Multiplatform Computational Analysis of Mast Cells in Adrenocortical Carcinoma Tumor Microenvironment

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ABSTRACT:

Introduction: Immunotherapeutic response failure of adrenocortical carcinomas (ACC) highlights a need for novel strategies. Recent studies have alluded to tumor mast cell (MC) infiltration and improved patient outcomes. In this study, we examine MC activity within the ACC microenvironment to offer new insights for novel therapeutic targets.

Methods: CIBERSORTx immunogenomic deconvolution of ACC tumors (TCGA, N=79). estimated tumor immune infiltrating MCs. We evaluated MC signaling genes for their prognostic potential characterizing pro- and anti-tumor signatures and impact on overall (OS) and disease-free(DFS) survival in ACC.

Results: MC infiltration was strongly associated with favorable DFS in ACC patients. Anti-tumor (ALOX5, CCL2, CCL5, CXCL10, HDC, IL16, TNF, TPSAB1, VEGFD) and pro-tumor (CXCL1, CXCL3, CXCL8, IL4, IL13, PTGS3, TNSF4, VEGFD) expression signatures were compiled from genes with significantly prolonged and shortened prognosis (OS, DFS $p<0.05$). Anti-tumor MC signature emerged as the predominant phenotype associated with improved OS and DFS and notably downregulated in immunotherapy resistant cortisol-secreting ACC (CS-ACC).

Conclusion: Increased mast cell infiltration in ACC microenvironment correlates with a favorable prognosis. Mast cells play complex mixed anti- and pro-tumor roles in ACC, with predominantly anti-tumor activity. Modulating tumor mast cell infiltration and activity may offer a potential target for novel synergistic immunotherapies for the treatment of CS-ACC.

KEYWORDS: Adrenocortical carcinoma, tumor microenvironment, tumor immunology, mast cells, immunogenomic deconvolution

INTRODUCTION

Adrenocortical carcinoma (ACC) is among the rarest and most aggressive cancers. There has been little improvement in overall mortality over the past several decades[1]. Surgical resection remains the mainstay for patients with ACC. Most patients, however, present with unresectable metastasis and resort to medical management[1]. Mitotane therapy, a cytotoxic agent developed in the late 1950s, remains the only approved ACC treatment by the U.S. Food and Drug Administration[2]. Although in recent years, immunotherapy has made great strides, the impact of immunotherapy in ACCs has been less encouraging[3,4]. Response to immunotherapy is dependent on the interaction between tumor cells and tumor microenvironment (TME). Increasing evidence has demonstrated tumor phenotypes and immunotherapy susceptibility to being determined by the complex interaction of various cell types in the TME, especially the tumor-infiltrating immune cells (TIICs)[4]. Within the TME, mast cells (MC) are highly proinflammatory and actively recruit cells of the innate immune system, mainly neutrophils, macrophages, and eosinophils and cells of the acquired immune system (B and T cells) to orchestrate immune responses[5].

MC-mediated signaling is complex and possesses both pro-and antitumorigenic properties across different tissues and cancer types[6,7]. Current studies revealed that the function, prognostic value, and degree of tumor-infiltrating mast cell (TIMC) varies greatly with the type of tumor[8]. A recent study by Tian et al. identified tumor mast cell infiltration (TMCI) to be associated with improved outcomes in ACC patients using CIBERSORT immunogenomic TIIC estimation of the TCGA database and validated the positive prognostic value of TIMCs in a single-center cohort[9]. Our study aims to further dissect MC activity within the ACC

microenvironment by analyzing the expression of the MC signaling gene to characterize MC pro- and anti-tumor effects and offer new insights for potential therapeutic targets.

METHODS

Data Acquisition

We utilized the RNA sequencing count table data of Adrenal Cortical Carcinomas (N=92) from The Cancer Genome Atlas (TCGA) Firehose Legacy Cohort[10] through the cBioPortal (https://www.cbioportal.org/) (Study ID: 5fe222f5e4b015b63e9d16e8)[11]. Within the TCGA dataset, 79 (86%) had reported mRNA expression values and were included in the study cohort.

Prognostic Value of Tumor Mast Cell Infiltration

CIBERSORTx immunogenomic deconvolution platform was used to estimate tumor-infiltrating immune subsets from mRNA expression profiles (including B cells, $CD4^+$T cells, $CD8^+$T cells, dendritic cells, macrophages, natural killer cells, neutrophils, etc). *CIBERSORTx* is a computational immunogenomic platform, a publicly available web-based deconvolution program (https://cibersortx.stanford.edu)[12]. Tumor mast cell infiltration (TMCI) was analyzed using univariate cox regression analysis for its relation to patient OS and DFS. The predictive prognostic value of TMCI in OS and DFS were determined by a maximally selected log-rank statistic test[13]. All survival and optimal cut-off analyses were performed using the 1.1.383 R statistics software (R Core Team Vienna, Austria).

Patient Demographics & Tumor Pathology by Tumor Mast Cell Infiltration

The American Joint Commission on Cancer Staging Manual, seventh edition, was used to determine TNM classification. Overall survival (OS) and disease-free survival (DFS) were defined as the time from the date of index operation to the date of death or date of documented disease recurrence or death, respectively. Categorical variables were presented as frequency and

percentages and compared using Chi-square or Fisher's exact test, as appropriate. Continuous variables were reported as median values with interquartile range (IQR) and compared using the Kruskal–Wallis test. OS and DFS were calculated using the Kaplan–Meier method and compared using the log-rank test. Individual gene expression profiles were analyzed using univariant Cox regression methods. Significance was set at a *p*-value less than 0.05.

Immunogenomic Deconvolution and Tumor-Infiltrating Immune Cells

All mast cell signaling gene (MCSG) expression and tumor-infiltrating immune cell (TIIC) intercorrelations were constructed in heatmap format to represent all potential associations. Correlations were calculated using Spearman rank correlation coefficient and evaluated using the Pearson correlation formula. Correlational significance was set at a *p*-value less than 0.01 and correlations with significant correlations indicated by heatmap shading. Statistically insignificant heat map correlations ($p \geq 0.01$) were void of shading.

Mast Cell Signaling Gene mRNA Expression Signature Calculation and Proportional Predominance

Cumulative anti- and pro-tumor signatures were normalized to positive values (0-5) and weighted against their sum to quantity the proportion of anti- and pro- mRNA expression signatures in each patient. Expression predominance was determined by proportional expression greater than 50%. OS and DFS were compared according to signature predominance using Kaplan-Meier and Log-rank test.

RESULTS

Optimal Mast Cell Infiltration

Tumor mast cell infiltration (TIMC) was significantly associated with prolonged disease-free survival (Hazard Ratio (HR) 0.02, 95% Confidence Interval (95% CI) 0.00 – 0.64 p=0.026)

(**Figure 1a**). TIMC was not significantly associated with overall survival (HR 0.27, 95% CI 0.02 – 4.99, p=0.378) (**Figure 1b**). Maximally selected log-rank statistic test identified mast cell infiltration >0.15au (arbitrary units) to be increasingly associated with prolonged DFS and an optimal TMCI value of 0.21 to carry the maximum DFS prognostic value (**Figure 1b-c**). Patients with TIMC > 0.21 were deemed "high TIMC" group and patients with TIMC <0.21 were considered "low TIMC" group. Low TIMC was associated with significantly shortened DFS (HR 5.51, 95% CI 1.32 – 22.97, p=0.019). No optimal threshold of mast cell inflammation was found to be a significant predictor of OS and OS log-rank comparison using optimal DFS optimal threshold did not reach statistical significance (p=0.17) (**Figure 1d**). DFS was significantly shortened for ACC patients with suboptimal TIMCs (5-year DFS 85.7 vs. 37.1%, p=0.005, **Figure 1e)**.

Patient Demographics and Tumor Pathology by Tumor Mast Cell Infiltration

Among the 79 patient cohorts, 64 (81%) had low TMCI and 15 (19%) had high TMCI. The groups were similar in age at diagnosis (p=0.871), sex (p=0.718) tumor stage T (p=0.622), nodal status N (p=0.791), metastasis M (p=1.000) and clinical stage (p=0.622). The high TIMC group had a significant portion of patients with an unreported rate (33.0 vs 9.4%, p=0.014). The type of excess adrenal steroid hormone oversecretion was significantly different between the low and high TIMC groups (p=0.047) with significantly higher rates of isolated cortisol-secreting ACC (CS-ACC) among the low TIMC group (25.4 vs 0.0%, p<0.001). Low and high TIMC groups demonstrated similar fractions of genome alteration (p=0.276), mutation count (p=0.222), mitotic rate (p=0.556), tumor necrosis (p=1.000), Weiss Score (p=0.545) (*), and rates of vascular invasion (p=0.849). Both groups reported similar resection margins (p=0.866) and underwent similar rates of unspecified neoadjuvant (p=1.000) and adjuvant (p=0.662) therapy, as

well as adjuvant radiation (p=0.866) and mitotane therapy (p=0.231). The low TIMC group experienced higher rates of ACC recurrence (55.9 vs 14.3%, p=0.012). Demographic, clinical, and pathologic features of the study cohort by cortisol secretion are summarized in **Table 1**.

Mast Cell Infiltration Positively Impacts Disease-free Survival Following Adrenocortical Carcinoma Resection

On multivariable Cox regression analysis controlling for cortisol-secretion, low TIMC was associated with significantly shortened DFS (HR 4.37, 95% CI 1.03 – 18.60, p=0.046) and cortisol secretion did not reach statistical significance (HR 1.94, 95% CI 0.99 – 3.77 p=0.053). The poor DFS prognosis related to low TMCI after controlling for cortisol-secretion and despite similar patient demographics, tumor pathology, and treatment protocols commonly associated with survival (patient age, cancer stage, Weiss Score, neoadjuvant/adjuvant therapy) is suggestive of a possible influential role of mast cell activity in ACC TME that may underlie disease biology and patient prognosis.

Mast Cell Signaling Genes

A summary of immune response signaling molecules characteristic but not limited to mast cell activity is displayed in **Figure 2**. The primary genes encoding for the signaling molecules or the rate-limiting step governing large molecule synthesis are listed in **Table 2**.

Anti-tumor Mast Cell Signaling Gene Expression and Tumor-Infiltrating Immune Cell Profile Correlations

Mast cell signaling genes (MCSGs) with significant positive prognostic value (Hazard Ratio (HR) < 1.00, p<0.05) in overall (OS) or disease-free survival (DFS) were deemed anti-tumor MCSGs. These genes included ALOX5 (DFS HR 0.70, 95% CI 0.50 - 0.97, p=0.033), CCL2 (DFS HR 0.65, 95% CI 0.47 – 0.91, p=0.011), CCL5 (DFS HR 0.61, 95% CI 0.42 – 0.87,

p=0.007), CXCL10 (DFS HR 0.63, 95% CI 0.43 – 0.93, p=0.020), HDC (DFS HR 0.51, 95% CI 0.30 – 0.86, p=0.012), IL16 (OS HR 0.65, 95% CI 0.44 – 0.96, p=0.031; DFS HR 0.56, 95% CI 0.40 -0.79, p=0.001), TNF (DFS HR 0.69, 95% CI 0.49 – 0.97, p=0.031), and VEGFD (OS HR 0.65, 95% CI 0.45 – 0.95, p=0.025; DFS HR 0.63, 95% CI 0.45 – 0.89, p=0.009) (**Figure 3a-b**). Cumulative anti-tumor MCSG signature expression was significantly associated with prolonged DFS (HR 0.893, 95% CI 0.84 – 0.95, p<0.001), however the univariate regression analysis did not meet statistical significance for OS (HR 0.936, 95% CI 0.88 – 0.100, p=0.054) (**Figure 3a-b**). The role of anti-tumor MCSGs are summarized in **Figure 3c**. There were several significant mRNA expression intercorrelations between all anti-tumor MCSGs (r>0.30) with the exception of VEGFD. VEGFD expression was positively correlated with HDC expression (r=0.32) (**Figure 3d**). Anti-tumor MCSG expression was positively correlated with the infiltration of $CD8^+$ T cells (r=0.54), T(reg) cells (r=0.53), M1 (r=0.54), M2 (r=0.63) macrophage, and total macrophage and monocyte (r=0.64) (p-values <0.01) (**Figure 3e**). Expression and TIIC correlations of pro-tumor MCSGs are further summarized in **Figure 3d-e**.

Pro-tumor Mast Cell Signaling Gene Expression and Tumor-Infiltrating Immune Cell Profile Correlations

Mast cell signaling genes (MCSGs) with significant negative prognostic value (Hazard Ratio (HR) > 1.00, p<0.05) in overall (OS) or disease-free survival (DFS) were deemed pro-tumor MCSGs. These genes included CXCLl (OS HR 1.45, 95% CI 1.04 – 2.02, p=0.028), CXCL3 (OS HR 1.58, 95% CI 1.16 – 2.16, p=0.003), CXCL8 (OS HR 1.78, 95% CI 1.23 – 2.57, p=0.002; DFS HR 1.60, 95% CI 1.14 – 2.25, p=0.007), IL4 (OS HR 1.47, 95% CI 1.01 – 2.12, p=0.042; DFS HR 1.47, 95% CI 1.01 – 2.14, p=0.046), IL13 (OS HR 1.39, 95% CI 1.01 – 1.92, p=0.042), PTGS2 (OS HR 1.52, 95% CI 1.07 - 2.16, p=0.020), TNFSF4 (OS HR 1.42, 95% CI

1.01 – 1.99, p=0.041), and VEGFC (OS HR 1.54, 95% CI 1.00 – 2.35, p=0.048). Cumulative pro-tumor MCSG signature expression was significantly associated with shortened OS and DFS (OS HR 1.18, 95% CI 1.09 – 1.29, p<0.001; DFS HR 1.14, 95% CI 1.05 – 1.23, p<0.001) (**Figure 4a-b**). The role of pro-tumor MCSGs are summarized in **Figure 4c**. Univariate Cox regression survival analysis for each MCSGs is further summarized in **Supplemental Table 1.** Strong intercorrelations were found between CXCL1, CXCL3, CXCL8, and PTGS2 mRNA expression (r>0.55) (**Figure 4d**). Pro-tumor MCSG expression was positively correlated with the infiltration of M1 macrophage (r=0.53), activated mast cells (r=0.52) and neutrophil (r=0.53) infiltration (**Figure 4e**) (p-values <0.01). Expression and TIIC correlations of pro-tumor MCSGs are further summarized in **Figure 4d-e**.

Mast Cell Signaling Gene Expression by Excess Cortisol Secretion

Among the anti-tumor MCSGs associated with favorable prognosis, the mRNA expression of CCL5 (p=0.008), HDC (p<0.001), IL16 (p=0.002), and TPSAB1 (p<0.001) were downregulated in CS-ACCs compared to nonCS-ACCs (**Figure 5a**). Furthermore, the anti-tumor expression signature was significantly decreased in CS-ACC relative to nonCS-ACC patients (p=0.006). CS- and nonCS-ACCs demonstrated similar expression of the pro-tumor MCSGs and the pro-tumor cumulative signature (**Figure 5b**).

Predominant Mast Cell Signaling Gene Expression Signature Predicts Survival Outcomes

Of the entire cohort, 72% (n=57) were identified to predominantly express anti-tumor gene signature and 28% (n=22) were pro-tumor signature predominant (**Figure 6a**). Anti-tumor mast cell signaling predominance among the cohort further suggests that although mast cells may play a heterogenous role in ACC TME, they may be considered a favorable prognostic indicator with a beneficial impact on the TME overall. Anti-tumor and pro-tumor signature predominant groups

were made up of similar portions of CS- and nonCS-ACC patients (54.5 vs. 36.8%, p=0.24). Anti-tumor predominant MCSG expression was associated with prolonged 5-year overall (73.0 vs. 28.3%, p<0.001) and DFS (53.9 vs. 9.7%, p<0.001) survival compared to ACC patients with pro-tumor predominant MCSG expression (**Figure 6a-c**).

DISCUSSION

In this study, we utilized multiplatform immunogenomic deconvolution to detangle the role of MC tumor infiltration and signaling activity within ACC TME. Our findings are multifaceted in that they support previous research characterizing TIMC abundance as a favorable prognostic factor in ACC[9]. Our analysis dives deeper to identify the optimal degree of TIMCs in the ACC microenvironment, delineate prognostic pro- and anti-tumorigenic MC signaling genes, and lay a foundation for future mechanistic studies and the development of new therapeutic interventions.

We compared demographic, clinical, pathologic, and treatment parameters between ACC patients harboring optimal and suboptimal TMCI, to identify potential confounders underlying the improved prognosis associated with TMCI in ACC patients. Interestingly, excess cortical steroid secretion was the only parameter identified to be significantly different between groups with isolated excess cortisol secretion significantly associated with low TIMC. Because this finding may suggest an influential role of glucocorticoid-induced MC immunomodulation, we compared the anti- and pro-tumorigenic gene expression levels by cortisol-secretion and found several anti-tumor genes (CCL5, HDC, IL16, TPSAB1) to be downregulated in CS-ACCs compared to nonCS-ACCs, as was the cumulative anti-tumor MCSG signature. Excess cortisol secretion is well known for both immunosuppression in ACC TME[15] and as an independent risk factor for poor prognosis in ACC patients[16]. However, the DFS prognostic value of TMCI maintained statistical significance after controlling for excess cortisol secretion on multivariant

analysis, while excess cortisol secretion did not. These findings suggest that cortisol-induced suppression of anti-tumor MC signaling may contribute to diminished MC anti-tumor activity in ACC TME and the poor prognosis in patients with CS-ACC.

Previous studies have demonstrated a wide range of variability in mast cell role according to the type, grade, or stage of tumor and the prognostic role has varied across cancer types[8]. Mast cells appeared to play a pro-tumorigenic role in human and experimental tumors (thyroid, gastric, pancreas tumors)[17-20], whereas evidence for an anti-tumorigenic role for mast cells has also been found in other cancers (breast, ovarian, prostate tumors)[21-23]. Additionally, a limited number of studies indicate a non-contributory role of mast cells in certain tumors (lung, renal, colon tumors)[24-26]. This collage of evidence suggests a heterogenous pro-tumorigenic and anti-tumorigenic role of mast cells in the TME may vary on a spectrum across different tissue and cancer types and influence by differential signaling activity and gene expression. In this study, we assessed the prognostic value of the mRNA expression of genes attributable to MC signaling to characterized dichotomous MC activity as pro-and anti-tumorigenic identifying genes and signaling molecules for future *in vitro* and *in vivo* experiments to reveal potential underlying mechanisms. The anti-tumor MCSGs, associated with improved prognosis was found to be predominantly involved in promoting local inflammation (ALOX5/leukotriene B_4, HDC/histamine), TPSAB1/serotonin), as well as lymphocyte (CCL2, CCL5, CXCL10, TNF)[27-29] and phagocyte (IL-16)[30-34] chemotaxis and activation. Although VEGF is well known for its angio- and lymphogenic properties in TME, the role of differentially expressed VEGF subunits (-A, -B, -C, and -D) remain less understood. In this study, VEGF-D expression was associate with improved prognosis and VEGF-C with poor prognosis. A similar phenomenon has been observed in non-small cell lung cancer and attributed to the increased lymphogenic properties of

VEGF-C over VEGF-D, which is thought to promote lymphatic spread and dissemination[35].

The pro-tumor MCSGs, associated with poor prognosis were found to be mostly involved in the promotion of neutrophil recruitment and activation (CXCL1, CXCL3, CXCL8, IL-8)[36-38], and the expression of the pro-tumor signature was strongly correlated with neutrophil tumor infiltration. Substance P (PTGS2) is released upon mast cell degranulation and has been shown to induce tumor cell mitogenicity (proliferation, invasion, metastasis) through activation of the neurokinin (NK)-1 receptor system while NK-1 antagonists have been shown to negate such effects[39,40]. The anti- and pro-tumor MCSG signatures appeared to inversely impact prognosis to a similar degree, however, the study cohort was shown to predominantly express the anti-tumor signature over the pro-tumor MCSG signature and anti-tumor predominance was significantly associated with improved prognosis. These findings support both the notions of a heterogenous role of MC in TME and the favorable prognostic value of MC infiltration in ACC patients.

However, this study is not without limitations. Primarily, this is a retrospective study design and inherently limited from determining causal relationships. Secondly, it is challenging to clearly define the role of individual immune cell types due to overlap in function, gene expression, and immune interplay, and this study is no exception. This study does not include single-cell analysis and the expression of genes analyzed in this study are likely not entirely of MC origin. Instead, this study aims to identify the potential activity of (but not limited to) MCs that may underly the prognostic significance in ACC TME that has now been deemed consistent across multiple methods of immunogenomic and immunohistochemistry quantification[9].

In conclusion, our study provides interesting new insight into the nuanced role of mast cell infiltration in ACC TME and identifies potential areas for cogent immunotherapy drug targeting. The augmentation or supplementation of anti-tumor mast cell signaling may be an

efficient means of optimizing TME immune response and enhancing immunotherapy response in ACC TME.

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FIGURES

Figure 1. Prognostic Value of Tumor Mast Cell Infiltration in Adrenocortical Carcinoma. **A.** Univariate cox regression overall (OS) and disease-free survival (DFS) according to mast cell tumor infiltration. **B.** Optimal value of tumor infiltrating mast cell prognostic for DFS determined by a maximally selected log-rank statistic test. **C.** Distribution of suboptimal (low) and optimal (high) tumor mast cell infiltration within the study cohort. **D.** OS stratified by optimal and suboptimal mast cell infiltration. **E.** DFS stratified by optimal and suboptimal mast cell infiltration.

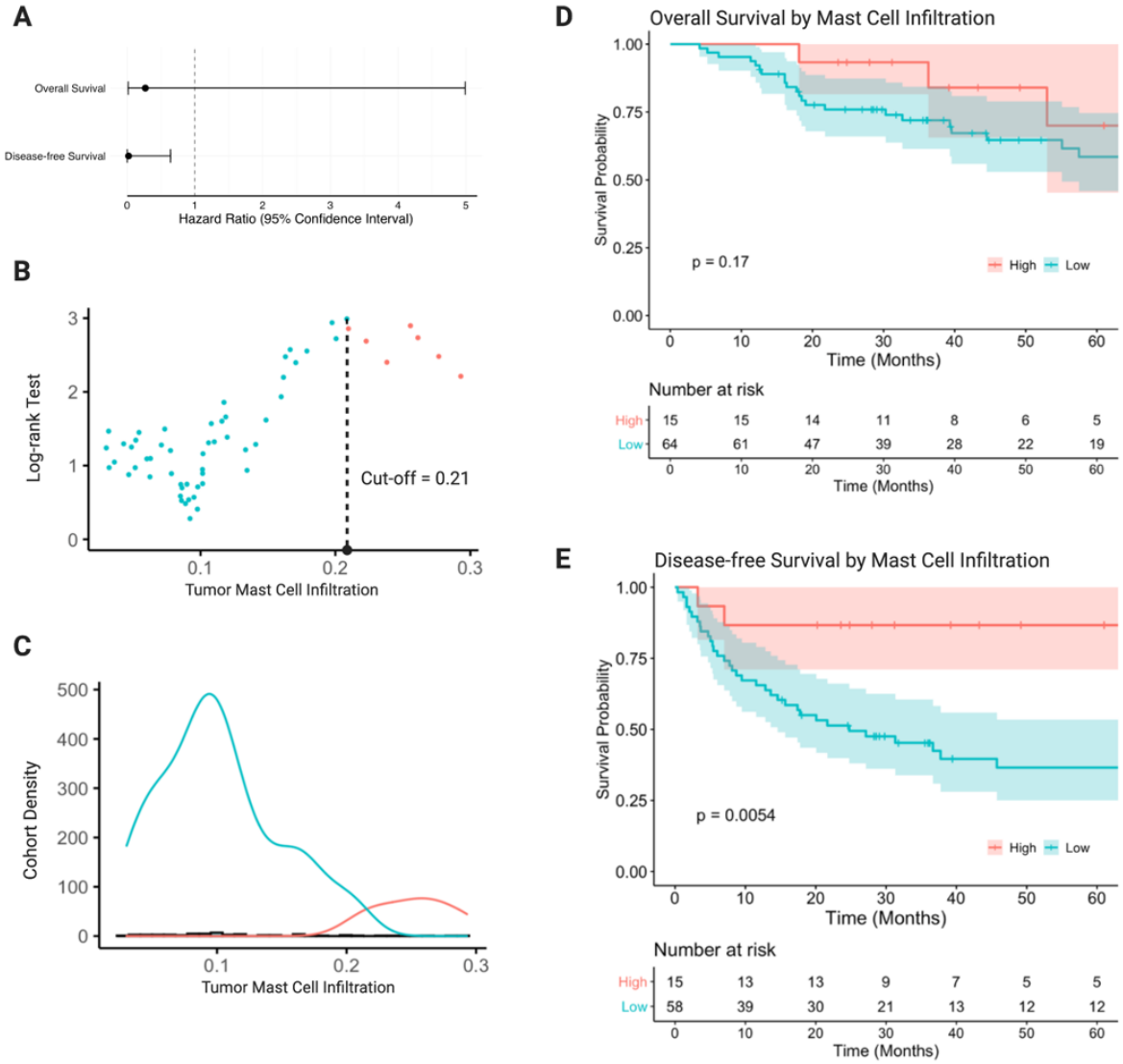

Table 1. Patient Demographic, Tumor Pathology, and Treatment Parameters of Adrenocortical Carcinoma. IQR = interquartile range, Age = age at diagnosis, Unknown = unreported data, HPF = high-power fields, RX = indeterminate margins.

		Low Mast Cells *N=64*	**High Mast Cells** *N=15*	**p-value**
Age (years) -Median [IQR]		48.0 [35.5;60.0]	50.0 [33.5;53.0]	0.871
Sex	Female	40 (62.5%)	8 (53.3%)	0.718
	Male	24 (37.5%)	7 (46.7%)	
Race	White	57 (89.1%)	9 (60.0%)	0.014
	Asian	1 (1.56%)	0 (0.00%)	
	Black	0 (0.00%)	1 (6.67%)	
	Unknown	6 (9.38%)	5 (33.3%)	
Clinical Stage	Stage I	7 (10.9%)	2 (13.3%)	0.622
	Stage II	28 (43.8%)	9 (60.0%)	
	Stage III	15 (23.4%)	1 (6.67%)	
	Stage IV	12 (18.8%)	3 (20.0%)	
	Unknown	2 (3.12%)	0 (0.00%)	
T Stage	T1	7 (10.9%)	2 (13.3%)	0.821
	T2	32 (50.0%)	10 (66.7%)	
	T3	7 (10.9%)	1 (6.67%)	
	T4	16 (25.0%)	2 (13.3%)	
	Unknown	2 (3.12%)	0 (0.00%)	
N Stage	N0	54 (84.4%)	14 (93.3%)	0.791
	N1	8 (12.5%)	1 (6.67%)	
	Unknown	8 (12.5%)	0 (0.00%)	
M Stage	M0	50 (78.1%)	12 (80.0%)	1.00
	M1	12 (18.8%)	3 (20.0%)	
	Unknown	2 (3.12%)	0 (0.00%)	
Excess Hormone Secretion	Cortisol	16 (25.4%)	0 (0.00%)	0.047
	Androgen + Cortisol	13 (20.6%)	3 (20.0%)	
	Androgen	8 (12.7%)	0 (0.00%)	
	Estrogen	1 (1.59%)	1 (6.67%)	
	Mineralocorticoids	2 (3.17%)	1 (6.67%)	
	Mineralocorticoid + Cortisol	1 (1.59%)	0 (0.00%)	
	None	21 (33.3%)	9 (60.0%)	
Excess Cortisol (including mixed) Secretion	No	34 (53.1%)	12 (80%)	0.108
	Yes	30 (46.9%)	3 (20.0%)	
Laterality	Left	38 (59.4%)	7 (46.7%)	0.545
	Right	26 (40.6%)	8 (53.3%)	
Fraction of Genome Altered -Median [IQR]		0.60 [0.38;0.91]	0.42 [0.25;0.83]	0.276
Mutation Count -Median [IQR]		87.0 [72.5;118]	81.5 [57.5;92.8]	0.222
Mitoses Count -Median [IQR]		7.00 [5.00;23.0]	7.00 [4.00;15.0]	0.556
Mitotic Rate	< 5/50 HPF	24 (37.5%)	5 (33.3%)	0.777
	> 5/50 HPF	32 (50.0%)	9 (60.0%)	
	Unknown	8 (12.5%)	1 (6.8%)	
Tumor Necrosis	Absent	14 (21.9%)	3 (20.0%)	1.00
	Present	46 (71.9%)	11 (73.3%)	
Weiss Score -Median [IQR]		5.50 [3.75;7.25]	7.00 [5.00;7.00]	0.545
Venous Invasion	Absent	31 (48.4%)	8 (53.3%)	0.849
	Present	26 (40.6%)	5 (33.3%)	
	Unknown	7 (10.9%)	2 (13.3%)	
Neoadjuvant Therapy	No	63 (98.4%)	1 (1.56%)	1.000
	Yes	1 (1.56%)	0 (0.00%)	
Resection Margin	R0	43 (67.2%)	12 (80.0%)	0.866
	R1	5 (7.81%)	1 (6.67%)	
	R2	7 (10.9%)	2 (13.3%)	
	RX	6 (9.38%)	0 (0.00%)	
	Unknown	3 (4.69%)	0 (0.00%)	
Adjuvant Therapy	No	20 (31.2%)	5 (33.3%)	0.662
	Yes	42 (65.6%)	9 (60.0%)	
	Unknown	2 (3.12%)	1 (6.67%)	
Adjuvant Mitotane Therapy	No	21 (32.8%)	5 (33.3%)	0.231
	Yes	41 (64.1%)	8 (53.3%)	
	Unknown	2 (3.12%)	2 (13.3%)	
Adjuvant Radiation Therapy	No	47 (73.4%)	12 (80.0%)	0.866
	Yes	14 (21.9%)	1 (6.67%)	
	Unknown	3 (4.69%)	2 (13.3%)	
New Neoplasm Following Initial Treatment	No	26 (44.1%)	12 (85.7%)	0.012
	Yes	33 (55.9%)	2 (14.3%)	

Figure 2. Mast cell Signaling and Target Cell Types. A summary of immune response signaling molecules characteristic but not limited to mast cell activity.

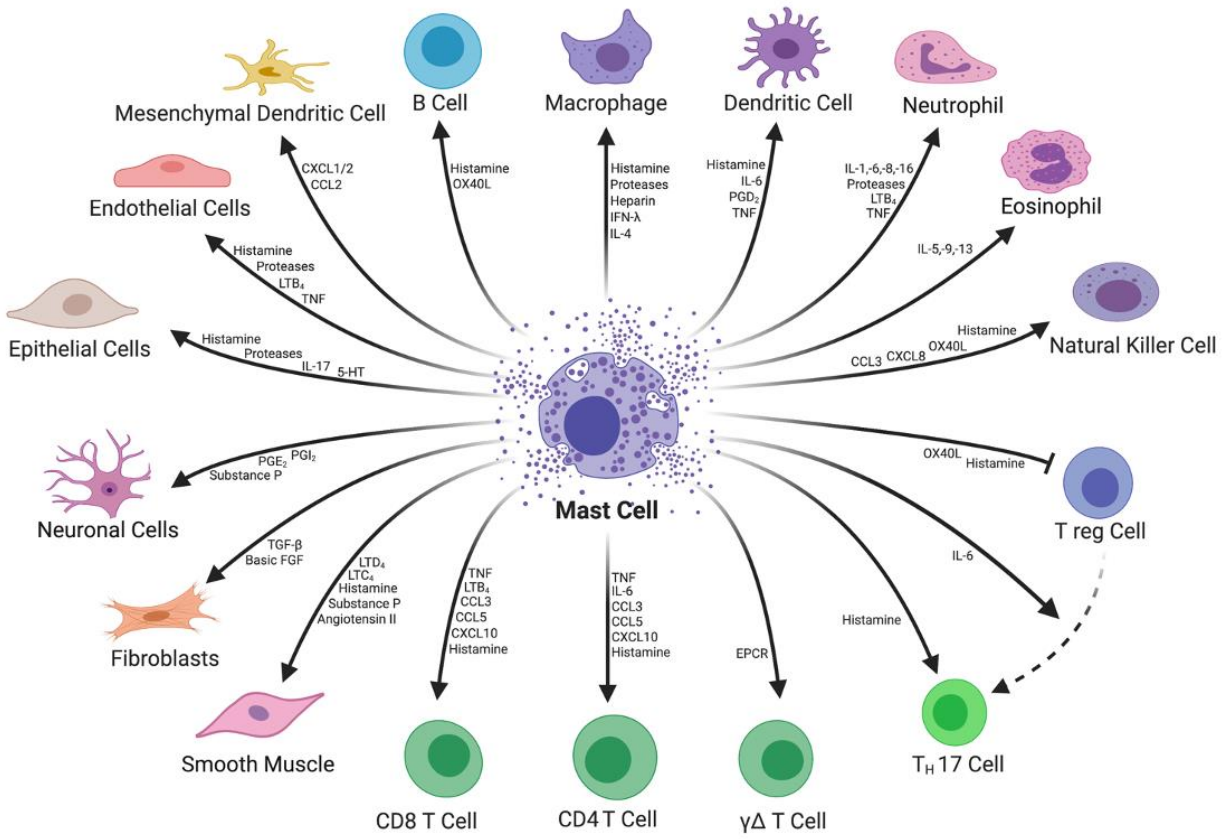

Table 2. Mast Cell Signaling Genes. The primary genes encoding for the signaling molecules or the rate-limiting step governing large molecule synthesis.

Mast Cell Signal	Primary Gene
5-HT	TPH1
Angiotensin II	ACE
Carboxypeptidase	CPE
CCL2	CCL2
CCL3	CCL3
CCL5	CCL5
Chymase	CMA1
CXCL1	CXCL1
CXCL10	CXCL10
CXCL2	CXCL2
CXCL3	CXCL3
EPCR	PROCR
Heparin	SERPIND1
Histamine	HDC
IFN-G	IFNG
IL-1	IL1A
IL-13	IL13
IL-16	IL16
IL-17	IL17A
IL-4	IL4
IL-5	IL5
IL-6	IL6
IL-8	CXCL8
LTB	ALOX5
OX40L	TNFSF4
PGD2	PTGDS
PGE2	PTGES
PGI2	PTGIS
Substance P	PTGS2
TGF-B	TGFB1
TNF	TNF
Tryptase	TPSAB1
VEGF-A	VEGFA
VEGF-B	VEGFB
VEGF-C	VEGFC
VEGF-D	VEGFD

Figure 3. Anti-tumor Mast Cell Signaling Genes in Adrenocortical Carcinoma. A-B. Significant prognostic indicators of mast cell signaling genes with significant associations for overall or disease-free survival (OS or DFS (HR <1.00, p<0.05). **C.** Heatmap anti-tumor mast cell signaling gene expression showing Spearman rank correlation coefficients with significant correlations (p<0.01) shaded. **D.** Diagram of anti-tumor mast cell activity and anti-tumor mast cell gene signaling roles. **E.** Heatmap of individual gene expression contributing to anti-tumor mast cell signaling signature with tumor-infiltrating immune cell (TIIC) subtypes. Significant correlations (p<0.01) shaded.

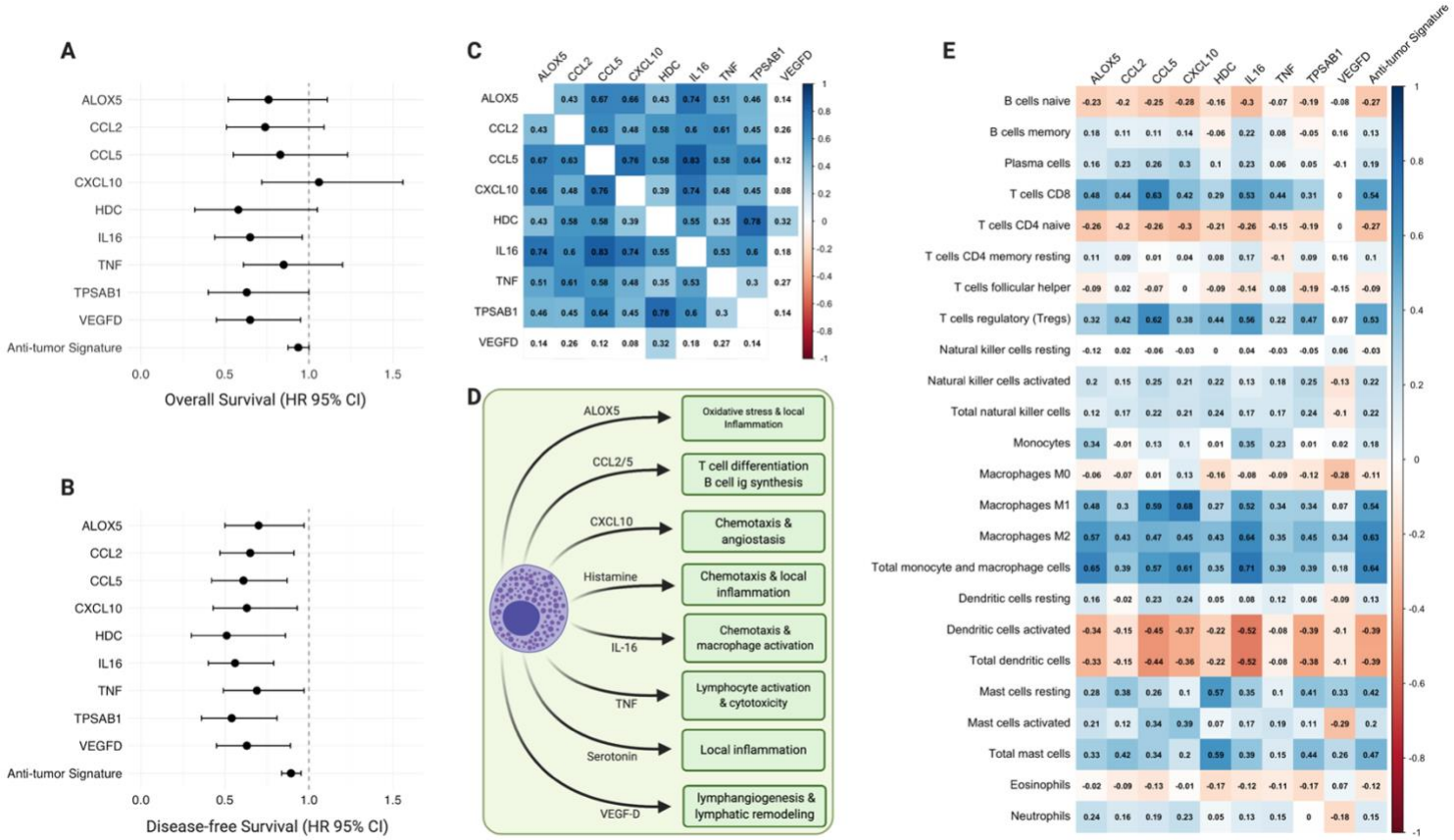

Figure 4. Pro-tumor Mast Cell Signaling Genes in Adrenocortical Carcinoma

A-B. Significant prognostic indicators of mast cell signaling genes with significant associations for overall and disease-free survival (OS or DFS (HR >1.00, p<0.05).

C. Heatmap pro-tumor mast cell signaling gene expression showing Spearman rank correlation coefficients with significant correlations (p<0.01) shaded.

D. Diagram of pro-tumor mast cell activity and pro-tumor mast cell gene signaling roles

E. Heatmap of individual gene expression contributing to pro-tumor mast cell signaling signature with tumor-infiltrating immune cell (TIIC) subtypes. Significant correlations (p<0.01) shaded.

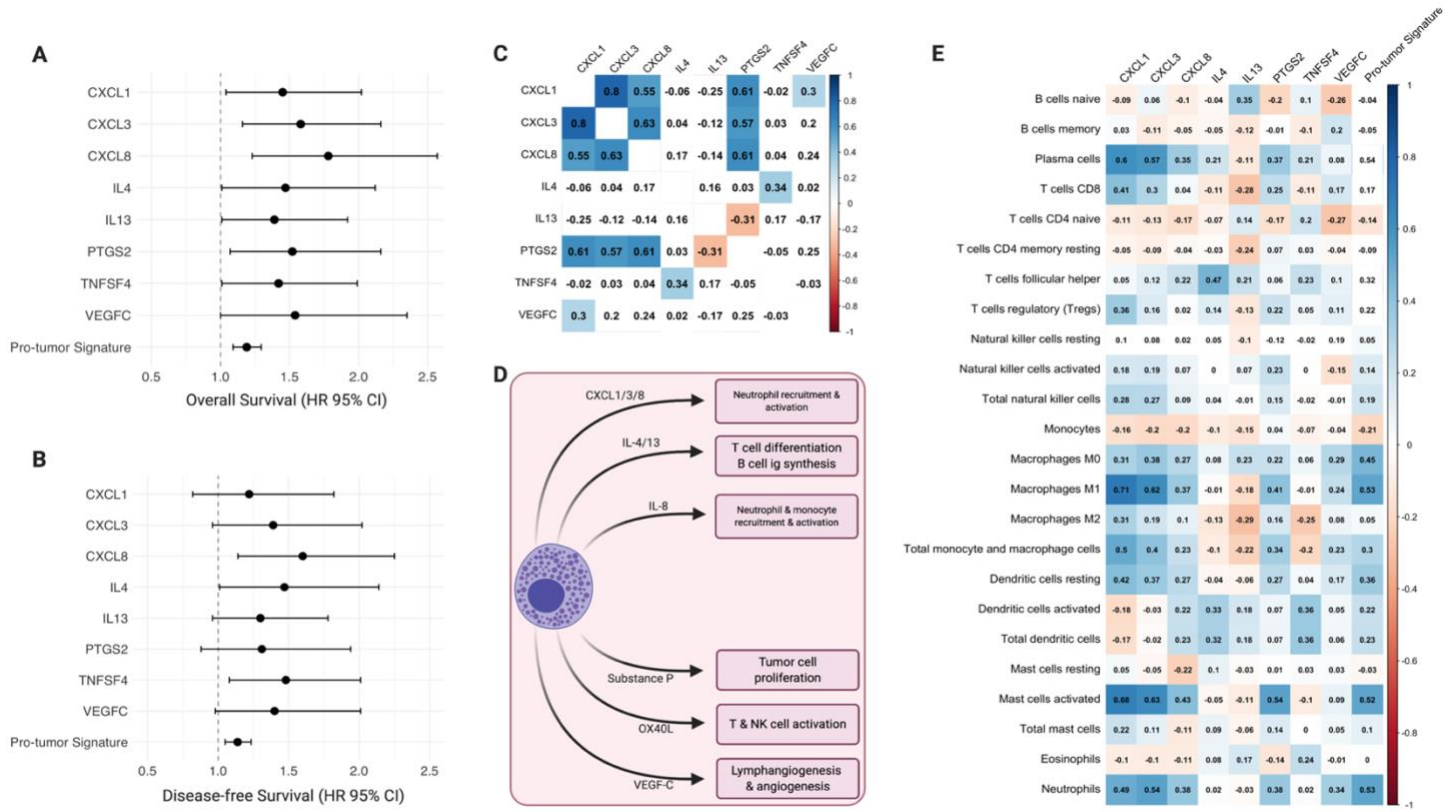

Figure 5. Differential Mast Cell Signaling Gene Expression By Cortisol Secretion In Adrenocortical Carcinoma. A. Anti-tumor mast cell signaling genes mRNA expression in ACC tumors and stratified into subgroups by cortisol secretion. **B.** Pro-tumor mast cell signaling genes mRNA expression in ACC tumors and stratified into subgroups by cortisol secretion. ns = p value ≥0.05; * = p value <0.05, ** = p value <0.01, *** = p value <0.001, **** = p value <0.0001.

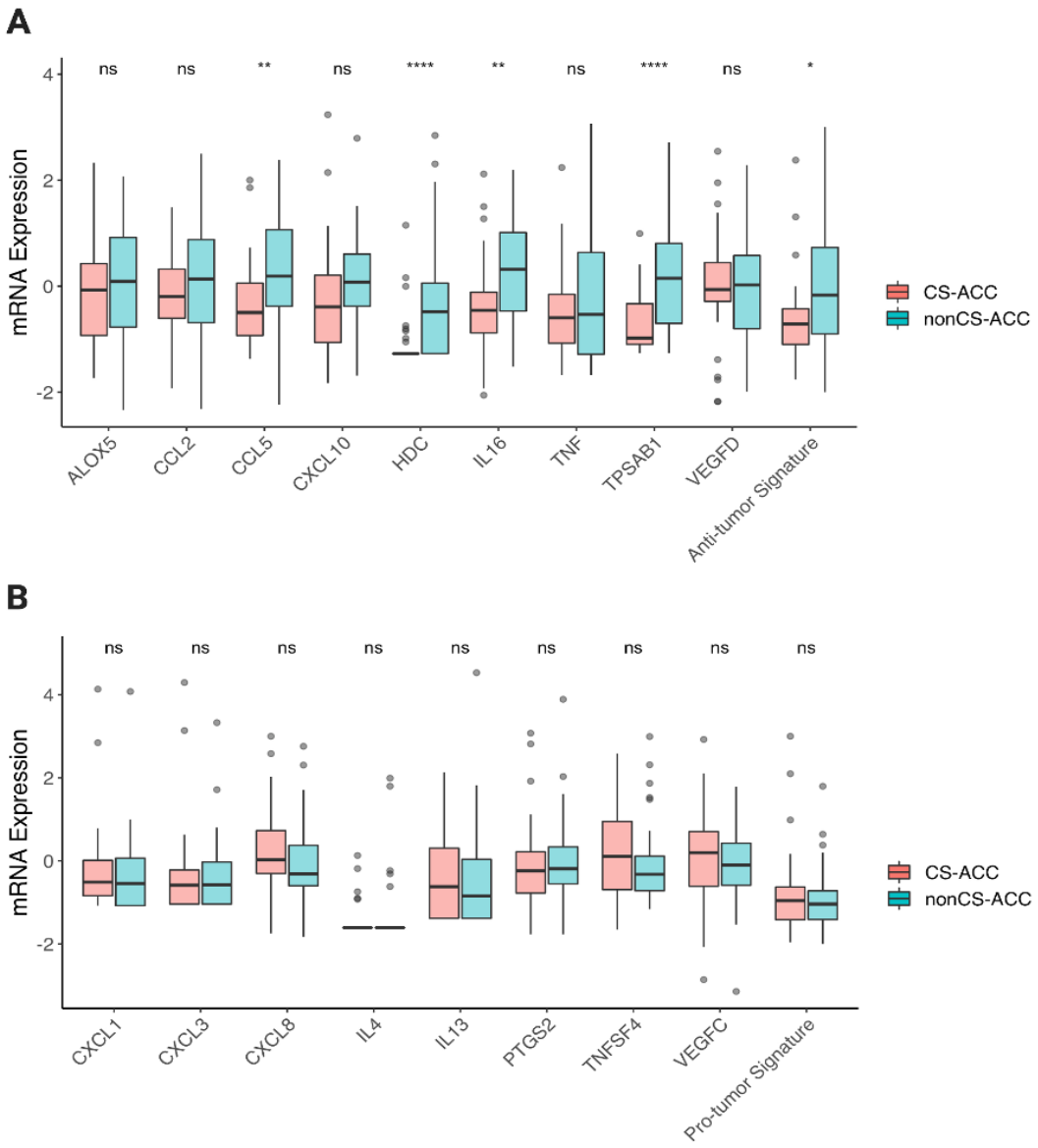
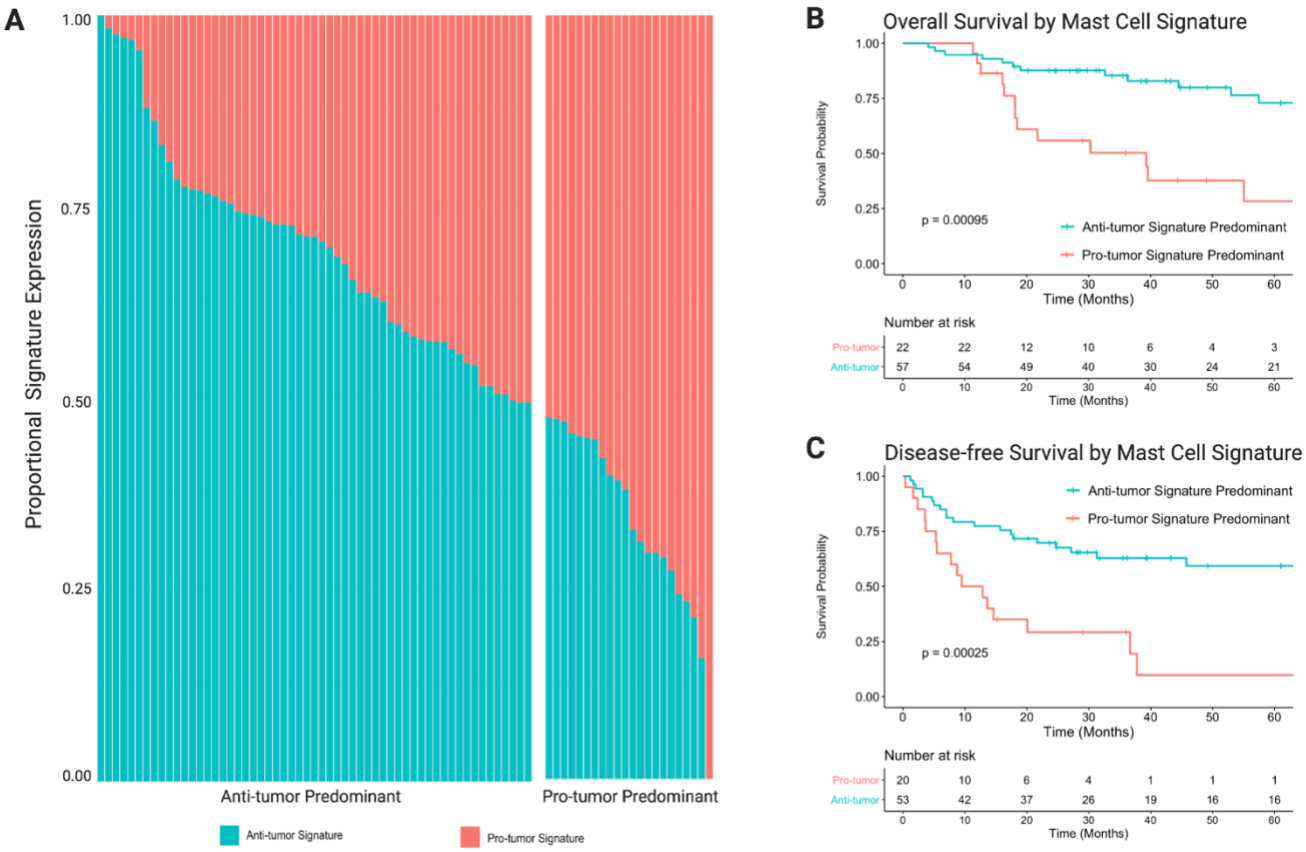
Figure 6. Mast Cell Signaling Gene mRNA Expression Signature Predominance. A. Proportional mRNA expression of anti- and pro-tumor signatures in each patient. **B.** Overall survival comparing anti- and pro-tumor signature expression predominance **C.** Disease-free survival comparing anti- and pro-tumor signature expression predominance.