironment. The PD-1/PD-L1 immune checkpoint pathway, a major contributor to this immunosuppression, inhibits tumor cell apoptosis and facilitates the conversion of effector T cells into Tregs. PD-L1 (programmed death-ligand 1, also known as CD274 and B7-H1) is a transmembrane protein encoded by the *PDCDL1* gene (18). It comprises IgV and IgC extracellular domains, a hydrophobic transmembrane domain, and a cytoplasmic domain that functions as a signal transducer. *PD-L1* is expressed in various cells, including tumor cells, antigen-presenting cells, B lymphocytes, and parenchymal cells, while its receptor PD-1 is found on activated T-cells. The interaction between PD-L1 and PD-1 facilitates immune escape by suppressing T-cell activation and proliferation, reducing cytokine production, and inducing T-cell exhaustion (19). Besides T-cell suppression, PD-L1 also contributes to epithelial-mesenchymal transition, chemoresistance, and the maintenance of stem cell properties (19). Numerous clinical trials are evaluating anti-PD-L1 drugs for GBM treatment. However, results have been poor and inconsistent. Some trials showed no significant benefit, while others indicated modest activity, particularly when combined with temozolomide and radiotherapy (20-23). PD-L1 is frequently expressed in GBM, but the level of expression varies, as the median number of positive cells is 2.77 % and is ranging from 0 - 86.6 % (24). So far, the results from the clinical trials targeting PD-L1 in GBM patients are not promising. In one clinical trial, it showed no benefit (20), while in other, it showed modest activity in combination with temozolomide and radiotherapy (23). Specifically, the anti-PD-L1 drug Avelumab has failed to demonstrate any survival benefit for patients, suggesting that PD-L1 may not be an optimal target for immunotherapy in GBM. (21, 22). In response to these challenges, this study constructs and analyses a PD-L1 interaction network, identifying over-represented pathways and examining the gene expression levels of pivotal genes through both *in silico* analyses using data from the CGGA, TCGA, and Rembrandt databases, and experimental validation using human GBM, lower-grade glioma, and normal brain tissue samples. This comprehensive approach aims to elucidate potential therapeutic targets and pathways that could enhance the efficacy of GBM treatment strategies. Building on the groundwork laid by previous research, this study specifically aims to dissect the complexity of the PD-L1 interaction network within GBM. Our objective is to bridge the gap between the promising concept of immunotherapy and theimprovement of treatment outcomes for GBM patients, potentially paving the way for more effective, targeted treatment strategies.

MATERIALS AND METHODS

Network construction and over-representation analysis

Two networks of PD-L1 (CD274) were constructed. The first was created using Cytoscape (25) and Pubmed query with keywords "PD-L1" and "glioblastoma". The confidence was set to 0.7 and no more than 100 interactors were included. The second network was constructed by String (26) with search term "PD-L1". Confidence was set to 0.7 and no more than 50 interactors were included. Both networks were then combined in String network to show their connection, without adding new neighbors. Over-representation analysis was performed with Webgestalt online tool (27), using KEGG (28), Panther (29) and Reactome (30), using genome protein-coding reference set and cutoff FDR lower than 0.05.

In silico analysis

Several datasets were included in *in silico* analysis: Chinese Glioma Genome Atlas (CGGA mRNAseq 693 RSEM), Cancer Genome Atlas obtained from Gliovis (TCGA; GBMLGG dataset) and Rembrandt obtained from Gliovis. The CGGA database includes 693 glioma patients, of those 249 with glioblastoma, 188 with LGG and 255 with glioma grade 3. Patients were not discriminated on sex, age and mutation status. The TCGA includes 669 glioma patients of various grades, of those 152 patients with glioblastoma, 226 patients with LGG and 244 with glioma grade 3. Astrocytomas with no known grade were removed from the study. Patients were not discriminated on sex, age and mutation status. Rembrandt dataset includes 558 subjects, among those 219 patients with GBM, 100 patients with LGG, 85 with glioma grade 3 and 28 non-tumor samples. Subjects with no known histology were removed from the study.

For CGGA and TCGA, gene expression was determined for GBM, grade lll glioma and lower-grade glioma (LGG). For Rembrandt, gene expression was determined for GBM, grade lll glioma, LGG and non-tumor tissue. The differential gene expression analysis was performed in R (version 4.3.0) and RStudio (version 2023.03.1), with One-way ANOVA statistical test using function aov of stats package. For each gene, survival was performed for GBM in R (version 4.3.0) and RStudio (version 2023.03.1). The two subgroups, with higher and lower expression, were divided based on the optimal cutoff calculated by surv_cutpoint (package survminer). The plot was constructed with

ggsurvplot. ROC curve was constructed from Rembrandt dataset, including GBM patients and non-tumor subjects, in R (version 4.3.0) using RStudio (version 2023.03.1) and pROC package.

Protein expression of CD44 was determined using results from the Human Protein Atlas (31, 32). The statistical analysis of protein expression was performed in GraphPad prism.

Human tissue samples and gene expression analysis (qPCR)

The use of human tissue samples was approved by the National Medical Ethics Committee of the Republic of Slovenia (Numbers: 92/06/12, 89/04/13 and 95/09/15). Written informed consent was signed by the patients prior to their surgery. Reference samples were obtained during autopsies, following the legal regulations valid for the Republic of Slovenia. All of the samples used in this study are anonymous.

Expression levels of genes identified *in silico* were determined by qPCR in 29 GBM tissues, 7 LGG tissues and 12 reference tissues. The RNA extraction has been already performed (33, 34). Afterwards, 500 ng of RNA was treated with DNAse 1 (Roche) with the following settings: incubation for 15 min at 30°C and 10 min at 75°C. Next, RNA was transcribed to cDNA with High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher) and RNAse inhibitor (1 µl/reaction; cat. n. N8080119, Thermofisher) was added. The following settings were used: incubation for 10 min at 25°C, 120 min at 37°C and 5 min at 85°C. The reaction mixture for qPCR was composed of 0.25 µL of TaqMan assay, 2.5 µL of TaqMan mastermix, 2 µL of H_20 and 0.25 µL of cDNA. The settings were as follows: 20 s at 95 °C; 45 cycles of 1 s 95 °C, 20 s 60 °C; hold 4 °C. The experiment was performed in three technical replicates. The reference genes were *GAPDH* and *HPRT1*. The results were analyzed as described before (35). The list of TaqMan probes is given in Table 1.

Association of gene expression, immune cells infiltration, correlation with immune genes and expression across different tumors

The association analysis of gene expression and immune cells infiltration was performed with TIMER 2.0 (36) online tool. Only results from CIBERSORT, a method to characterize cell composition, were included. The correlation with immune genes was performed in in R (version 4.3.0) and RStudio (version 2023.03.1) using stats package and Spearman statistical test. Genes

were retrieved from TISIDB online database (37). Gene expression across different tumors was retrieved from GEPIA online database (38).

RESULTS

Immune processes were identified as the most over-represented in the network and gene set enrichment analysis

In the first step we constructed a network of PD-L1 using Cytoscape and String (Figure 1). The pathway analysis (Table 2) revealed that the genes are mostly implicated with different immune processes. The 20 most over-represented pathways are presented in Table 2 with top five being 1) PD-1 signaling, 2) Translocation of ZAP-70 to Immunological synapse, 3) Phosphorylation of CD3 and TCR zeta chains, 4) RUNX1 and FOXP3 control the development of regulatory T lymphocytes (Tregs) and 5) Allograft rejection.

In silico selection of a set of genes overexpressed in GBM and correlated to worse overall survival

In the next step, we analysed gene expression and correlation with survival. Three datasets were included, CGGA, TCGA and Rembrandt. The genes included were from STRING and Cytoscape analysis, determined in 3.1 part. From the results obtained, we selected genes for further analysis based on two criteria: 1) overexpressed in GBM compared to WHO 3 glioma, LGG and normal brain tissues (for Rembrandt only) across at least two datasets and 2) their higher expression was related to worse overall survival of GBM patients also across at least two datasets. The genes that met these criteria were: *CASP4*, *CD40*, *CD44*, *CD163*, *CD276*, *CMTM6*, *FKBP5*, *HOXD13*, *ITGAM*, *PVR*, *RAB42*, *TNFSF14*, *TNFRSF14*, *TMEM205*, and *TSTD1*.

CD44, *TNFSF14* and *HOXD13* are overexpressed in GBM tissue samples and linked to worse overall survival

Among 15 genes whose expression levels were analyzed by qPCR (Figures 2-4, Figure S1), only three genes in particular *CD44*, *TNFSF14* and *HOXD13* were found to be overexpressed in GBM *versus* both LGG and normal brain tissue as presented in Figures 2d, 3d, and 4d. *CD44* and

TNFSF14 were overexpressed in GBM compared to WHO Grade 3 glioma (confirmed by TCGA, CGGA and Rembrandt), to LGG and normal brain tissue (confirmed by Rembrandt and qPCR analysis). Beside the gene expression, CD44 was also found to be expressed on protein level in GBM patients compared to control (Figure 2e). *HOXD13* was overexpressed in GBM compared to WHO Grade 3 glioma (confirmed by TCGA and CGGA), LGG (confirmed by TCGA, CGGA, Rembrandt and qPCR) and normal brain tissue (confirmed by Rembrandt and qPCR). Moreover, higher expression levels of all three genes were related to worse overall survival across all three datasets, TCGA, CGGA and Rembrandt (Figure 5, Table 3).

CD44 and *TNFSF14* distinguish between GBM and normal brain tissue and are associated with mesenchymal subtype

From the Rembrandt dataset we calculated how well can the selected genes distinguish between GBM and normal brain tissue. From the ROC curve (Figure 6) we can observe, that *CD44* and *TNFSF14* can distinguish between GBM and normal brain tissue with high sensitivity and specificity (AUC above 0.8), while *HOXD13* (AUC is 0.553) cannot distinguish between GBM and normal brain tissue well. Next, we investigated the relationship between *CD44*, *TNFSF14* and *HOXD13* and specific GBM subtypes (Figure 7). The results show that *CD44* and *TNFSF14* are more related to mesenchymal subtype (ME) in TCGA and Rembrandt subtype. On the other hand, *HOXD13* is not related to any of the subtypes.

CD44 and *TNFSF14* are positively associated with Treg, resting NK cells and PD-L1 expression

We also analyzed the relationship between the expression of *CD44*, *TNFSF14* and *HOXD13* and different immune cells (Table 4). *CD44* and *TNFSF14* are positively associated with regulatory T cells and NK cell resting as well as negatively associated with NK cells activated. Additionally, *CD44* is negatively associated with macrophage type M2 and positively associated with CD4+ T cells memory resting. At last, we checked expression association of all three genes to *PD-L1* (Figure 8). Results show that *CD44* is moderately and positively associated with PD-L1 (rho is 0.43). *TNFSF14* is weakly and positively correlated with *PD-L1* expression (rho is 0.296), while there is weak negative association between PDL-1 and HOXD13 association (rho is -0.21).

CD44, *TNFSF14* and *HOXD13* are related to both immunoinhibitory and immunostimulatory genes

In the next step we analyzed the relation of *CD44*, *TNFSF14*, and *HOXD13* to immunoinhibitory and immunostimulatory genes. Immunoinhibitory and immunostimulatory genes were selected from TISIDB repository portal (37). Results show that all three genes are positively related to most of the immunostimulatory as well as immunoinhibitory genes. The strongest positive correlation was with immunostimulatory genes *TNFRSF14*, *CD276*, *CD40*, and *CD48* (Figure 9). For immunoinhibitory genes, the strongest correlation was with genes *PVRL2*, *IL10RB*, and *PDCD1LG2* (Figure 10).

Expression of *CD44, TNFSF14* and *HOXD13* across different tumors and corresponding reference tissues

In the last step we focused on the gene expression patterns of *CD44*, *TNFSF14*, and *HOXD13* across a range of tumors compared to normal tissues (Figure 11) obtain from online available database GEPIA (38). For *CD44*, we observed a general trend of higher expression in tumor tissues across most of the tumors studied (Figure 11a). Notably, the greatest differences where the expression was higher in normal compared to tumor tissues were seen in thymoma (THYM), uterine corpus endometrial carcinoma (UCEC), and uterine carcinosarcoma (UCS). In contrast, *TNFSF14* showed predominantly higher expression in normal tissues, with a general trend of downregulation in tumor cells (Figure 11b). Interestingly, there were exceptions in nine types of tumors where *TNFSF14* expression was elevated in tumor tissues compared to normal brain tissue, including GBM. At last, *HOXD13* (Figure 11c) displayed low or negligible expression in various tumors. However, it was highly expressed in normal tissues relative to tumor tissues in cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), and prostate adenocarcinoma (PRAD). Conversely, in several tumors, including GBM, head and neck squamous cell carcinoma (HNSC), lung squamous cell carcinoma (LUSC), skin cutaneous melanoma (SKCM), and UCS, *HOXD13* was markedly expressed, while it was low or absent in corresponding normal tissues.

DISCUSSION

GBM is one of the deadliest cancers with median survival rate of only 15 months (8). Despite advancements in treatment, survival rates have not significantly improved, emphasizing the urgent need for novel therapies. Immunotherapy, a rapidly developing field in cancer treatment, has shown limited success in GBM. Some of the reasons why current therapeutic approaches are not successful is high tumor heterogeneity, lack of neoantigens and immunosuppressive environment (39-42).

However, recent studies suggest potential benefits of immunotherapy in treating this aggressive cancer.

In our study we aimed to explore the PD-L1-related gene network to identify potential new therapeutic targets. We constructed a PD-L1 network and analyzed data from three datasets (TCGA, CGGA, and Rembrandt). These three datasets were included because they comprise a large number of samples with detailed clinical reports, ensuring the robustness of our analysis.

Our analysis identified 15 genes overexpressed in GBM compared to WHO Grade 3 glioma, LGG, and normal brain tissue. These genes are also associated with shorter overall survival. Validating these findings with tissue samples from our biobank, we confirmed higher expression levels of three genes—*CD44*, *TNFSF14*, and *HOXD13*—in GBM. CD44 is a transmembrane glycoprotein, expressed in different isoforms, and is involved in several key cancer processes, such as carcinogenesis, progression and resistance to therapy (43). It is a well-established biomarker of glioblastoma stem cells and is frequently co-expressed with CD133, another important glioblastoma stem cell biomarker (44). Interestingly, CD44 is not exclusively glioblastoma-specific; it is also expressed in astrocytes under normal physiological conditions (45). Beyond glioblastoma, CD44 has been implicated in aggressive behavior and tumorigenicity in multiple cancer types, such as breast, kidney, pancreatic, prostate, and gastrointestinal cancers (46). TNFSF14, another protein overexpressed in GBM as shown in our study, is expressed on activated T cells, NK cells and immature DC cells. It exists both as a soluble protein and a type II transmembrane protein, interacting with two distinct receptors: HVEM and TLβR. The LIGHT-HVEM axis is particularly significant for its anti-tumor immune activity, as it facilitates the infiltration of CD8+ T cells into tumors, enhancing anti-cancer immune responses (47). Notably, Long et al. observed that the level of TNFSF14 in combination with immune-check inhibitor genes can predict survival prognosis for patients with GBM (48). Furthermore, TNFSF14 has been

associated with disulfidptosis, a novel programmed cell death mechanism, and identified as one of the disulfidptosis-related immune checkpoints. Alongside genes such as CD276, TNFRSF14, TNFSF4, CD40, and TNFRSF18, TNFSF14 has been incorporated into a comprehensive overall survival prediction model designed to evaluate sensitivity to immunotherapy (49). Of the three genes discussed, the available data on HOXD13 is the most limited. HOXD13 is known to contribute to the pathogenesis of acute myeloid leukemia (AML) through the formation of a fusion protein with NUP98. Its expression patterns in cancer vary, with either overexpression or downregulation observed depending on the cancer type (50). In glioblastoma, however, research on HOXD13 remains sparse. A study by Zhang et al. highlighted that HOXD13 is regulated by miR-7156-3p, a tumor suppressor, whereas HOXD13 itself exhibits oncogenic activity by promoting glioma stemness and tumor progression (51).

Even though it was found to be overexpressed in GBM, *HOXD13* did not show the ability to differentiate between GBM and normal brain tissue, and was not related to any specific GBM subtype. On the other hand, *CD44* and *TNFSF14* showed a distinct ability to differentiate between GBM and normal brain tissue and are associated with the mesenchymal subtype of GBM. *CD44* has been previously linked to this subtype, as well as to increased invasion and proliferation in GBM (52-55). *TNFSF14* is also overexpressed in GBM and correlates with shorter overall survival and the mesenchymal subtype (48, 56, 57). Our *in-silico* study, incorporating three independent large datasets, reaffirms these associations. The association of both CD44 and TNFSF14 with the mesenchymal subtype is particularly significant, as this glioblastoma subtype is characterized by heightened inflammation, poorer survival rates, and the worst prognosis compared to other subtypes (53, 59, 60). The mesenchymal subtype is also linked to increased infiltration of tumor-associated macrophages, which further worsens its aggressive nature (61). Notably, in cases of recurrence, the proneural subtype frequently transitions to the mesenchymal subtype, leading to a more aggressive tumor phenotype and worse clinical outcomes (62).

CD44 and *TNFSF14* are both associated with resting NK cells. NK cells, when in active state, have high antitumor and antimetastatic activity (63). However, tumors can evade surveillance of NK cells, when these are not in active state but are so-called tumor-exposed NK cells and have similar gene expression profile as resting NK cells. In GBM, the positive association between *CD44* and *TNFSF14* expression and resting NK cells suggests that these genes may be involved in immune evasion and suppression. Additionally, *CD44* and *TNFSF14* were found to be positively associated

with regulatory T (Treg) cells, which are known to suppress the immune response and promote tumor growth. Namely, Treg cells are one of the major contributors to immune-suppressive environment (64-66). Hu et al. developed a prognostic model based on natural killer T (NKT) cells, incorporating single-cell RNA sequencing (scRNA-seq) data from the GEO database, as well as datasets from TCGA and CGGA. This model includes two key NKT markers, CD44 and TNFSF14 (67). Xiao et al. further highlighted the role of CD44 in glioma immunity, demonstrating that CD44+ tumor cells are predominantly in a mesenchymal-1-like cellular state. Additionally, CD44+ T cells exhibit high expression levels of PD-1 and PD-L1, emphasizing its role in immune regulation (68). Notably, CD44 expression is positively correlated with PD-L1 levels in glioblastoma patients, a relationship also observed in triple-negative breast cancer (TNBC) patients. CD44 has even been proposed as a potential target for modulating PD-L1 function, as it triggers transcriptional activation of PD-L1 through its intracytoplasmic domain (69). In TNBC, tumors with high PD-L1 expression are associated with enhanced immune and cancer stemness pathways, further elucidating its connection to CD44. Besides CD44, a weaker but positive correlation between TNFSF14 and PD-L1 expression has also been observed. Similarly, Yang et al. reported a significant association between TNFSF14 and immune checkpoints such as the PD-1/PD-L1 pathway, TIM-3, and B7-H3 .

To fully understand these findings, functional studies are essential. Co-culture experiments of glioblastoma cells with immune cells and gene knockout combined with advanced techniques like single-cell sequencing, could provide deeper insights into the mechanisms underlying these correlations and their potential for therapeutic exploitation.

CONCLUSION

In this study, by integrating three independent datasets we revealed insights into the molecular behavior of GBM, particularly highlighting the roles of *CD44*, *TNFSF14*, and *HOXD13*. *CD44* and *TNFSF14* emerged as promising therapeutic targets in GBM due to their significant overexpression in GBM compared to normal brain tissue and their association with the mesenchymal subtype, which is linked to heightened inflammation, poorer prognosis, and immune suppression. Both genes also correlate with immune evasion mechanisms involving resting NK cells and regulatory T cells, highlighting their role in shaping the tumor microenvironment. CD44 and TNFSF14 are closely associated with PD-L1 expression, highlighting their potential role in

regulating immune checkpoint pathways. To confirm the role of CD44 and TNFSF14 as potential targets, functional studies, such as co-culture experiments of glioblastoma cells with immune cells, gene knockout models, and advanced techniques like single-cell sequencing are required. These studies could provide deeper mechanistic insights and support the development of novel, more effective therapeutic strategies for GBM.

Conflicts of interest: Authors declare no conflicts of interest.

Funding: This research was funded by Research Programme Grants P1-0390 and postdoctoral project Z3-4510 (to A. Z) from the Slovenian Research Agency (SRA).

Data availability: Data is available at the corresponding author upon request.

Submitted: 3 December 2024

Accepted: 15 January 2025

Published online: 13 February 2025

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TABLES AND FIGURES WITH LEGENDS

Table 1. List of TaqMan probes used in the study.

Gene	Assay (ThermoFisher)
GAPDH	Hs99999905_m1
HPRT1	Hs02800695_m1
CASP4	Hs01031951_m1
CD276	Hs00987207_m1
CD44	Hs01075864_m1
RAB42	Hs05288343_g1
TNFRSF14	Hs00187058_m1
CD163	Hs00174705_m1
FKBP5	Hs01561006_m1
TNFSF14	Hs00542477_m1
CD40	Hs01002915_g1
ITGAM	Hs00167304_m1
PVR	Hs00197846_m1
TMEM205	Hs00414441_m1
TSTD1	Hs00939899_g1
CMTM6	Hs00215083_m1
HOXD13	Hs00171253_m1

Table 2. List of 20 most over-represented pathways. Over-representation analysis was performed with Webgestalt online tool (27), using KEGG (28), Panther (29) and Reactome (30), using genome protein-coding reference set and cutoff FDR lower than 0.05.

Description	**Enrichment Ratio**	**FDR**	**Database**
PD-1 signaling	95.1	0.0E+00	pathway_Reactome
Translocation of ZAP-70 to Immunological synapse	90.9	0.0E+00	pathway_Reactome
Phosphorylation of CD3 and TCR zeta chains	83.7	0.0E+00	pathway_Reactome
RUNX1 and FOXP3 control the development of regulatory T lymphocytes (Tregs)	57.6	4.4E-07	pathway_Reactome
Allograft rejection	54.5	0.0E+00	pathway_KEGG
Generation of second messenger molecules	52.3	0.0E+00	pathway_Reactome
GRB2 events in EGFR signaling	49.3	5.1E-04	pathway_Reactome
Graft-versus-host disease	44.9	0.0E+00	pathway_KEGG
Asthma	44.6	0.0E+00	pathway_KEGG
Costimulation by the CD28 family	44.4	0.0E+00	pathway_Reactome
SHC1 events in EGFR signaling	43.2	7.6E-04	pathway_Reactome
Interleukin-6 signaling	41.9	5.2E-05	pathway_Reactome
Type I diabetes mellitus	40.2	0.0E+00	pathway_KEGG
Intestinal immune network for IgA production	39.9	0.0E+00	pathway_KEGG

CD28 dependent Vav1 pathway	38.4	7.3E-05	pathway_Reactome
Autoimmune thyroid disease	36.9	0.0E+00	pathway_KEGG
TNFs bind their physiological receptors	35.7	9.7E-11	pathway_Reactome
CTLA4 inhibitory signaling	32.9	6.9E-07	pathway_Reactome
GAB1 signalosome	31.4	2.0E-03	pathway_Reactome
Inflammatory bowel disease (IBD)	30.1	0.0E+00	pathway_KEGG

Table 3. Survival of glioblastoma patients. The table presents median survival (months) in of glioblastoma patients across three datasets (TCGA, CGGA, and Rembrandt) and divided into two groups (high and low gene expression). For CGGA, the median survival is presented in months and in brackets in days.

	TCGA		CGGA		Rembrandt	
	high	low	high	low	high	low
CD44	12.6	15.1	15.1 (459)	28.4 (866)	11.7	17
TNFSF14	11	16.1	13.2 (401)	19.8 (603)	12.9	15.8
HOXD13	11.3	16.1	14.4 (438)	23.8 (723)	11.5	15.6

Table 4. Association of *CD44*, *TNFSF14* and *HOXD13* with immune cells in GBM. Data was obtained from online available platform TIMER 2.0, which provides information about association of immune infiltrates and genetic or clinical features (36). Only results obtained from CIBERSORT were included. Statistically significant results ($p<0.05$) are shown in orange (positive association) or blue (negative association).

	CD44	*TNFSF14*	*HOXD13*
CD 8+ T cells	-0.061	-0.079	0.003
CD4+ T cells naïve	-0.147	-0.088	-0.018
CD4+ T cells activated	0.056	0.059	0.072
CD4+ T cells memory resting	0.269	-0.032	-0.123
Treg	0.314	0.178	-0.043
Macrophage M0	-0.012	-0.017	0.144
Macrophage M1	-0.158	-0.08	-0.032
Macrophage M2	-0.214	-0.004	0.014
Myeloid DC activated	0.13	0.124	-0.058
Myeloid DC resting	0.041	-0.084	-0.09

NK cells activated	-0.277	-0.176	0.002
NK cells resting	0.291	0.188	-0.061

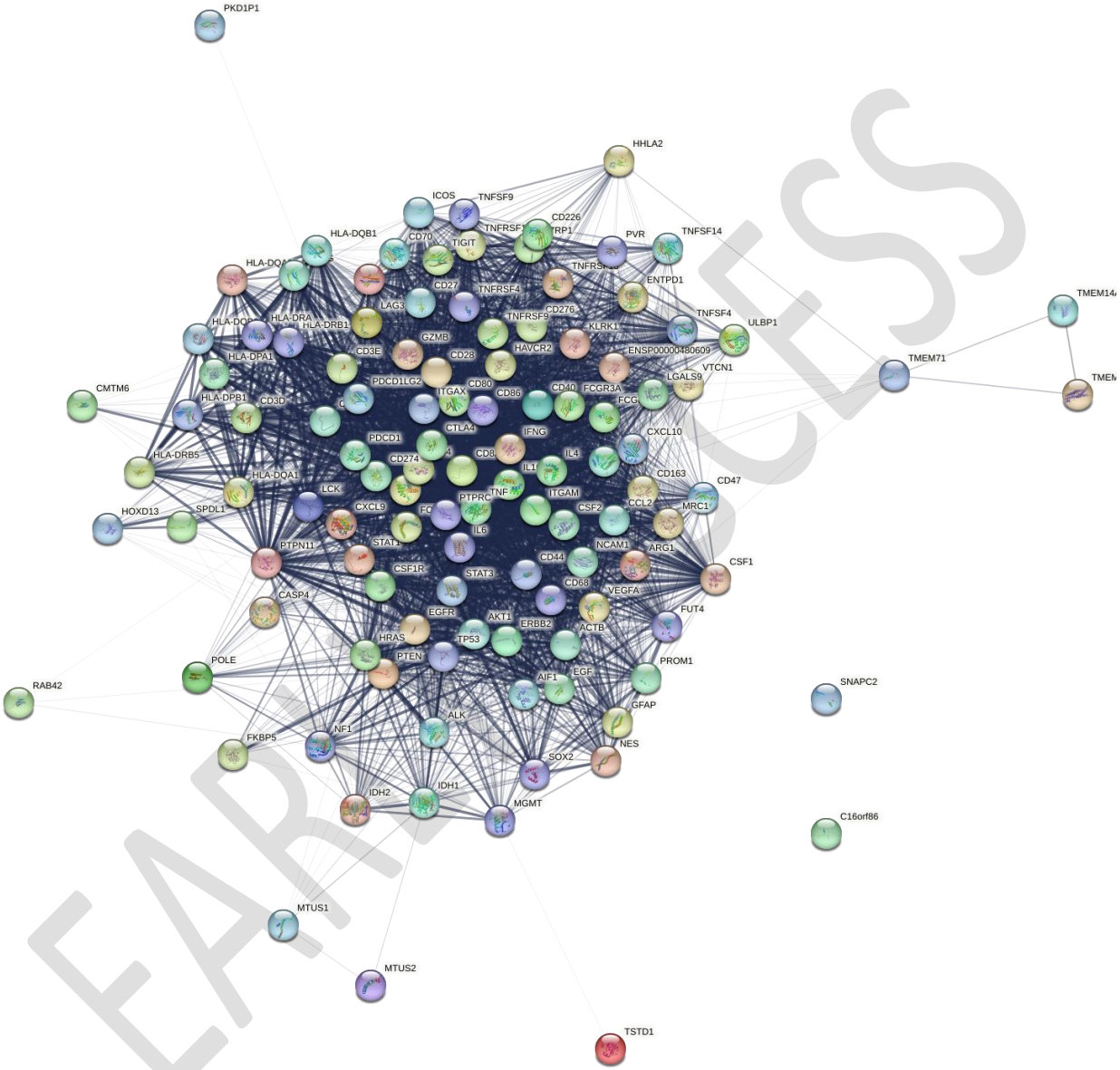
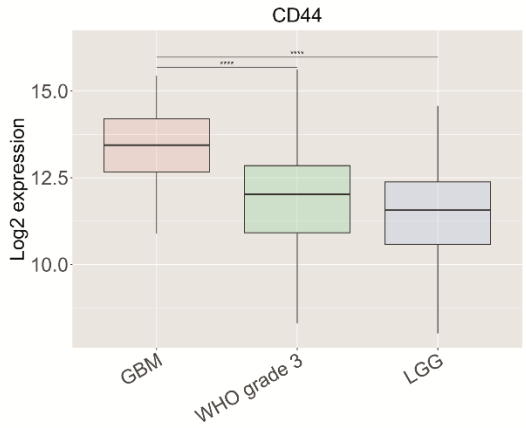


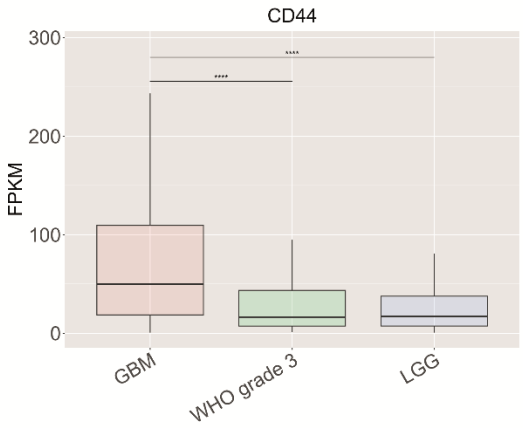
Figure 1. STRING network of PD-L1 associated proteins. It is a combination of two networks, performed with 1) Cytoscape and 2) String analysis analysis. The first network was created using Cytoscape and Pubmed query with keywords “PD-L1” and “glioblastoma”. The second network was constructed by String with the search term “PD-L1”. Confidence was set to

0.7 and no more than 50 interactors were included. Both networks were then combined in String to show their connection, without adding new neighbors.

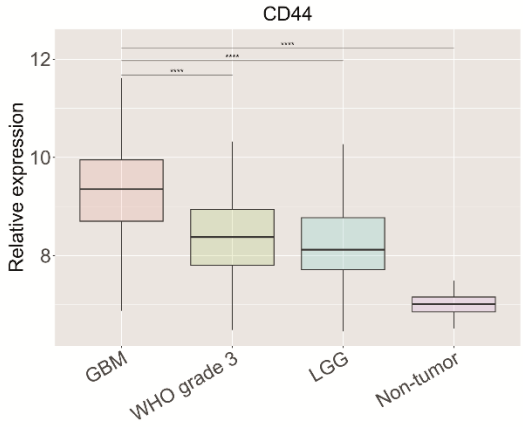
a) TCGA



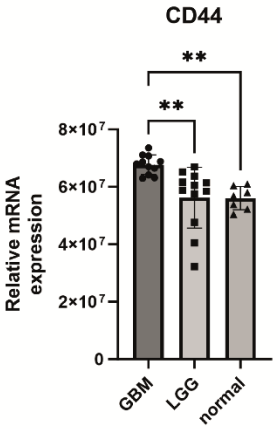
b) CGGA



c) Rembrandt



d) qPCR



e) Protein expression

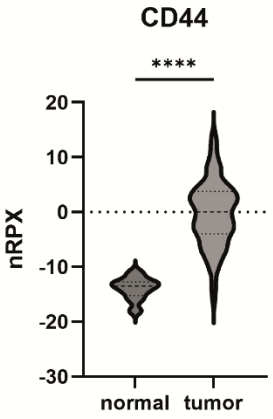


Figure 2. Gene expression of *CD44*. Gene expression was determined across three different datasets, a) TCGA, b) CGGA and c) Rembrandt. Gene expression levels were d) experimentally validated by qPCR in human tissue samples. e) Protein expression of CD44 is presented, results were obtained from the Human Protein Atlas (31, 32). Results are presented as mean +/- SD. * p<0.05, ** p<0.01, *** p<0.001, *** p<0.0001.

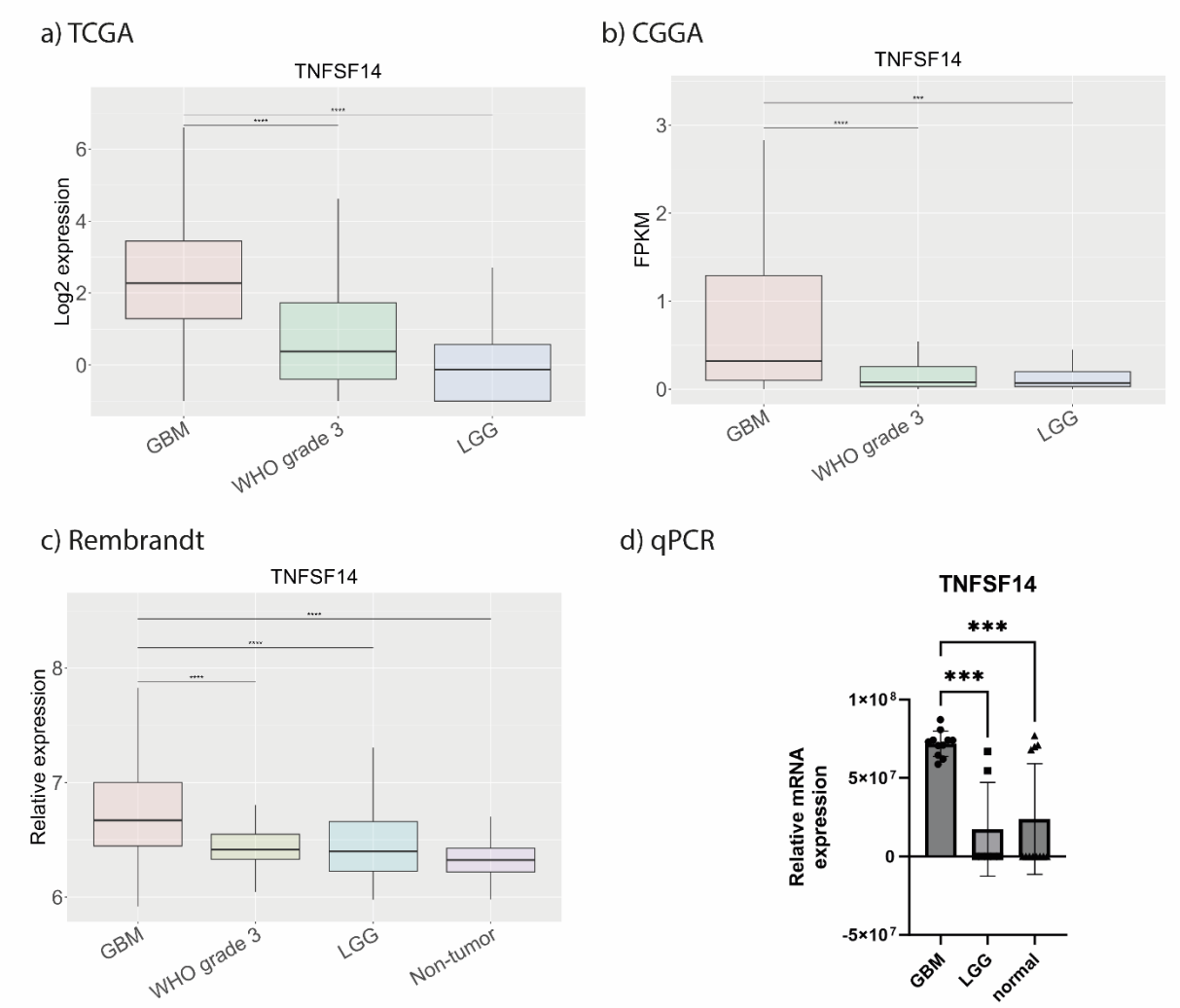


Figure 3. Gene expression of *TNFSF14*. Gene expression was determined across three different datasets, a) TCGA, b) CGGA and c) Rembrandt. Gene expression levels were d) experimentally

validated by qPCR in human tissue samples. Results are presented as mean +/- SD. * p<0.05, ** p<0.01, *** p<0.001, *** p<0.0001.

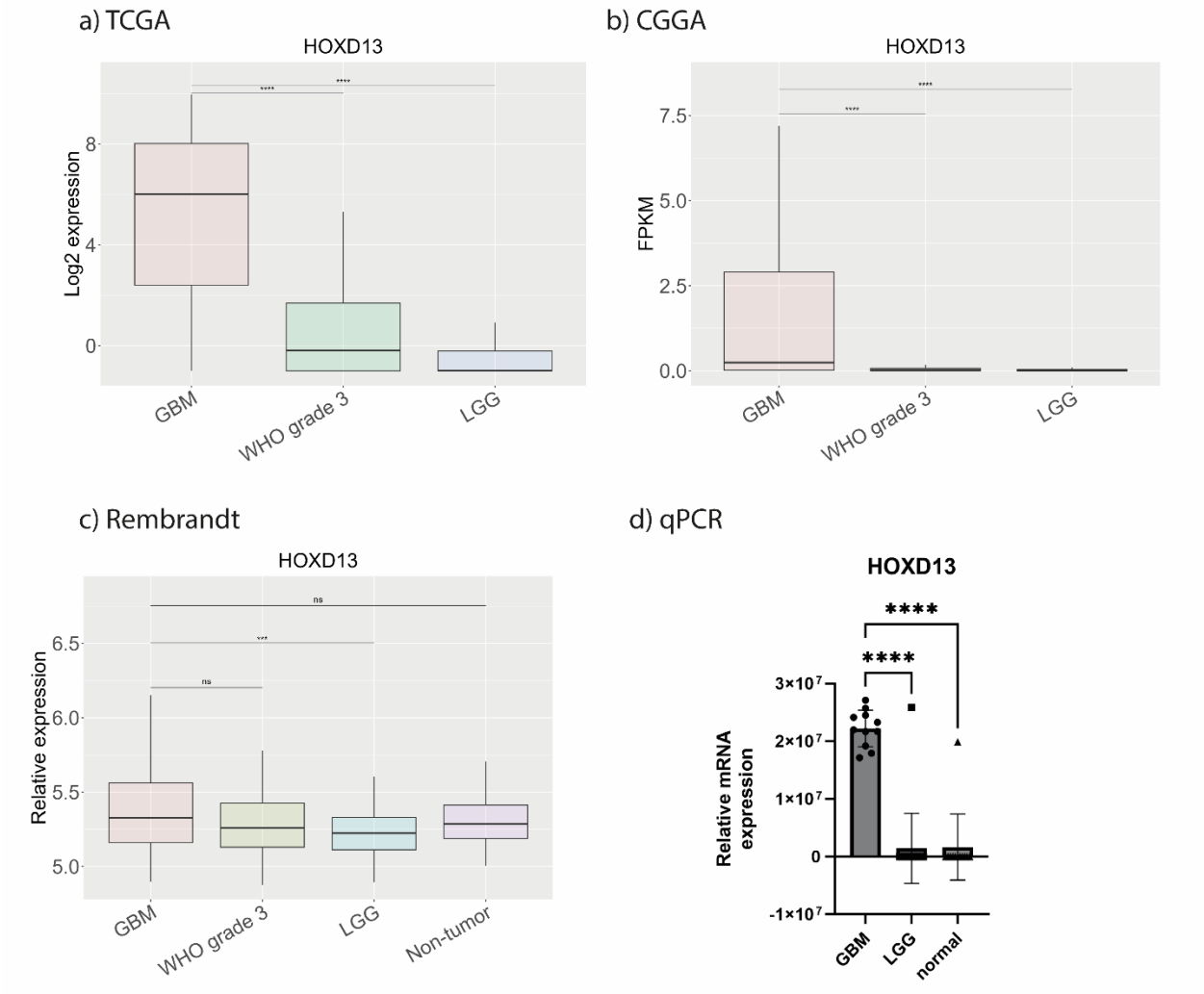


Figure 4. Gene expression of *HOXD13*. Gene expression was determined across three different datasets, a) TCGA, b) CGGA and c) Rembrandt. Gene expression levels were d) experimentally validated by qPCR in human tissue samples. Results are presented as mean +/- SD. * p<0.05, ** p<0.01, *** p<0.001, *** p<0.0001.

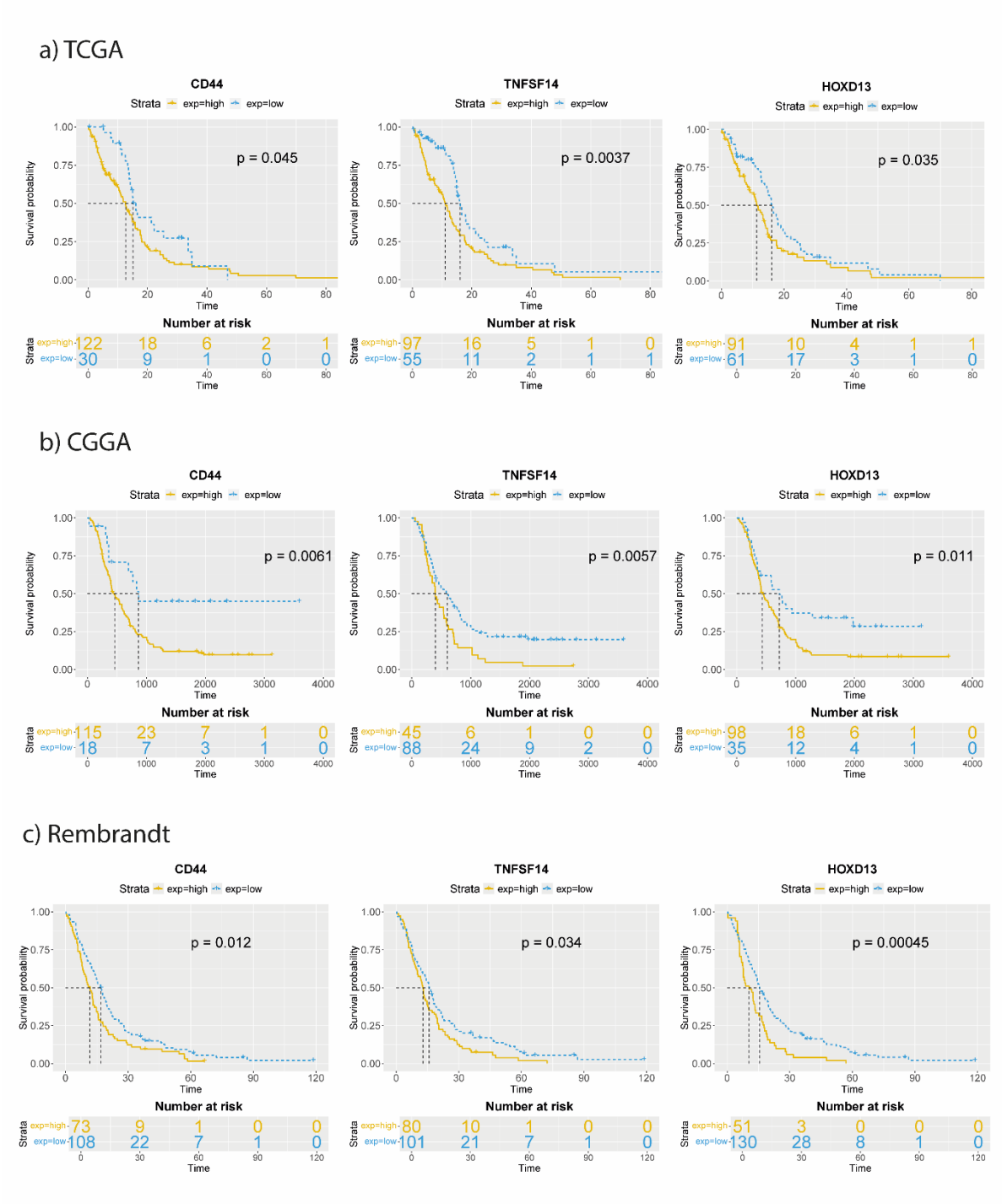


Figure 5. Survival analysis of *CD44*, *TNFSF14* and *HOXD13*. Survival of CD44, TNFSF14 and HOXD13 was determined across a) TCGA, b) CGGA and c) Rembrandt datasets.

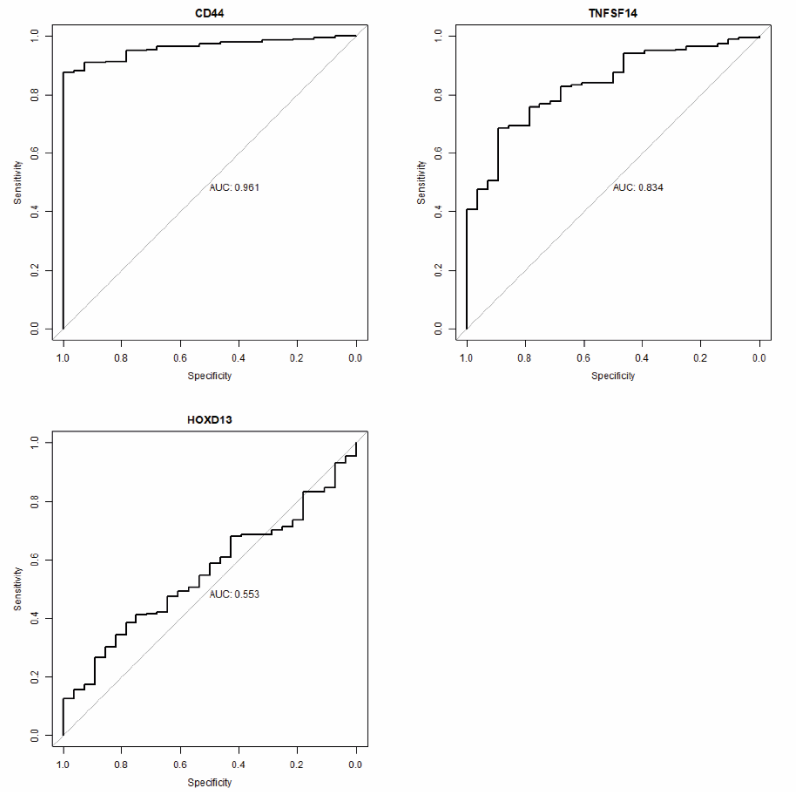


Figure 6. ROC curve of *CD44*, *TNFSF14* and *HOXD13*. The AUC for *CD44* is 0.961, for *TNFSF14* 0.834 and for *HOXD13*, 0.553.

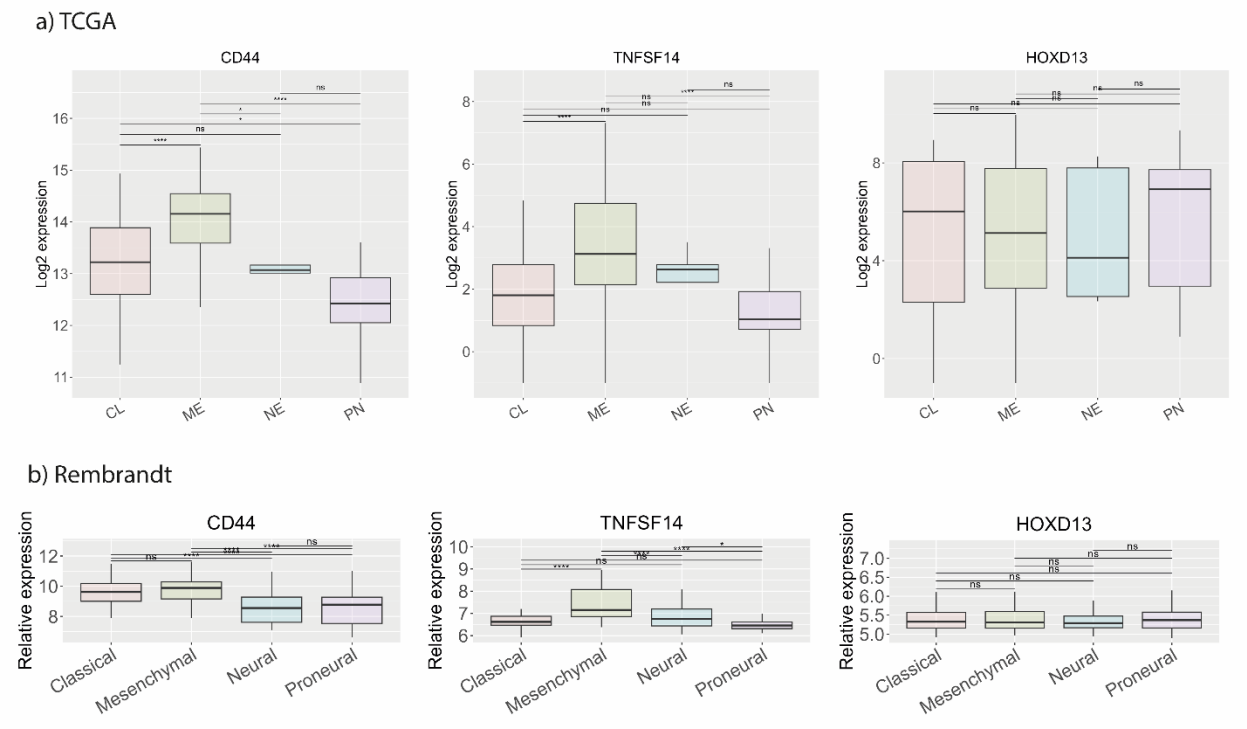


Figure 7. Gene expression levels of *CD44*, *TNFSF14* and *HOXD13* across different GBM subtypes. Two datasets were included in the study, a) TCGA and b) Rembrandt. Four different subtypes were included, classical (CL), mesenchymal (ME), neural (NE), and proneural (PN). Results are presented as mean +/- SD. * p<0.05, ** p<0.01, *** p<0.001, *** p<0.0001.

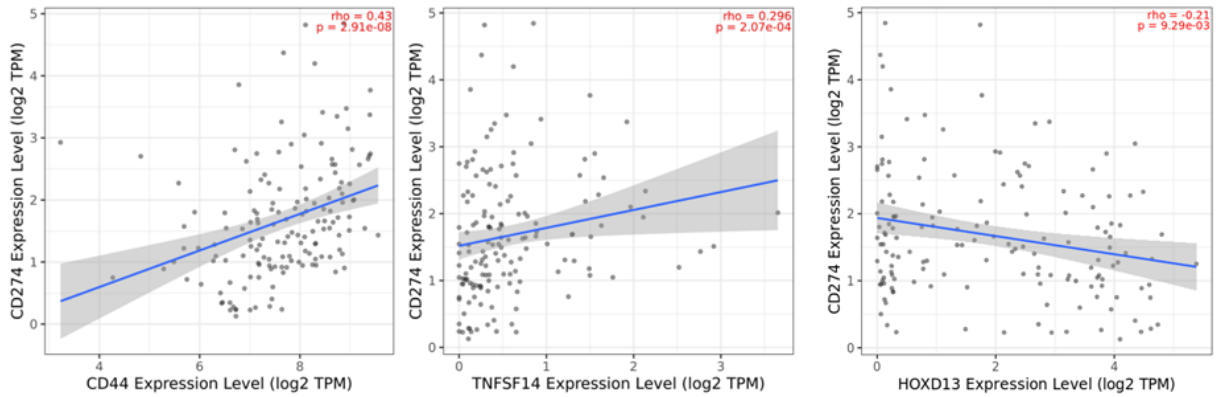


Figure 8. The association of *CD44*, *TNFSF14* and *HOXD13* with *CD274* (PD-L1) expression. For CD44, the rho was 0.43, for TNFSF14 0.296, and for HOXD13 -0.21.

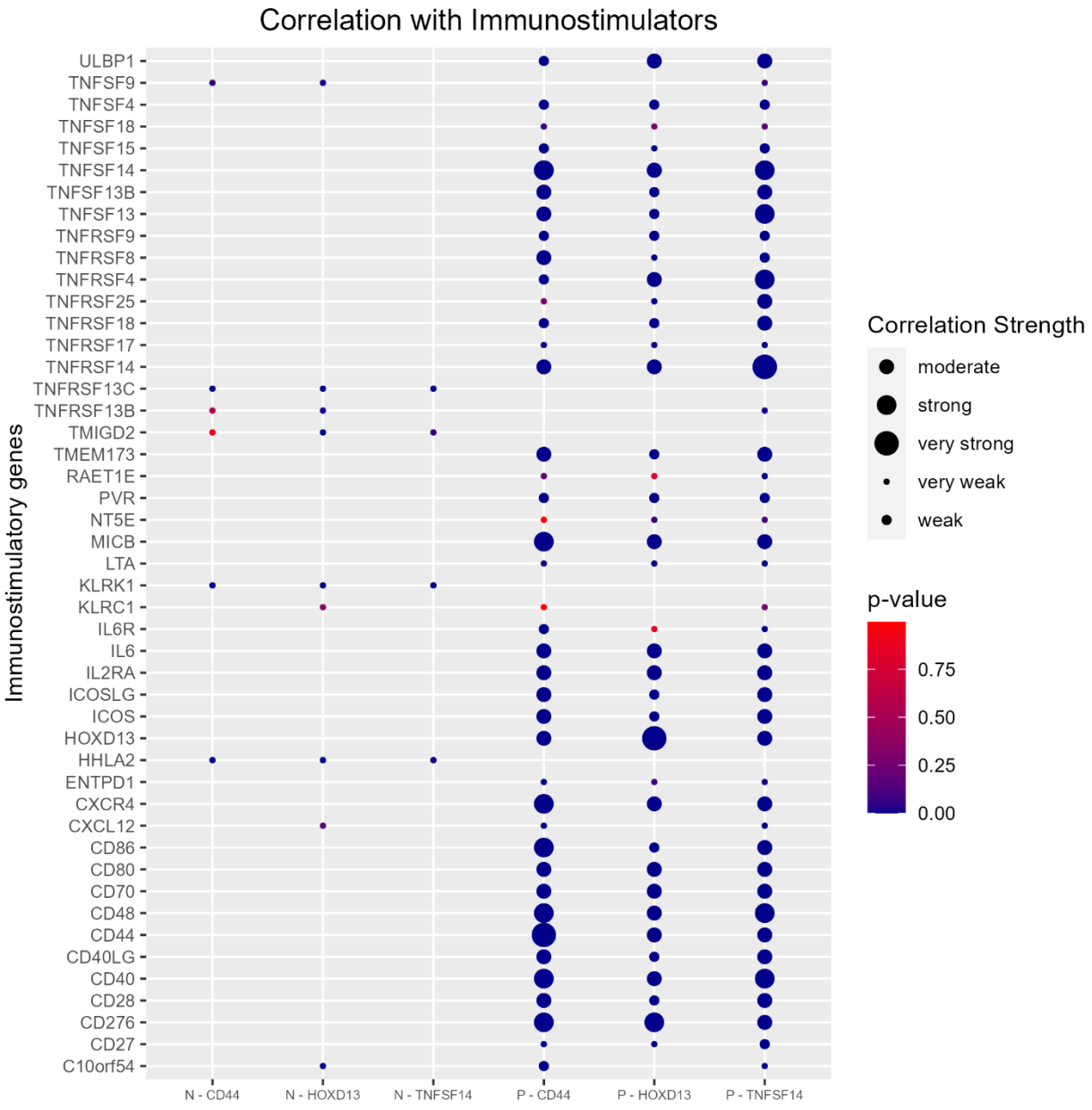


Figure 9. Spearman correlation of *CD44*, *HOXD13* and *TNFSF14* with immunostimulatory genes. Genes were retrieved from TISIDB online database (37).

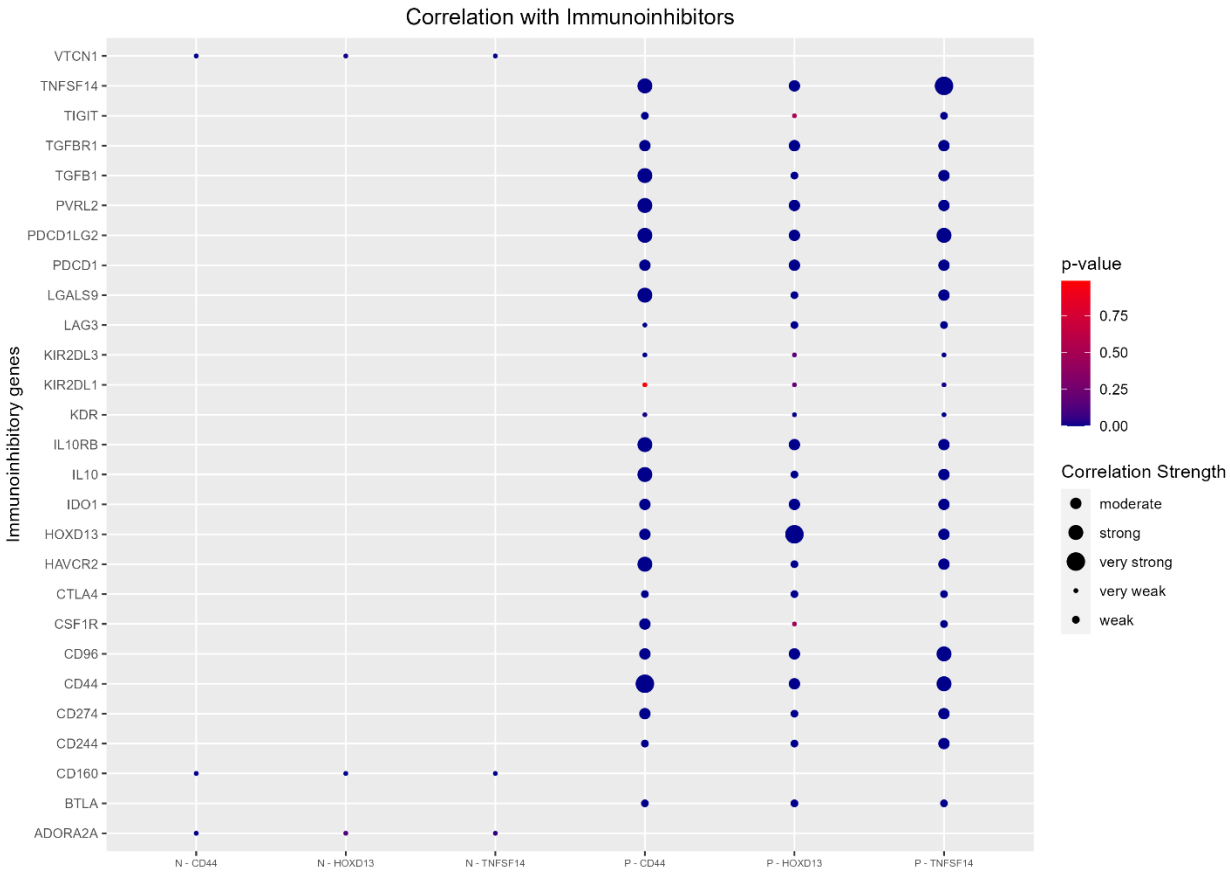
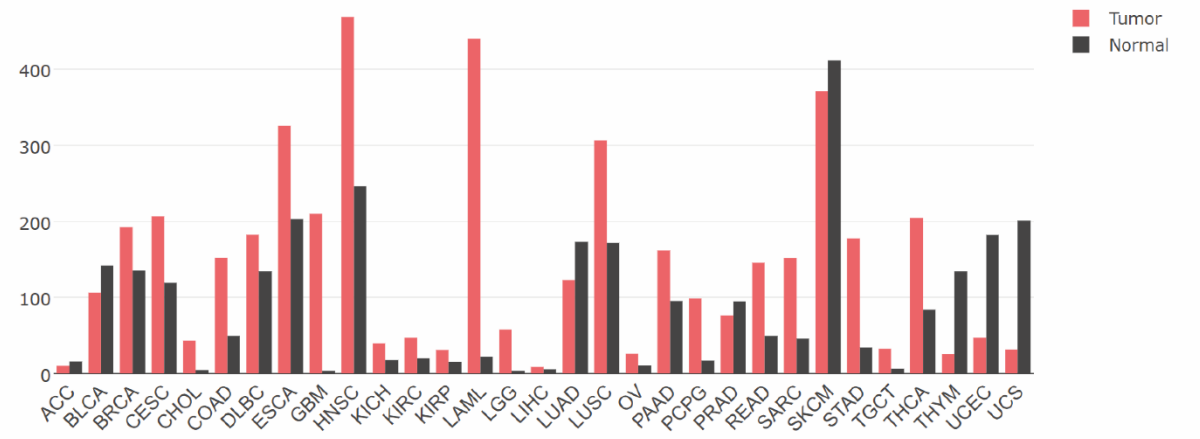
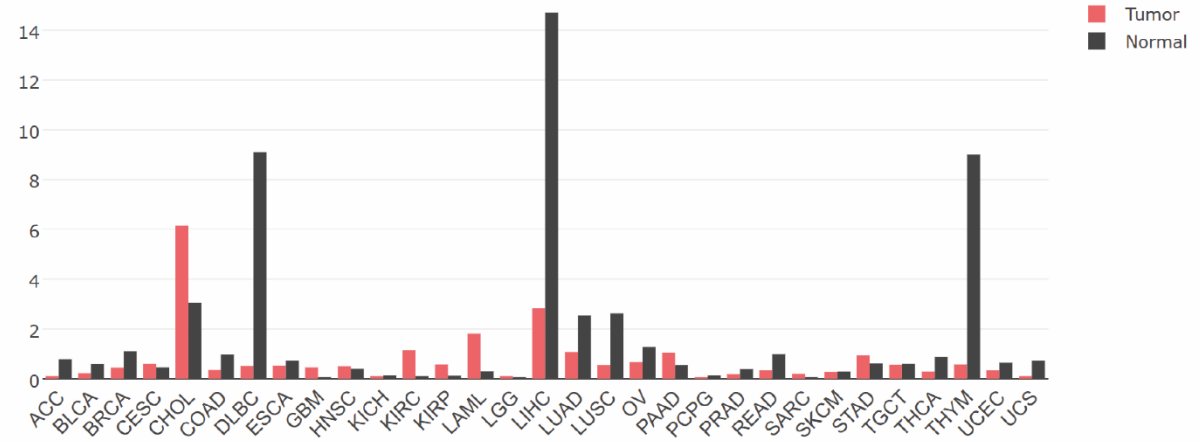


Figure 10. Spearman correlation of *CD44*, *HOXD13* and *TNFSF14* with immunostimulatory genes. Genes were retrieved from TISIDB online database (37).

a) CD44



b) TNFSF14



c) HOXD13

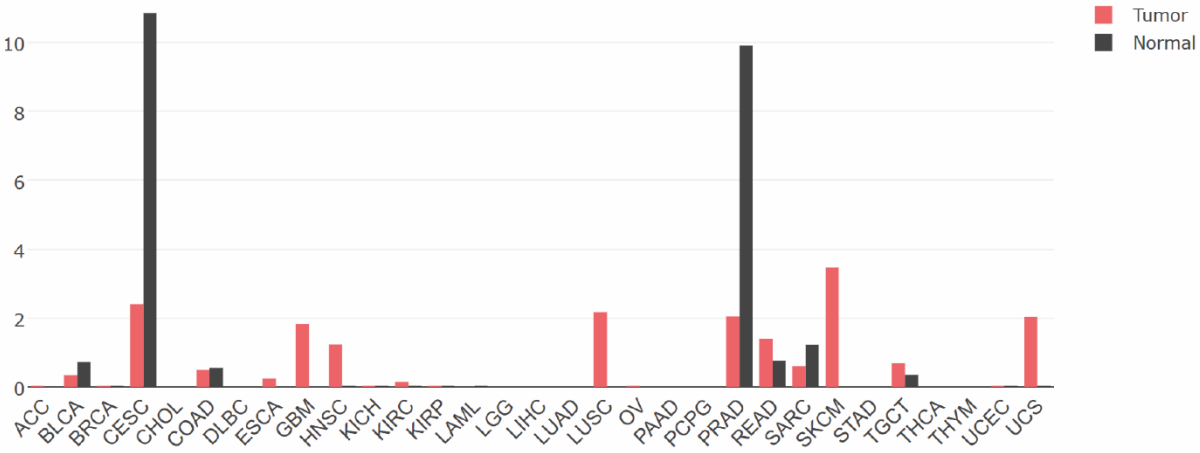


Figure 11. Differential expression of a) *CD44*, b) *TNFSF14* and c) *HOXD13*. Graphs were obtained from GEPIA online tool (38).

SUPPLEMENTAL DATA

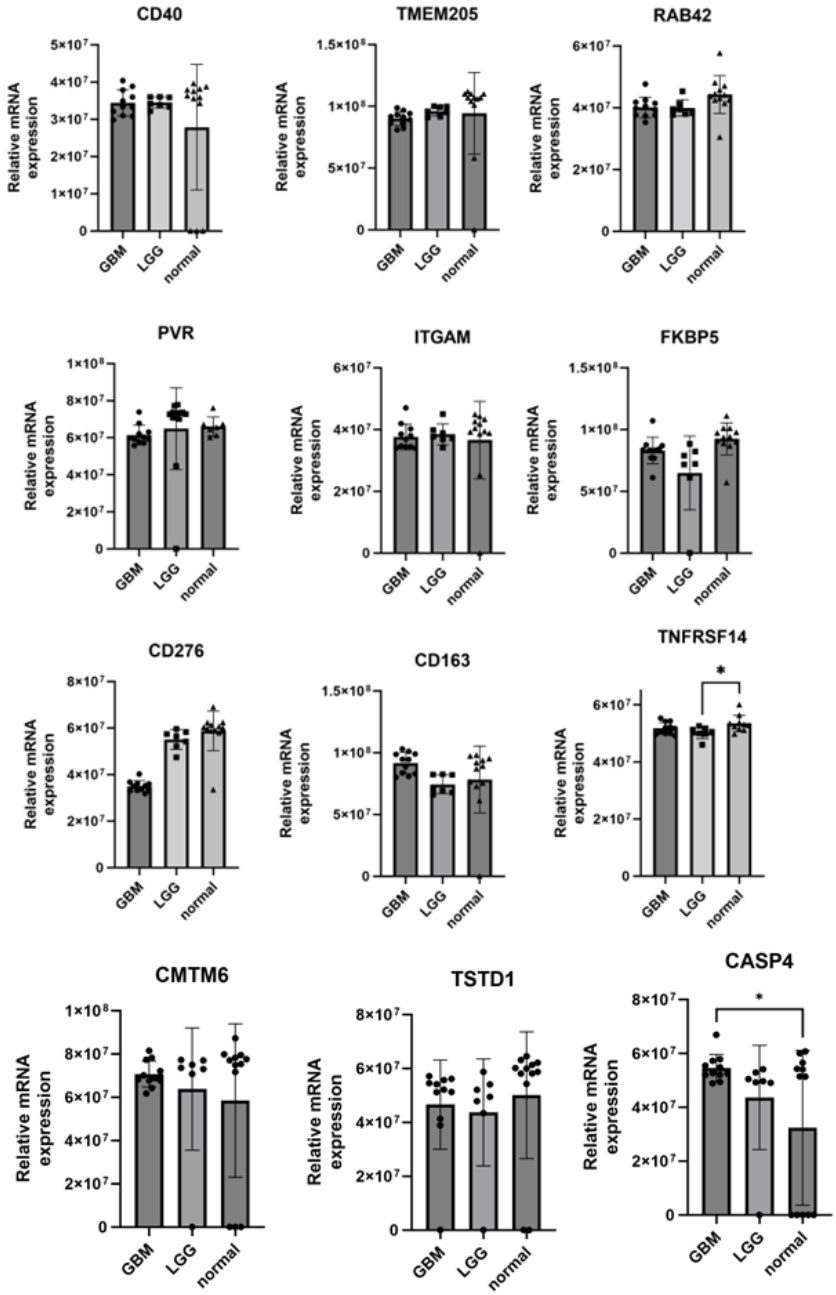


Figure S1. Gene expression in human tissue samples. Results are presented as mean +/- SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, *** $p < 0.0001$.