

The Cancer Stem Cell Marker CD44 Play Key Roles in Immune Suppression/Evasion, Drug Resistance, Epithelial-Mesenchymal Transition, and Metastasis in several human cancers

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Abstract: One of the most used markers of cancer stem cells in several cancers, including colorectal cancer and breast cancer, is CD44. CD44 is a glycoprotein that traverses the cell membrane and binds to many ligands including hyaluronan resulting in activation of signaling cascades. Several reports have shown conflicting data on the expression of CD44 and that the expression depends on modes of investigations and subtypes of cancers. In addition, the correlation between CD44 expression and drug resistance, immune infiltration, EMT, metastasis and patients prognosis in several cancer types remains unclear. This study investigated CD44 expression in several cancers and explored its relationship with tumorigenesis using various publicly available databases, including The Cancer Genome Atlas, GEPIA, Oncomine, Genomics of Drug Sensitivity in Cancer and Tumor Immune Estimation Resource. Our analysis reveals that CD44 is differentially expressed in different cancers. CD44 expression is significantly associated with cancer patients' survival in gastric, pancreatic and colorectal cancers. In addition, CD44 expression is closely linked with immune infiltration and immune suppressive features in pancreatic, colon adenocarcinoma and stomach cancer. High CD44 expression was significantly correlated with the expression of drug resistance-, EMT- and metastasis- linked genes. Tumors expressing high CD44 have higher mutation burden and afflict older patients than tumors expressing low CD44. Cell lines expressing high CD44 are more resistant to anti-cancer drugs compared to those expressing low CD44. Protein-protein interaction investigations and functional enrichment analysis showed that CD44 interacts with gene products related to cell-substrate adhesion, migration, platelet activation, and cellular response to stress. KEGG pathway analysis revealed that these genes play key roles in biological adhesion, cell component organization, locomotion, G- α -signaling and the response to stimulus. Overall, this investigation reveals that CD44 play significant roles in tumorigenesis, can be used as a prognostic biomarker in several cancers and can be therapeutically targeted in cancer therapy.

Keywords: CD44, Cancer Stem Cells, Tumorigenesis, Drug Resistance, Immune Markers, Epithelial to Mesenchymal Transition, and Therapeutic Targeting.

Introduction

Tumors are a mass of cancer cells, stromal cells, and the extracellular matrix (ECM), forming a heterogeneous mixture of cells from multiple sources [1-4]. Several reports have shown that tumor cells, stromal cells and the ECM change during the different stages of cancer development [5-10]. The main contributors to tumor heterogeneity are tumor cell evolution, stromal component contribution, and spatial differences occurring during tumor cell differentiation [11-16]. Based on the hierarchical organization of tumor cells, cancer stem cells are tumor-initiating cells capable of self-renewing and differentiating into non-tumorigenic cells [14, 17, 18]. Thus, cancer stem cells propel tumor formation, its progression, and the formation of secondary tumors [19-21]. In addition, cancer stem cells have been implicated in drug resistance and cancer relapse [14, 18, 20]. Various reports have demonstrated the presence of CSCs in many tumors, including those of the colon, brain, esophageal, cervix and skin [22-25]. While investigations are continuing on the role of CSCs, recent reports demonstrate that they are linked to poor prognosis in several cancers [14, 17, 18, 26]. We recently demonstrated that isolated CSC-like cells via the side population technique formed more colonies on ECMs compared to normal cancer cells, even when they are both challenged with drugs [1]. Thus, CSCs display drug resistance properties when challenged with commonly used drugs, including cisplatin and 5-fluorouracil [1]. Other studies have shown that while drugs destroy cancer cells, CSCs remain and may re-form the tumors or form tumors in other body regions [27-31]. The targeting of CSCs with therapeutic agents is gaining ground and, together with cytotoxic drugs, will usher in novel strategies to treat and manage cancers. The characterization of CSCs and their properties is of paramount importance in the treatment of cancer.

The characterization of CSCs entails the identification of several cell surface markers, including CD44, CD133, and ALDH1 [17-19]. Many studies have shown that CD44, for example, is expressed highly in many cancers, coupled with low expression of CD24 [14, 17, 18, 32-35]. In addition, CD133 is a well-known marker of colorectal CSCs [36, 37]. As recently reviewed by Dzobo and colleagues, other markers used to characterize CSCs in different cancers are CK17, CD105, ABCG2, EpCAM, and BCRP1 [18]. One limitation of using CSC markers is that markers are not universal, and the expression of different markers cannot be correlated with each other. Furthermore, subtypes of the same cancer may also express CSC markers differently. Overall, there are limitations on using CSC markers in different cancers, making further studies on CSCs and their properties warranted.

CD44 belongs to a family of transmembrane glycoproteins expressed by various cells, including stem cells [38, 39]. CD44 is a CSC marker and exists in several isoforms [40-42]. The standard form of CD44 is referred to as CD44s. The binding of CD44 to its main ligand, hyaluronic acid, through the ligand-binding domain causes structural changes leading to many signaling cascades being activated, with many cascades involved in cellular proliferation, adhesion, and migration (Figure 1) [43, 44]. Besides binding to its ligands, CD44 has been shown to sequester growth factors and thereby participates in growth factor signaling [45]. CD44 has been implicated in various processes in cancer cells, such as epithelial to mesenchymal transition, drug resistance, and metastasis [46, 47].

By playing key roles, including maintenance of stemness and promotion of tumorigenesis, CD44 has been suggested to be a relevant diagnostic and prognostic biomarker in several cancers.

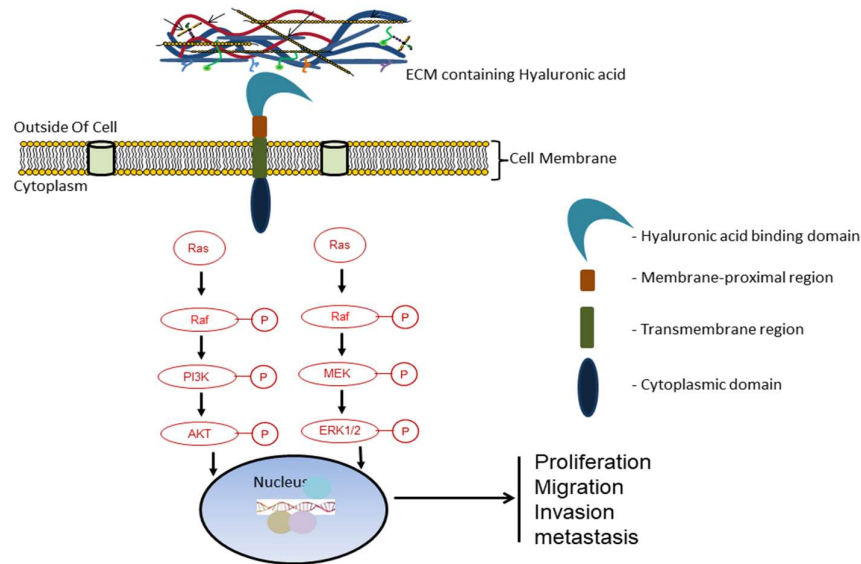


Figure 1. CD44 glycoprotein contains the ligand-attaching domain, the membrane-proximal domain, the transmembrane section as well as the cytoplasmic tail. The binding of its ligand, for example, hyaluronic acid, results in the activation of various downstream signaling cascades involved in cellular proliferation, migration, invasion, and metastasis. The figure is adapted from [48].

Several studies have shown that the expression of CSC markers can be linked to properties of tumors such as invasiveness and metastatic behavior. For example, Abraham and colleagues demonstrated that the expression of CD44 is associated with the formation of distant metastases by breast cancer cells [49]. Several other studies demonstrated similar results, clearly proving that CD44 expression can be linked to certain properties of a sub-population of cancer cells [50-52]. However, a more in-depth and systemic analysis of the relationship between CSC markers and the properties of cancer cells are needed. In addition, studies on the relationship between CSC markers expression and tumorigenesis can be limited due to the lack of tumor samples for certain cancers.

Furthermore, extensive studies have shown that stromal cells such as CAFs, CAMs, and ECM, impact tumor cells' response to therapy, including chemotherapy [1, 53]. Stromal components regulate cancer cell behavior, including CSCs properties via mutual interactions [11, 13, 18, 30]. In this study, we utilized publicly available databases and bioinformatic techniques to analyze the possible role of CSC markers in tumorigenesis and drug resistance. This study investigated the link between CSC markers expression and markers of inflammation, stromal, and immune infiltration as well as drug resistance markers. Our data show that CD44 expression is closely linked with immune infiltration and immune suppressive features in pancreatic, colon adenocarcinoma and stomach cancer and high CD44 expression was significantly correlated with the expression of drug resistance-, EMT- and metastasis- linked genes.

Materials and Methods

Databases and RNA-seq / Gene Expression Analysis

This study utilized datasets from publicly available databases including TCGA (<http://cancergenome.nih.gov>), GEPIA (<http://gepia.cancerpku.cn>) [54], Oncomine database (<https://www.oncomine.org>), PrognScan database (<http://www.abren.net/PrognScan>), Tumor Immune Estimation Resource (TIMER) database (<http://cistrome.org/TIMER/>) and The Human Protein Atlas (www.proteinatlas.org), as previously described and did not involve human subject recruitment or animal studies. Whole-genome messenger RNA expression levels of several genes involved in inflammation, drug resistance, metastasis, CAF, EMT, and immune markers were examined in tumor and normal adjacent tissues (Match TCGA normal data) in relation to CD44 gene expression as well as patients' survival outcomes. Databases were accessed for different cancers in 2020 through the respective portals.

RNA-seq and Correlation Analysis

Three databases namely: GEPIA (<http://gepia.cancer-pku.cn>), Oncomine (<https://www.oncomine.org>), and the PrognScan databases (<http://www.abren.net/PrognScan>), were used for mRNA/gene expression analysis in normal versus tumor tissues in several cancers. Furthermore, these same databases were also used to study CD44 expression's correlation with patients' prognosis in several cancers [55-59]. Parameters were set as fold change required: >1.5; Gene rank: 10%; $p < 0.001$ for Oncomine analysis. For correlation analysis using the PrognScan analysis, the threshold for p-value was set at $p < 0.05$.

Kaplan-Meier Plot

This study utilized the Kaplan-Meier survival analysis method to associate gene expression with survival data in several cancers (<https://kmplot.com/>) [60]. Survival graphs (overall and Disease-free) were generated for cancer patients displaying low and high CD44 gene expression. P values (Log-rank) were used to evaluate statistical significance.

Tumor Infiltration Analysis

To investigate immune cell infiltration into tumors, the TIMER database (<http://cistrome.org/TIMER/>) [61] was used. The TIMER database uses cancer data from the TCGA and calculates gene expression association with immune infiltration. In this study, CD44 was correlated with immune cell gene markers as indicated. Spearman's correlation was used to calculate correlation coefficients with gene expression shown as log2 RSEM.

Cancer Cell Lines Dose Responses

Dose responses of cancer cell lines was obtained from the Genomics of Drug Sensitivity in Cancer (GDSC) database (<https://www.cancerrxgene.org/>) [62]. Messenger RNA transcription levels of CD44 in cancer cell lines were obtained from the Cancer Cell Line Encyclopaedia (CCLE) (<https://portals.broadinstitute.org/ccle>) [63]. Cancer cell lines were grouped into low

and high CD44 expression and the Welch test was used to compare average differences in the z-score transformed IC50 values between the two groups for the anti-cancer drugs.

Protein-Protein Interaction Networks

The GeneMANIA database (<http://genemania.org>) [64] was utilized for protein-protein networks construction and investigate the functions of interactive genes. GeneMANIA displays genes that are co-expressed, co-localized and physically interact with CD44.

Gene Functional Enrichment Analysis

Metascape (<https://metascape.org>) was used to delineate pathway enrichment and to annotate biological processes associated with CD44. This website integrates data from different sources such as Kyoto Encyclopaedia of Genes and Genomes and Gene Ontology to provide a comprehensive data analysis on enrichment and biological processes.

Data Availability

Data used for the analysis shown in this study are available from the following databases: The Cancer Genome Atlas (TCGA) (<https://www.cancer.gov/>); Gene Expression Profiling Interactive Analysis (GEPIA) (<http://gepia.cancer-pku.cn/index.html>); GeneMANIA (<https://genemania.org/>); Tumor IMMune Estimation Resource (TIMER) (<https://cistrome.shinyapps.io/timer/>); Genomics of Drug Sensitivity in Cancer (GDSC) (<https://www.cancerrxgene.org/>); Cancer Cell Line Encyclopaedia (CCLE) (<https://portals.broadinstitute.org/ccle>), and PrognoScan (<http://dna00.bio.kyutech.ac.jp/PrognoScan/>).

Statistical Analysis

GraphPad was used for all statistical analyses (GraphPad version 6). All survival curves were displayed with HR and p-values (log-rank test). Spearman's correlation was utilized for the comparison between CD44 and all other genes, including data from GEPIA and TIMER. For the Welch test was used to compare variables for normally distributed data. Statistical significance was considered significant when $p < 0.05$ or as indicated with data.

Results

The role of CD44 in tumorigenesis and as a prognostic marker in human cancers

Many reports have revealed differential expression of CD44 in different cancers. In this study, we determined CD44 mRNA expression in normal and in tumor tissues for several cancers via the use of the Oncomine database. Our analysis shows that CD44 expression was significantly higher in brain, CNS, colorectal, gastric, head and neck (HNSC) and kidney cancers, melanoma, and sarcoma tissues in comparison to adjacent normal tissues (Figure 2 A). Further analysis of RNA-seq data via the use of TIMER showed significantly higher CD44 expression levels in

tumor tissues compared to normal tissues in several cancers, including cholangiocarcinoma, colorectal, HNSC, esophageal carcinoma and stomach adenocarcinoma (Figure 2 B). Protein expression data based on The Human Protein Atlas database (<http://www.proteinatlas.org/>) [65, 66] mostly agree with RNA-seq data. For example, 10 out of 12 colorectal patients show medium to high CD44 protein expression, while 11 out of 11 melanoma patients show medium to high CD44 protein expression based on immunohistochemistry data. One of the most lethal cancers is pancreatic cancer, and protein data based on The Human Protein Atlas database show that 6 out of 10 pancreatic cancer patients have medium to high CD44 protein expression.

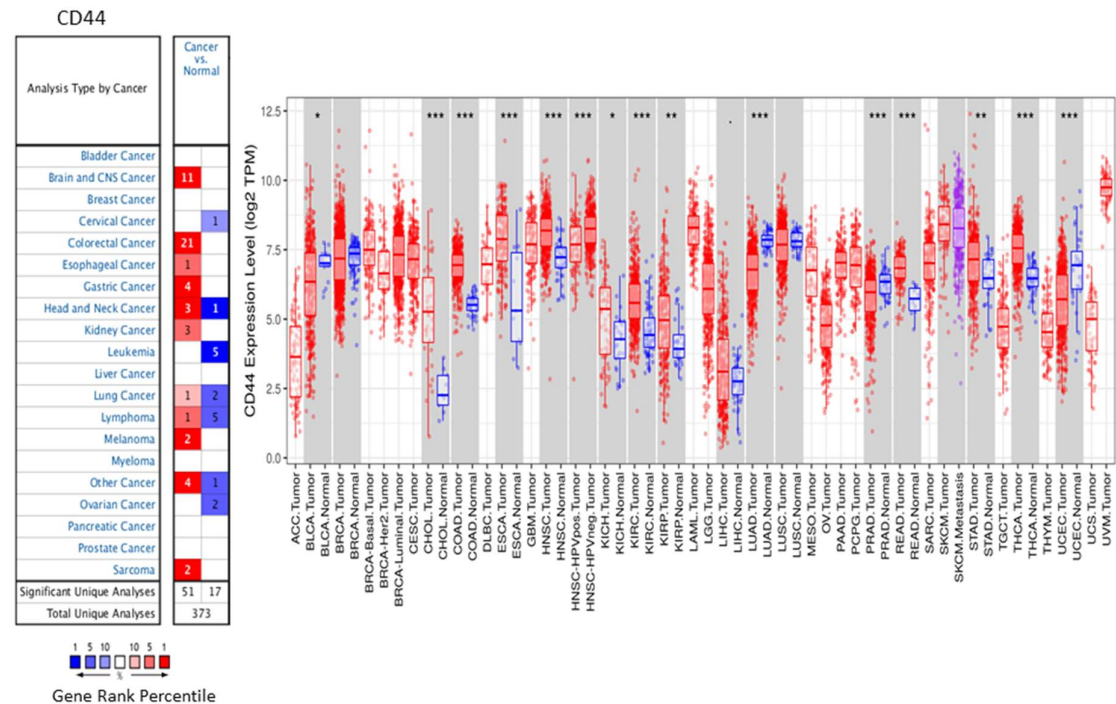
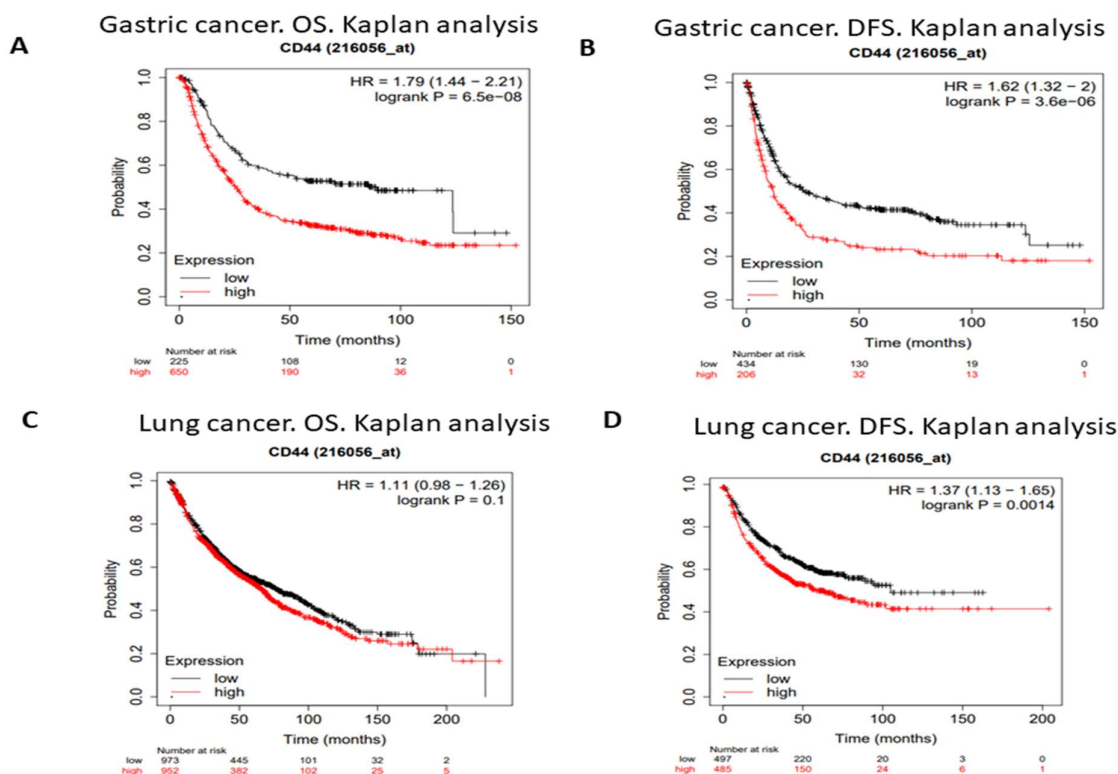


Figure 2. CD44 expression in different types of human cancers. (A) CD44 expression in tumors and normal tissues based on data from Oncomine database. P value was set as: p value < 0.0001; Only fold change > 2 was considered; Gene rank was set at 10%. (B) CD44 expression in tumors and adjacent normal tissues based on data from TIMER database (p: 0 ≤ *** < 0.001 ≤ ** < 0.01 ≤ * < 0.05 ≤ . < 0.1).

Association of CD44 expression and the prognosis of cancer patients revealed contrasting data of the patients' survival between patients: (i) those with tumors displaying high CD44 expression and (ii) those with tumors displaying low CD44 expression (Figure 3 A-H; Figure 4 A-F). Three different databases were used to cater to different cancers. Through the use of the Kaplan-Meier plotter, significant association between gastric cancer patients' survival and high CD44 expression was observed. Specifically, gastric patients with tumor displaying enhanced CD44 expression were associated with significantly low overall survival (HR = 1.79; 95% confidence interval = 1.44-2.21; p = 6.5 x 10⁻⁸) and disease-free survival (HR = 1.62; 95% confidence interval = 1.32-2.0; p = 3.6 x 10⁻⁶) compared to patients with tumors displaying low CD44 expression (Figure 3 A-B). In lung cancer, patients with tumors displaying enhanced CD44 expression had significantly lower disease-free survival (HR = 1.37; 95% confidence interval = 1.13-1.65; p = 0.0014) compared to those with low CD44 expression, with the overall survival (HR = 1.11; 95% confidence interval = 0.98- 1.26; p = 0.1) being similar for patients with high and low CD44 expression (Figure 3 C-D). Liver cancer analysis revealed interesting results, with

those showing high CD44 expression having significantly prolonged disease-free survival compared to those with low CD44 expression (HR = 0.71; 95% confidence interval = 0.51-0.99; $p = 0.042$) (Figure 3 F). Overall survival of liver cancer patients was similar regardless of level of CD44 expression (HR = 1.42; 95% confidence interval = 0.97 – 2.07; $p = 0.069$) (Figure 3 E). Through the use of GEPIA database, our analysis showed that both breast cancer and esophageal cancer patients show similar survival (Overall, Disease-free) regardless of CD44 expression levels (Figure 3 G-H; Figure 4 A-B). Pancreatic cancer patients with tumors displaying low and high CD44 expression showed significant differences in overall survival. Specifically, pancreatic cancer patients with tumors displaying enhanced expression of CD44 were linked to poor overall survival (HR = 1.8; $p = 0.0045$) (Figure 4 C-D). CD44 expression and disease-free survival in pancreatic cancer patients showed no significant association. Lastly, using the Prognoscan analysis, our data show that colorectal cancer patients with tumors displaying enhanced CD44 expression had significantly reduced overall survival compared to those with low CD44 expression (HR = 7.20; Cox $p = 0.038$) (Figure 4 E). Disease-free survival was not significantly different between colorectal patients with tumors displaying low and high CD44 expression (Figure 4 F).



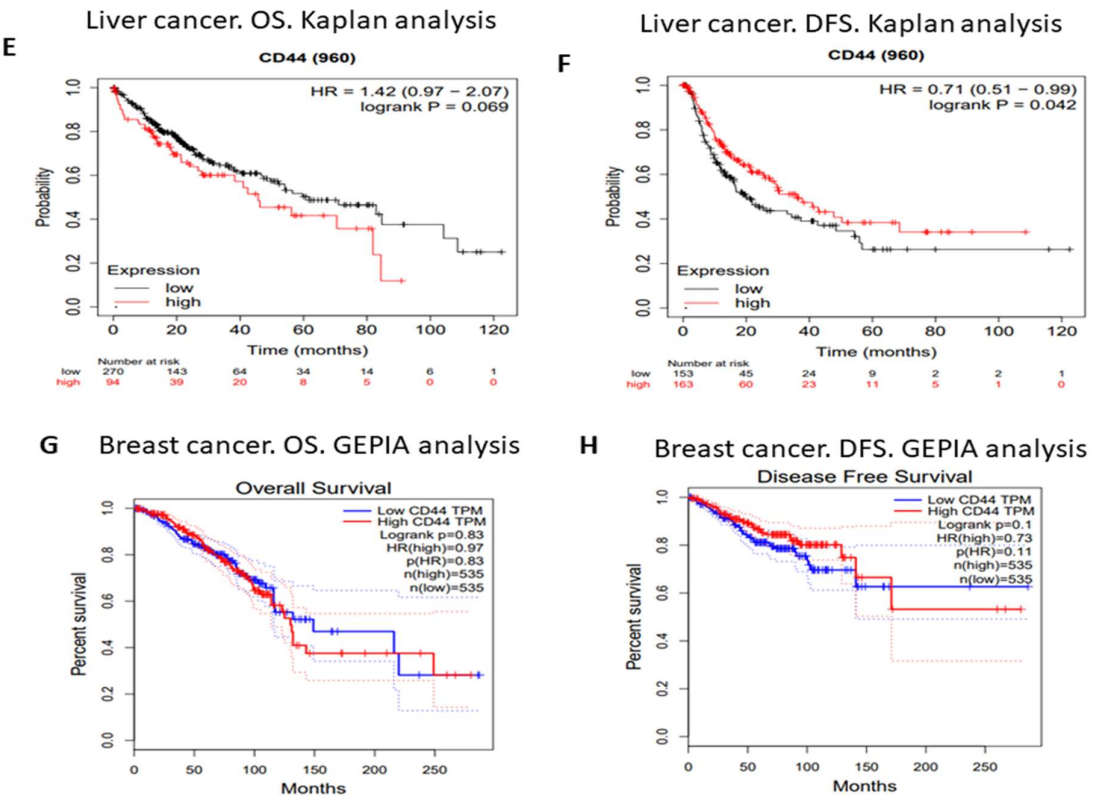
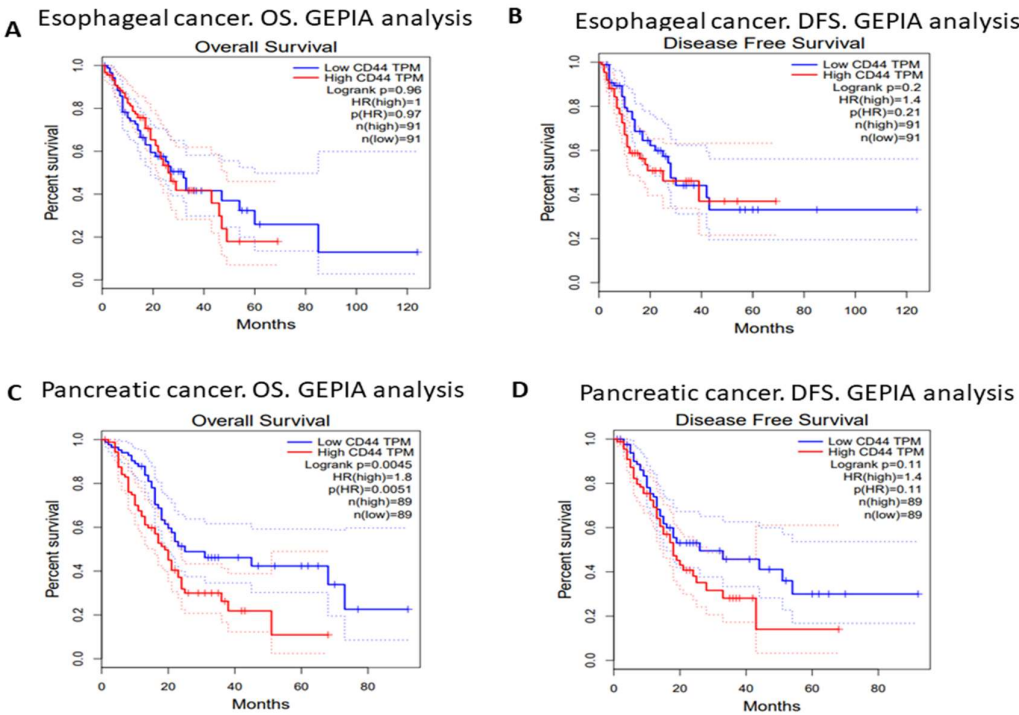


Figure 3 (A-B). Correlation between expression of CD44 and survival of gastric cancer patients based on data from Kaplan-Meier plotter. (C-D). Correlation between expression of CD44 and survival of lung cancer patients based on data from Kaplan-Meier plotter. (E-H). Correlation between expression of CD44 and survival of liver cancer patients based on data from Kaplan-Meier plotter. (G-H). Correlation between expression of CD44 and survival of breast cancer patients based on data from GEPIA plotter.



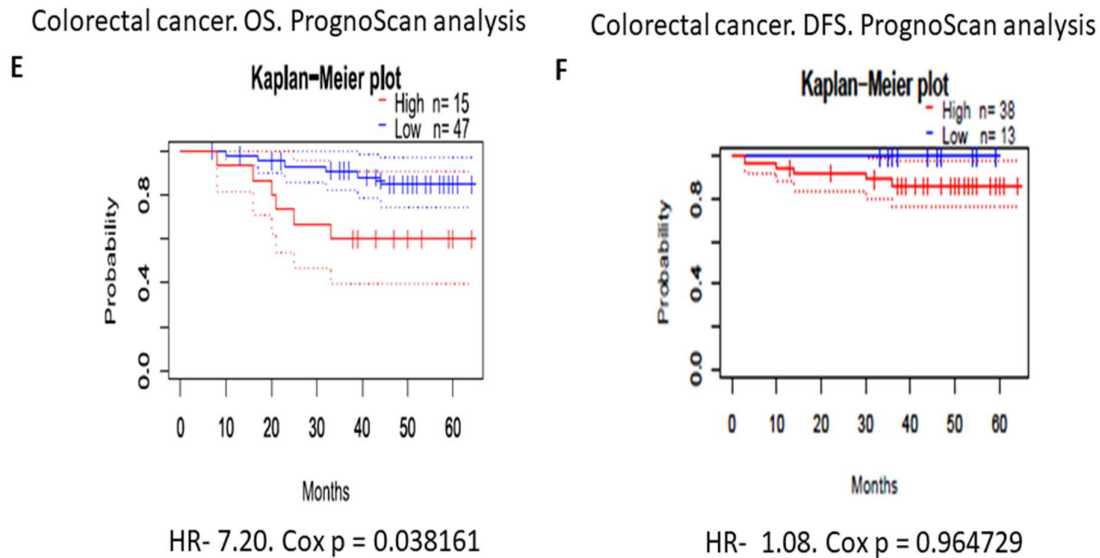


Figure 4 (A-B) Correlation between expression of CD44 and survival of esophageal cancer patients based on data from GEPIA plotter. (C-D) Correlation between expression of CD44 and survival of pancreatic cancer patients based on data from GEPIA plotter. (E-F) Correlation between expression of CD44 and survival of colorectal cancer patients based on data from PrognScan plotter.

Overall, our analysis revealed that colorectal and pancreatic cancer patients with tumors displaying enhanced CD44 expression were linked to poor overall survival, and its expression may be useful as a biomarker for prognosis prediction.

CD44 expression is linked to infiltration of immune cells and immunosuppression in human cancers

Several studies have demonstrated the link between cancer stem cells, tumors, immune suppression, and evasion [67-70]. Therefore, this study investigated the link between the expression of CD44, immune cell infiltration into tumors, and immune markers using TIMER. Our data show that tumor purity and CD44 expression were positively correlated in COAD and ESCA ($p < 0.05$) (Figure 5 A-B). The expression of CD44 was significantly correlated ($p < 0.05$) with infiltration of all immune cells used in the analysis (B cell, CD8+ T cell, CD4+ T cell, macrophage, neutrophil and dendritic cell) in COAD (Figure 5 A). In ESCA, CD44 was significantly correlated ($p < 0.05$) with B cell, CD4+ T cell and dendritic cell infiltration (Figure 5 B). In HNSC, CD44 expression was significantly correlated ($p < 0.05$) with infiltration of all immune cells used in the analysis except macrophages (B cell, CD8+ T cell, CD4+ T cell, neutrophil and dendritic cell) (Figure 5 C). CD44 expression was significantly correlated ($p < 0.05$) with B cell, CD8+ T cell, macrophage, neutrophil and dendritic cell infiltration in pancreatic adenocarcinoma (Figure 5 D).

Immune cells infiltrating into tumors have been linked to various pro-tumorigenic processes including aiding tumor cells to evade immune detection. The link between CD44 expression and immune suppressive features in human cancers was investigated via correlation analysis between CD44 expression and immune cell markers' expression (Figure 6, Table 1, 2, 3). For this analysis,

pancreatic adenocarcinoma, colon adenocarcinoma, and stomach adenocarcinoma were chosen and analyzed using the TIMER database. Correlation analysis was performed with adjustment for tumor purity. CD44 expression was significantly correlated with FOXP3, a Treg marker, in PAAD, COAD, and STAD ($p < 0.05$) (Figure 6 A). In addition, CD44 was significantly correlated with MRC1 expression, a myeloid cell marker, in PAAD, COAD, and STAD ($p < 0.05$) (Figure 6 B). Finally, CD44 expression was significantly correlated with the expression of

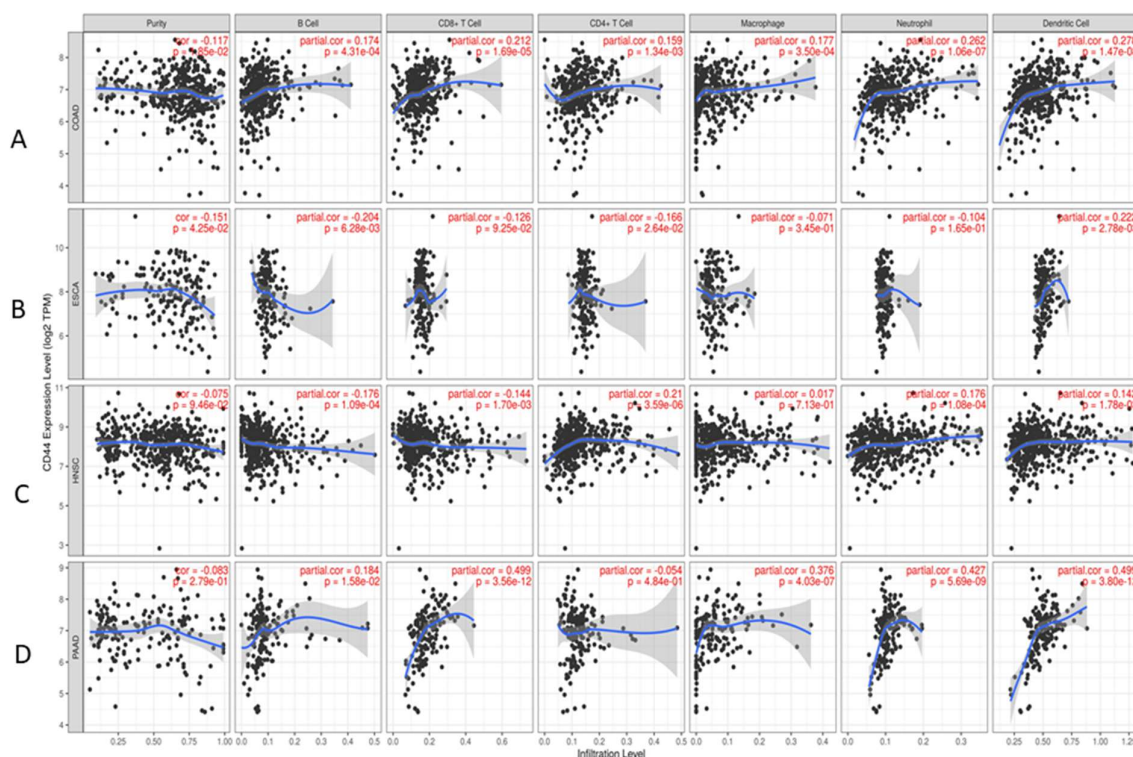


Figure 5 (A-D). Correlation of expression of CD44 with immune infiltration level in several tumors as analyzed using TIMER. (A) Colon adenocarcinoma (COAD), (B) Esophageal carcinoma (ESCA), (C) Head and Neck cancer (HNSC), (D) Pancreatic adenocarcinoma (PAAD).

immune regulatory factors CTLA4 and CCL2 expression in PAAD, COAD, and STAD ($p < 0.05$) (Figure 6 C-D). This study investigated the link between forms of CD44 copy number and immune cells in several cancers and this was shown to be significant. In PAAD, CD44 arm-level deletion was significantly correlated with CD4+ T cell ($p < 0.0001$) and neutrophil ($p < 0.01$) infiltration (Figure 7 A). In COAD, CD44 arm-level gain was significantly correlated with CD8+ T cell ($p < 0.0001$) and neutrophil ($p < 0.01$) infiltration (Figure 7 B). In STAD, CD44 arm-level deletion, and arm-level gain were significantly correlated with immune cell infiltration (Figure 7 C). This study also investigated the link between the age of patients and the mutation load of tumors afflicting them and found a significant positive correlation (Figure 7 D). This means tumors expressing high CD44 afflict, mostly older patients than those expressing low CD44, and the high CD44 tumors have a higher mutation burden than low expressing tumors (Figure 7 D).

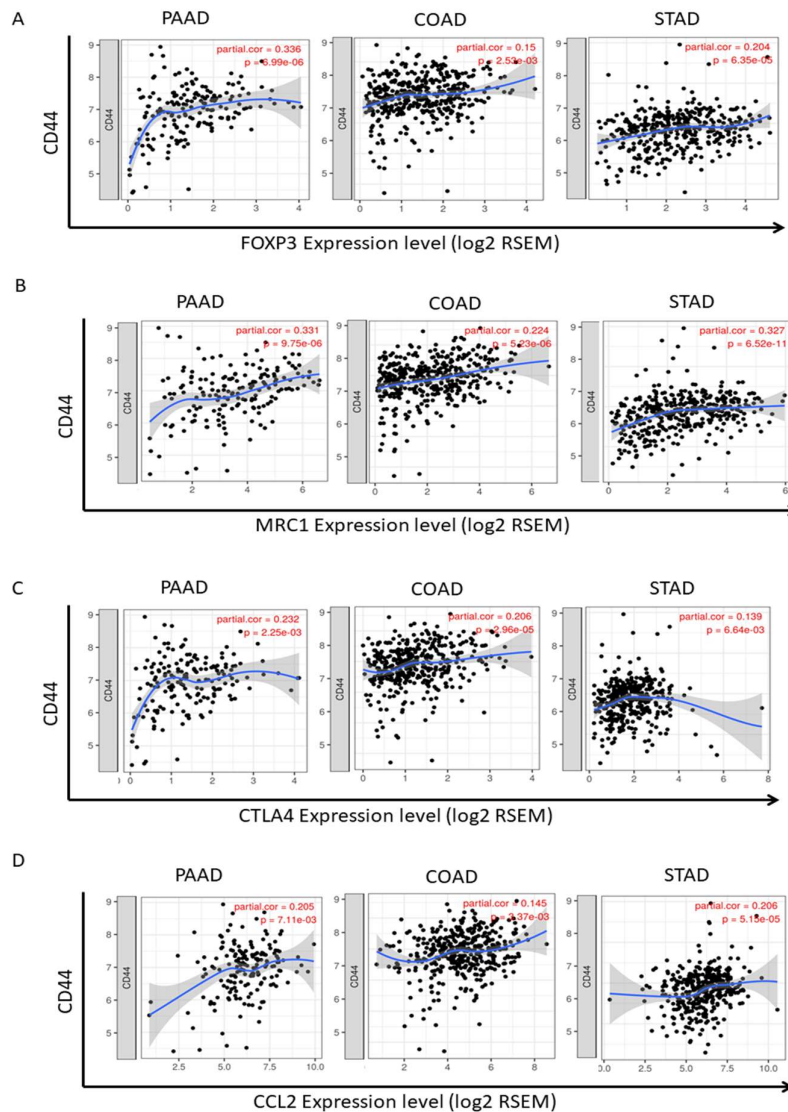


Figure 6. Correlation analysis between expression of CD44 and immune markers in PAAD, COAD and STAD using TIMER. (A) Correlations between CD44 expression and FOXP3 expression in PAAD, COAD and STAD (B) Correlations between CD44 expression and MRC1 expression in PAAD, COAD and STAD (C) Correlations between CD44 expression and CTLA4 expression in PAAD, COAD and STAD (D) Correlations between CD44 expression and CCL2 expression in PAAD, COAD and STAD.

In summary, our data show that CD44 expression was significantly correlated with immune cell infiltration into tumors and immune markers' expression (Figures 5, 6; Tables 1, 2, 3). Overall, these data show that CD44 expression is closely linked with immune infiltration and immune suppressive features of human tumors, promoting tumorigenesis.

Table 1. Correlation analysis between CD44 and immune cell marker gene expression in PAAD based on data from TIMER.

| Gene | Gene Marker | Correlation conditioned on tumor purity | p |
|------|--------------|---|-------|
| CD44 | CD19 | 0.061 | 0.429 |
| CD44 | CD68 | 0.387 | *** |
| CD44 | CD86 | 0.392 | *** |
| CD44 | IL10 | 0.204 | * |
| CD44 | COX2 (PTGS2) | 0.388 | *** |
| CD44 | CD163 | 0.369 | *** |
| CD44 | CCR7 | 0.169 | * |
| CD44 | CCR8 | 0.395 | *** |
| CD44 | CD8A | 0.235 | ** |
| CD44 | GATA3 | 0.31 | *** |
| CD44 | PDCD1 | 0.201 | * |
| CD44 | IL2RA | 0.452 | *** |
| CD44 | STAT1 | 0.502 | *** |
| CD44 | STAT3 | 0.468 | *** |
| CD44 | STAT4 | 0.171 | * |
| CD44 | STAT6 | 0.298 | *** |
| CD44 | TGFB1 | 0.297 | *** |
| CD44 | KIR2DL3 | 0.226 | ** |
| CD44 | KIR3DL1 | 0.077 | 0.316 |
| CD44 | HLA-DRA | 0.349 | *** |

*P <0.05; **<0.005; ***P<0.0005

Table 2. Correlation analysis between CD44 and immune cell marker gene expression in COAD based on data from TIMER

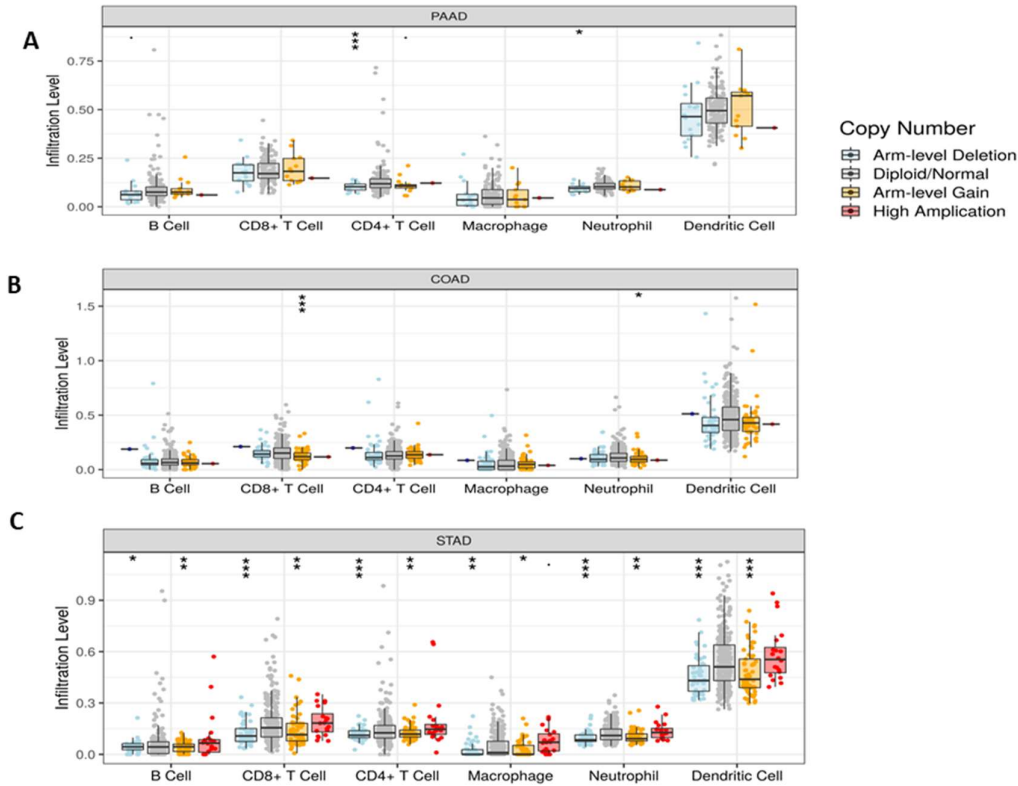
| Gene | Gene Marker | Correlation conditioned on tumor purity | p |
|------|--------------|---|-------|
| CD44 | CD19 | 0.107 | * |
| CD44 | CD68 | 0.141 | ** |
| CD44 | CD86 | 0.248 | *** |
| CD44 | IL10 | 0.177 | *** |
| CD44 | COX2 (PTGS2) | 0.228 | *** |
| CD44 | CD163 | 0.235 | *** |
| CD44 | CCR7 | 0.116 | * |
| CD44 | CCR8 | 0.171 | ** |
| CD44 | CD8A | 0.176 | *** |
| CD44 | GATA3 | 0.11 | * |
| CD44 | PDCD1 | 0.163 | ** |
| CD44 | IL2RA | 0.259 | *** |
| CD44 | STAT1 | 0.234 | *** |
| CD44 | STAT3 | 0.369 | *** |
| CD44 | STAT4 | 0.256 | *** |
| CD44 | STAT6 | 0.103 | * |
| CD44 | TGFB1 | 0.113 | * |
| CD44 | KIR2DL3 | 0.045 | 0.368 |
| CD44 | KIR3DL1 | -0.003 | 0.959 |
| CD44 | HLA-DRA | 0.248 | *** |

*P <0.05; **<0.005; ***P<0.0005

Table 3. Correlation analysis between CD44 and immune cell marker gene expression in STAD based on data from TIMER

| Gene | Gene Marker | Correlation conditioned on tumor purity | p |
|------|--------------|---|-----|
| CD44 | CD19 | 0.137 | * |
| CD44 | CD68 | 0.226 | *** |
| CD44 | CD86 | 0.311 | *** |
| CD44 | IL10 | 0.219 | *** |
| CD44 | COX2 (PTGS2) | 0.146 | ** |
| CD44 | CD163 | 0.323 | *** |
| CD44 | CCR7 | 0.233 | *** |
| CD44 | CCR8 | 0.332 | *** |
| CD44 | CD8A | 0.228 | *** |
| CD44 | GATA3 | 0.145 | ** |
| CD44 | PDCD1 | 0.168 | ** |
| CD44 | IL2RA | 0.333 | *** |
| CD44 | STAT1 | 0.21 | *** |
| CD44 | STAT3 | 0.393 | *** |
| CD44 | STAT4 | 0.273 | *** |
| CD44 | STAT6 | 0.338 | *** |
| CD44 | TGFB1 | 0.251 | *** |
| CD44 | KIR2DL3 | 0.142 | * |
| CD44 | KIR3DL1 | 0.176 | ** |
| CD44 | HLA-DRA | 0.284 | *** |

*P <0.05; **<0.005; ***P<0.0005



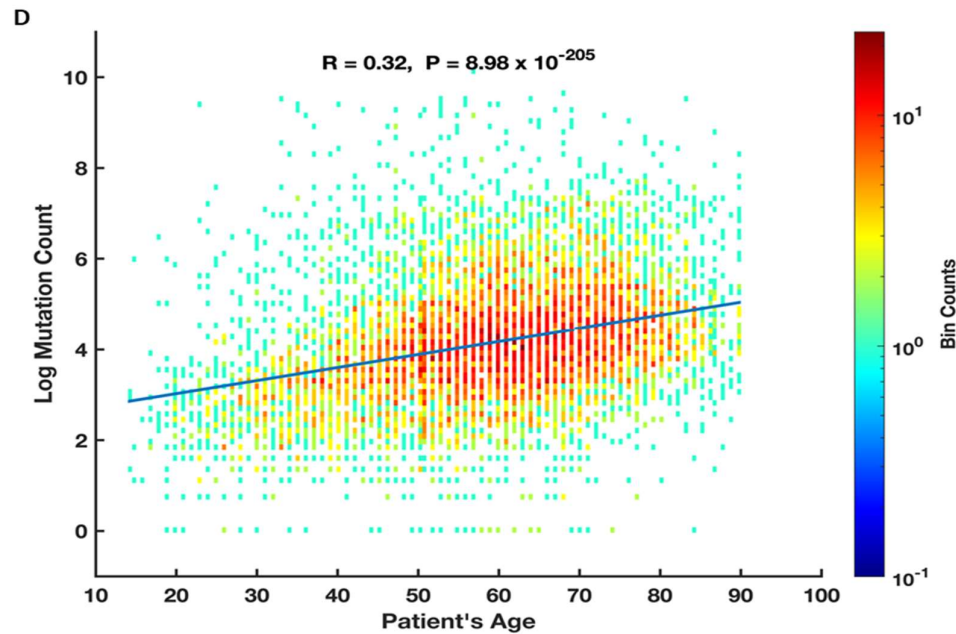


Figure 7. A-C Association between CD44 copy number variation and immune cells infiltration in PAAD, COAD and STAD. P: ***: < 0.0001; **: 0.001; * < 0.01. (D) Pearson's linear correlation between age of patients and mutations revealed in tumors of patients shown as a binned scatter plot.

CD44 correlation with Drug Resistance, EMT, and Metastasis

We further investigated the association between CD44 expression and drug resistance and metastasis. Through the use of the TIMER database, our analysis revealed a significant positive correlation between CD44 expression and ABCC1, ABCC2, ABCC3, and ABCB1 expression in PAAD ($p < 0.05$) (Figure 8 A). In COAD, a significant positive correlation was observed between CD44 expression and ABCC1 gene expression only ($p < 0.05$) (Figure 8 B). Our analysis shows a significant positive correlation between CD44 expression and ABCC1, ABCC3, and ABCB1 expression in STAD ($p < 0.05$) (Figure 8 C). Further analysis of the response of different cell lines expressing both low and high CD44 expression show that those expressing high CD44 mostly have high IC50 compared to those with low IC50, confirming the association between high CD44 expression and drug resistance (Figure 8 D and E). Specifically, dose-responses of cancer cells from the GDSC database to small molecule inhibitors in combination with CD44 mRNA transcription from the CCLE database was used to examine how CD44 transcript levels in cancer patients' tumors could influence anti-cancer drug effectiveness. Cancer cells expressing high CD44 were more resistant to anti-cancer drugs than those expressing low CD44 (Figure 8 D). In addition, high CD44 expressing cancer cells were mostly resistant to top 30-ranked drugs (Figure 8 E). CD44 expression may be important in predicting response of patients' tumors to therapeutic drugs. The acquisition of the EMT phenotype results in cancer cells with enhanced invasive abilities and being metastatic. Data from many research investigations point to the association between CSCs and epithelial to mesenchymal transition (EMT). Also, reports indicate that acquisition of the EMT phenotype is linked to drug resistance. We, therefore, investigated the correlation between CD44 expression and EMT markers. Our investigations showed a significant positive correlation ($p < 0.05$) between CD44 expression and TGF-B1, TWIST1, VIMENTIN, and ZEB1 expression in PAAD and STAD (Figure 9 A, C). In

COAD, a significant positive correlation ($p < 0.05$) was observed between CD44 expression and TGF-B1 and VIMENTIN expression only (Figure 9 B).

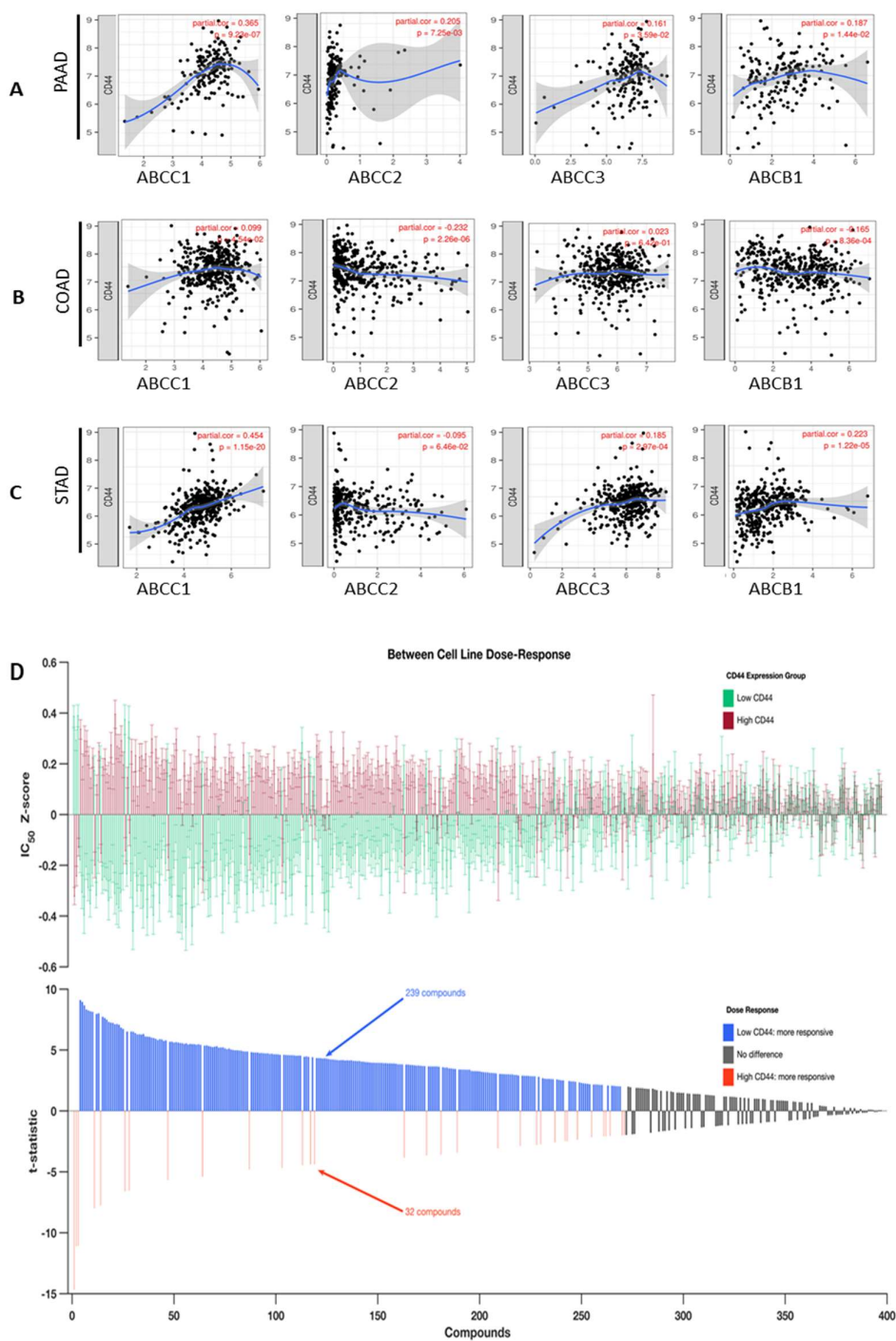




Figure 8. A-C Correlation between CD44 expression and Drug resistance genes in PAAD, COAD and STAD using TIMER. (A) Correlations between CD44 expression and ABCC1, ABCC2, ABCC3 and ABCB1 expression in PAAD (B) Correlation between CD44 expression and ABCC1, ABCC2, ABCC3 and ABCB1 expression in COAD (C) Correlation between CD44 expression and ABCC1, ABCC2, ABCC3 and ABCB1 expression in STAD. (D) Data from GDSC cancer cell lines showing dose-responses between cells expressing high and low CD44. Top panel show IC50 values for cells expressing high CD44 (red) and those expressing low CD44 (green). Bottom panel show Welch test for t-value for anti-cancer drugs used in the dose-response analysis. Drugs shown in bottom panel are matched to columns in the top panel. High-CD44 cancer cells lines respond more to drugs indicated in orange whilst low CD44 cancer cells lines respond more to drugs indicated in blue. Grey is used to indicate drugs to which both high- and low-CD44 cancer cells do not respond. (E) Top 30 drugs demonstrating enhanced significant differences in dose response between low- and high-CD44 cancer cell lines. Standard error of median logarithm IC50 for cancer drugs is shown as error bars. *** $p < 0.001$.

It is known that many proteins that are characteristic of highly metastatic tumors play crucial roles during tumorigenesis and dissemination of tumor cells. Our analysis shows a significant positive correlation between CD44 and SNED1, MMP1, S100A2, and EGLN1 expression in PAAD as well as in STAD ($p < 0.05$) (Figure 10 A, C). In COAD, a significant positive correlation between CD44 expression and SNED1, MMP1, and EGLN1 expression ($p < 0.05$) (Figure 10 B). Thus, these identified biomarkers can serve as prognostic as well as diagnostic tools in these cancers.

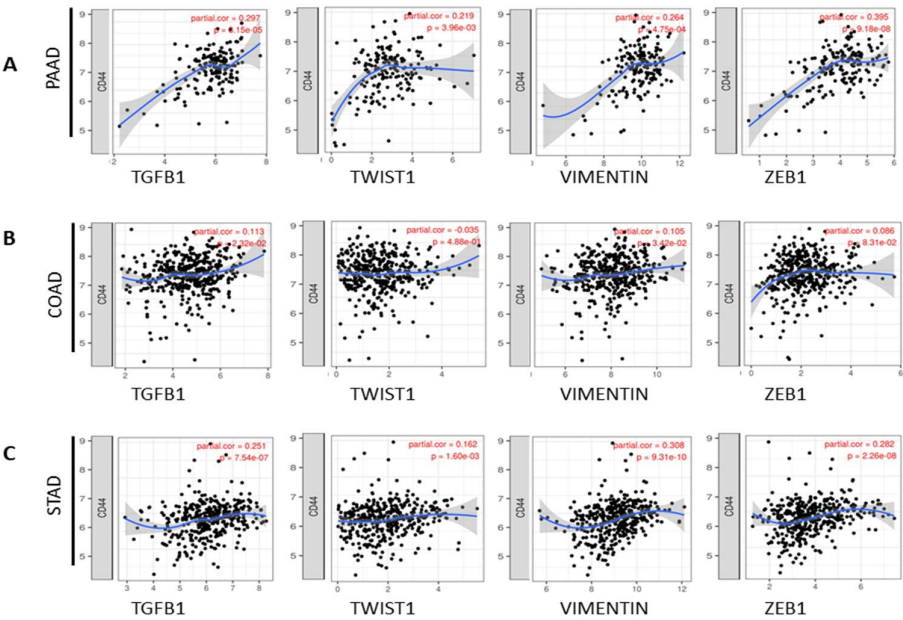


Figure 9. A-C Correlation analysis between CD44 expression and EMT-linked genes in PAAD, COAD and STAD using TIMER. (A) Correlations between CD44 expression and TGFB1, TWIST1, VIMENTIN and ZEB1 expression in PAAD (B) Correlations between CD44 expression and TGFB1, TWIST1, VIMENTIN and ZEB1 expression in COAD (C) Correlations between CD44 expression and TGFB1, TWIST1, VIMENTIN and ZEB1 expression in STAD.

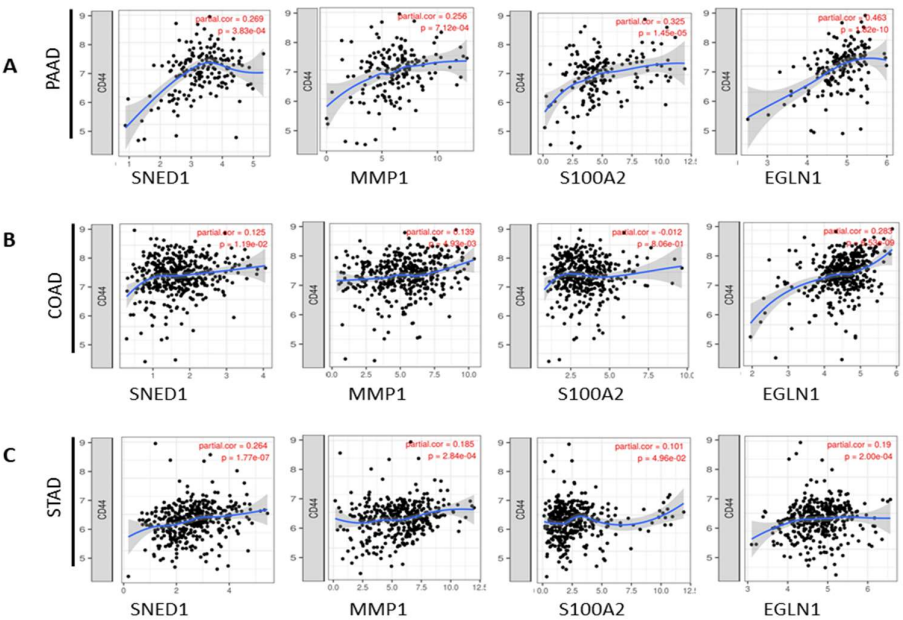


Figure 10. A-C Correlation analysis between CD44 expression and metastasis-linked genes in PAAD, COAD and STAD using TIMER. (A) Correlations between CD44 expression and SNED1, MMP1, S100A2 and EGLN1 expression in PAAD (B) Correlations between CD44 expression and SNED1, MMP1, S100A2 and EGLN1 expression in COAD (C) Correlations between CD44 expression and SNED1, MMP1, S100A2 and EGLN1 expression in STAD.

CD44 Protein-Protein Interaction Network and Functional Enrichment

It is important to identify proteins interacting with CD44 and the functional information of genes interacting with CD44. The protein-protein interaction network of CD44 was analyzed using GeneMANIA. Our analysis shows that CD44 interacts with ROCK2, NFS1, SLC3A2, DPP8, CD9, BAG1, TIAM1, PHRF1, DMP1, ITGA4, SLC7A11, COL14A1, ITGB7, COL6A6, VCAN, MMP15, TIAM2, ACAN, VAV2, and AIM1 (Figure 11 A). Functional enrichment analysis was performed using GO and KEGG pathway analysis via MetaScape. Based on Gene Ontology analysis, CD44 interacts with gene products related to cell-substrate adhesion, migration, platelet activation, and cellular response to stress (Figure 11 B). KEGG pathway analysis revealed that these genes are involved/play key roles in biological adhesion, cell component organization, locomotion, G- α -signaling, regulation of biological processes, and the response to stimulus (Figure 11 B). Network diagrams were also built based on the enriched terms (Figure 11 C).

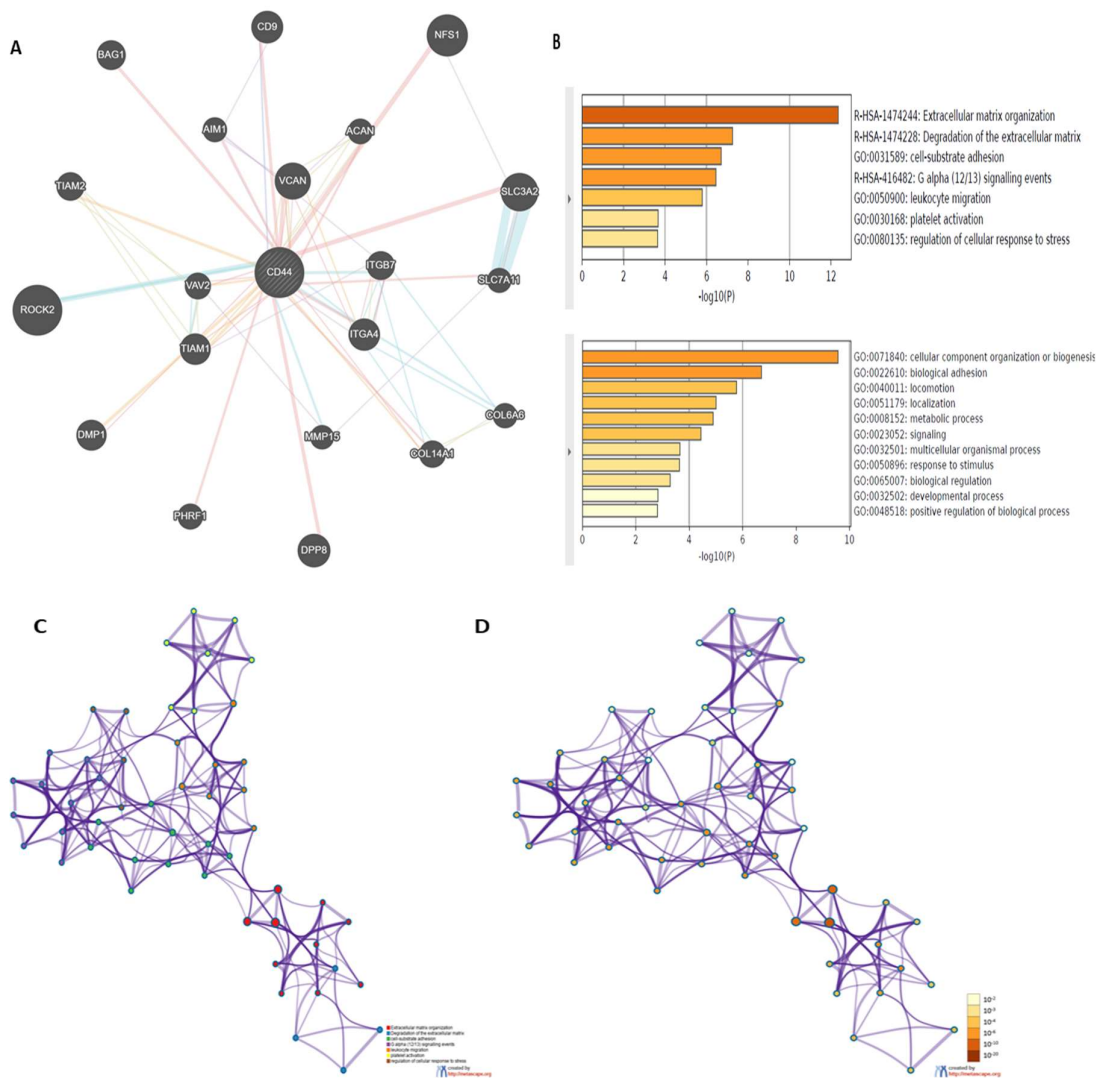


Figure 11. CD44 PPI networks and functional enrichment analysis; (A) PPI network of CD44 as depicted in GeneMANIA. (B) Gene Ontology and KEGG analysis of CD44 and its interactive genes. Enrichment

terms were coloured orange based on p values; (C-D) Interactive network showing top enrichment terms coloured by cluster ID.

Discussion

CD44 has been linked with pleiotropic functions in cancer. For example, in breast cancer, CD44 has been implicated as a ligand for P-selectin ligand and a receptor for fibrin in colon carcinoma cell adhesion [71]. While much information on CD44 is being revealed, several studies have associated enhanced CD44 expression with poor prognosis in several cancer types, including breast and colorectal cancers [35, 72-76]. In this study, we employed public databases to determine CD44 expression levels in different cancers and correlate its expression to tumorigenesis, immunosuppression, drug resistance, and metastasis. Overall, our analysis shows that CD44 is highly expressed in several cancers (tumors versus adjacent normal tissues), and its expression is associated with patient prognosis and, therefore, can serve as a prognostic biomarker in some cancers. Specifically, high CD44 expression was linked to worse overall survival in gastric, pancreatic, and colorectal cancers. Given the revealed role of CD44 in several cancer cell strategies to avoid death, targeting this cancer stem cell marker may reverse tumor immune resistance mechanisms. Other studies have shown that immune proteins, including PD-L1, are associated with CSC markers' expression in resistant colorectal cancer tissues [77]. In addition, several studies have shown that CD44 plays a key role in cytokine synthesis and secretion in LUAD [78]. Other CSC markers, such as Oct4 and Nanog, have been associated with drug resistance in cancers, including oral squamous cell carcinoma [79]. This study is the first of its kind to link CD44 expression in several cancers to cancer patients' prognosis as well as patients' tumor responses to therapeutic drugs.

Recent studies, including several of our own, have shown that tumors contain several stromal cells, including fibroblasts, macrophages, dendritic, and neutrophils [1, 2, 11, 13, 16, 80-83]. Importantly, recent reports indicate that several immune cells play key roles in tumorigenesis, drug resistance, and metastasis [84-87]. For example, regulatory T cells or Tregs have been shown to prevent the activation of CD4⁺ and CD8⁺ T cells and thus result in the suppression of anti-cancer cell immunity [88]. Our studies have shown that it is important to study the microenvironment surrounding tumor cells to understand tumorigenesis and drug resistance [1, 2]. This study therefore investigated the link between CD44 expression and immune cell infiltration and the expression of immune markers, with our analysis adjusted by tumor purity. Using COAD, ESCA, HNSC, and PAAD as our model cancers, our analysis reveals a significant positive correlation between CD44 expression and infiltration levels for several immune cells, including B cell, CD4⁺ T cell, CD8⁺ T cell, macrophage, and neutrophil. Furthermore, using PAAD, COAD, and STAD as examples of cancers, this study investigated the relationship between CD44 expression and immune markers such as FOXP3, MRC1, and CTLA4. Our data show a significant positive correlation between CD44 expression and FOXP3, MRC1, CTLA4, and CCL2 expression in PAAD, COAD, and STAD. As reviewed by Morath and colleagues, CD44 is more than a CSC marker as it is involved in several cancer cell processes such as migration and signaling [89]. Furthermore, it has been revealed that CSCs interact with immune cells via the release of cytokines and growth factors [90, 91]. Overall, our analysis shows a link between the expression of CD44 and immune cell tumor infiltration and the expression of immune markers. This may show that CD44 expression is linked to the recruitment of immune cells to tumors and the development of immunosuppression.

Cancer patients with high expression of CD44 have been associated with a worse prognosis before [92-95]. By being linked with a poor prognosis in several cancers, CD44 may therefore play key roles in several cancer processes such as EMT, cancer cell invasion, and metastasis. This study confirms this notion. Cancer cells achieve cellular plasticity via the process of epithelial and mesenchymal transition. Cancer cells transition from epithelial phenotype to a mesenchymal phenotype, allowing them to achieve enhanced migratory and invasive behavior [46, 96]. Also, cells undergoing EMT display stem-cell like properties, including chemoresistance [97-99]. Once cancer cells reach new sites, they become less migratory and invasive through the process of mesenchymal to epithelial transition (MET), allowing cancer cells to establish secondary tumors [100-102]. An increase in CD44 expression has been linked with a mesenchymal phenotype in several cancer cells, including colon and breast cancer cells [103-105]. Furthermore, CD44 knockdown has been associated with reduced expression of occluding and E-cadherin and the ability to migrate and invade surrounding tissues [106-108]. Some studies have shown that cancer cell treatment with drugs induce EMT, with the cells expressing high levels of CD44 and being drug-resistant (Figure 12) [109-112]. Knockdown of CD44 has been associated with reduced drug resistance and fewer cells expressing drug resistance genes such as ABCB1 [113-115].

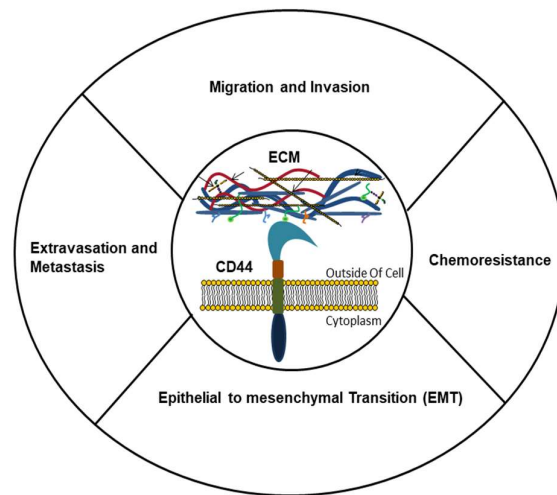


Figure 12. CD44 plays key roles in tumorigenesis through influencing cellular processes such as migration, invasion, epithelial to mesenchymal transition, chemoresistance, and metastasis.

This study provides insights into further work that may require CD44 knockdowns and animal studies. As demonstrated by the PPI network analysis, CD44 and its interacting proteins play key roles in ECM organization, cell-substrate adhesion, and cellular response to stress and signaling. CD44 may, therefore, be involved in cellular positioning, attachment, migration, and signaling. Therefore, patients with high CD44 expression may have high immune infiltration, leading to the expression of immune markers involved in immunosuppression and evasion. CD44 may aid in immune evasion and suppression through its interaction with immune cells and, therefore, promote tumor growth and metastasis. Several strategies have been developed to target CD44 and its ligands, including hyaluronic acid. For example, neutralizing antibodies to CD44 are being developed and under different stages of clinical trials. Bivatuzumab, KM201, U36, RG7356, and VFF18 are some of the antibodies against CD44 being studied. Several peptides and aptamers have been made with the ability to block the interaction between CD44 and its ligand, Hyaluronic acid, and these include PEP-1 and EphA2, respectively. In addition, pharmacological inhibitors of CD44 include Silibinin and Zerumbone. Naturally-derived compounds such as

curcumin and EGCG have also shown inhibiting activity against CD44. Lastly, CD44-Hyaluronic acid interaction can be blocked via the use of CD44 decoys.

Conclusion

This analysis shows that CD44 expression is upregulated in many cancers, and it may be used as a diagnostic and prognostic biomarker in several cancers such as PAAD, COAD, and STAD. In addition, CD44 may play key roles in immune evasion and suppression, EMT, drug resistance and metastasis. The specific mechanisms through which CD44 influences immune cells require further studies. The clinical relevance of our analysis lies in the targeting of CSCs in general and CD44 in particular in many cancers. With the introduction of immunotherapy, it will be important to develop therapeutic strategies targeting CD44 and immune markers to prevent immunosuppression and evasion.

Abbreviations

| | |
|--------|--|
| ALDH1 | Aldehyde dehydrogenase 1 |
| CAF | Cancer-associated Fibroblasts |
| CD44 | Cluster of Differentiation 44 |
| CI | Confidence interval |
| CNS | Central Nervous System |
| COAD | Colon adenocarcinoma |
| CSC | Cancer stem cells |
| DFS | Disease-free survival |
| ECM | Extracellular Matrix |
| EMT | Epithelial to mesenchymal Transition |
| ESCA | Esophageal carcinoma |
| GEPIA2 | Gene expression profiling interactive analysis 2 |
| GO | Gene ontology |
| HA | Hyaluronic acid |
| HNSC | Head and Neck Squamous Cell carcinoma |
| HR | Hazard ratio |
| KEGG | Kyoto encyclopaedia of genes and genomes |
| LIHC | Liver hepatocellular carcinoma |
| LUAD | Lung Adenocarcinoma |
| MET | Mesenchymal to Epithelial transition |
| OS | Overall survival |
| PAAD | Pancreatic adenocarcinoma |
| PPI | Protein-protein interaction |

| | |
|-------|----------------------------------|
| STAD | Stomach adenocarcinoma |
| TCGA | The cancer genome atlas |
| TIMER | Tumor Immune Estimation Resource |
| TME | tumor microenvironment |

Author Contributions

The study was conceptualized by K.D, and M.S. K.D and M.S performed the formal analysis of the data. K.D and M.S drafted the manuscript. K.D and M.S edited and reviewed the manuscript. All the authors approved the final draft manuscript.

Acknowledgments

None

Competing interests

The authors declare that they have no competing interests

Ethics Approval

This work is based on data from publicly available datasets and does not require ethical approval.

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