Discovery of a glucocorticoid receptor (GR) activity signature correlates with immune cell infiltration in adrenocortical carcinoma

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ABSTRACT

Background Adrenocortical carcinoma (ACC) is a rare and highly aggressive endocrine malignancy, of which >40% present with glucocorticoid excess. Glucocorticoids and glucocorticoid receptor (GR) signaling have long been thought to suppress immunity and promote tumor progression by acting on immune cells. Here, we provide new insights into the interaction between GR signaling activity and the immune signature of ACC as a potential explanation for immune escape and resistance to immunotherapy.

Methods First, GR immunohistochemical staining and immunofluorescence analysis of tumor-infiltrating lymphocyte (CD4 T, CD8 T cells, natural killer (NK) cells, dendritic cells and macrophages) were performed in 78 primary ACC tissue specimens. Quantitative data of immune cell infiltration in ACC were correlated with clinical characteristics. Second, we discovered a GR activity signature (GRsig) using GR-targeted gene networks derived from global gene expression data of primary ACC. Finally, we identified two GRsig-related subtypes based on the GRsig and assessed the differences in immune characteristics and prognostic stratification between the two subtypes.

Results GR was expressed in 90% of the ACC tumors, and CD8+ cytotoxic T lymphocytes were the most common infiltrating cell type in ACC specimens (88%, 8.6 cells/high power field). GR expression positively correlated with CD8+ T cell (Phi=0.342, p<0.001), CD4+ T cell (Phi=0.280, p<0.001), NK cell (Phi=0.280, p<0.001), macrophage (Phi=0.285, p<0.001), and dendritic cell (Phi=0.397, p<0.001) infiltration. Clustering heatmap analysis also displayed high immune cell infiltration in GR high-expressing tumors and low immune cell infiltration in GR-low tumors. High GR expression and high immune cell infiltration were significantly associated with better survival. Glucocorticoid excess is associated with low immune cell abundance and unfavorable prognosis. A GRsig comprising n=34 GR-associated genes was derived from Gene Expression Omnibus/The Cancer Genome Atlas (TCGA) data sets and used to define two GRsig-related subtypes in the TCGA cohort. We demonstrated distinct differences in the immune landscape and clinical outcomes between the two subtypes.

Conclusion GR expression positively correlates with tumor-infiltrating immune cells in ACC. The GRsig could serve as a prognostic biomarker and may be helpful for prognosis prediction and response to immunotherapy. Consequently, targeting the GR signaling pathway might be pivotal and should be investigated in clinical studies.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Adrenocortical carcinoma (ACC) is a rare endocrine malignancy, with more than 40% of patients presenting with tumor-related glucocorticoid excess.
⇒ Glucocorticoids and glucocorticoid receptor (GR) signaling may be associated with an immune profile and prognosis in patients with ACC.

WHAT THIS STUDY ADDS

⇒ Here, we demonstrated that GR expressions were significantly associated with immune cell infiltration and better survival in patients with ACC. Furthermore, a GR activity signature discovered herein could serve as a robust prognostic biomarker and may contribute to prognostic prediction and response to immunotherapy in patients with ACC.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our findings identify a GR activity signature that could characterize the tumor immune microenvironment and response to immune checkpoint blockade therapy, and targeting GR signaling may improve immunotherapy response and survival outcomes in patients with ACC.

INTRODUCTION

Adrenocortical carcinoma (ACC) is a rare cancer with an annual incidence of 0.5–2 new cases per million worldwide.1–2 ACC is characterized by an aggressive disease course, with almost two-thirds of the patients presenting with local recurrence and distant metastases despite complete surgical resection.3 The 5-year survival is approximately 0%–15% for advanced disease.4–5 Currently, mitotane monotherapy or mitotane+etoposide, doxorubicin and cisplatin remain the recommended standard treatment for recurrent and metastatic ACC based on the First International
Randomized Trial in Locally Advanced and Metastatic Adrenocortical Carcinoma Treatment (FIRM-ACT) trial, offering a promising treatment option, but not showing a significant improvement in overall survival (OS). Given the chemoresistant properties and lack of effective therapeutic targets, there is a critical need to explore new treatment modalities for prolonging patient lifespans. Immunotherapy by activating the immune response is the latest revolutionary therapy for multiple cancers. However, preliminary clinical trial results with immune checkpoint inhibitors showed modest antitumor activity in patients with ACC, with a heterogeneous response rate and survival benefit in different cases. Therefore, understanding the molecular and oncogenic pathways that impair the efficacy of cancer immunotherapy is important for devising strategies to improve immunotherapy and selecting patients who will benefit from this treatment.

It is well known that exogenous and endogenous glucocorticoids often result in immunosuppression and promote tumor progression through regulating the immune system. Notably, approximately 40% of ACCs have high glucocorticoid secretion with aggressive behavior and an unfavorable prognosis. Therefore, one potential cause of immunotherapy failure may be excess cortisol secretion by ACC tumors. The interplay between steroidogenesis and the immune profile in ACC has been uncovered by recent studies. Based on public transcriptome data, researchers established the molecular steroidogenic classification for ACC, and found that patients with a high steroid phenotype presented lower levels of immune cell infiltration and immune modulator genes than those with a low steroid phenotype. Moreover, other investigators have demonstrated that glucocorticoid excess is negatively associated with T helper cell infiltration, as evidenced by immunofluorescence staining of tumor-infiltrating immune cells in 146 ACC tissues. Overall, the current findings improve our understanding of the immune landscape of ACC tumors and highlight the impact of glucocorticoids on immune system activity, drawing attention to possible druggable targets.

Mechanically, the immunosuppressive effect of glucocorticoids is achieved primarily through glucocorticoid receptor (GR) interference with the activation of several transcription factors, including NF-kB, AP-1, STAT, and GATA-3. By targeting these transcription factors, they can downregulate the expression of major histocompatibility complex (MHC) molecule and proinflammatory cytokines while inducing the release of anti-inflammatory cytokine (interleukin (IL)-10), thereby impairing the antigen-presenting ability of macrophages and dendritic cells. Furthermore, GR signaling transactivates IL-10 and checkpoint receptor expression, promoting CD8+ tumor-infiltrating lymphocyte dysfunction. Consequently, GR signaling leads to immunosuppression and a poor response to immune checkpoint blockade. Beyond its indirect influence on tumor growth via the immune microenvironment, GR can directly influence tumor cell biology by controlling their survival pathways, and play different roles in different tumors with tissue cell specificity. For example, in pancreatic, liver, and bladder cancers, GR may act as a tumor suppressor gene, whereas in endometrial, colon, and ovarian cancers, GR may function as a tumor driver gene. Despite extensive studies on the functions of GR in many cancers, surprisingly little is known regarding its role in ACC. Our earlier study provided initial insights into the role of GR in ACC through immunohistochemistry and bioinformatics analysis and discovered that patients with low GR expression often exhibited excess glucocorticoid secretion and a dismal poor prognosis. These data emphasize the vital roles of GR signaling in the immune microenvironment composition.

To provide new insights into the interaction between GR signaling and the immune signature of ACC, we first comprehensively analyzed GR expression and the infiltration of CD4+ T cells, CD8+ cytotoxic T cells, CD16+ cytotoxic natural killer (NK) cells, dendritic cells and macrophages in primary ACC tumors using immunofluorescence and bioinformatics analysis. Furthermore, we identified a GR activity signature (GRsig) using GR-targeted gene networks derived from transcriptome data of primary ACC. Subsequently, we assessed the relevance of GRsig to the tumor microenvironment and its prognostic value for patients with ACC by using bioinformatics algorithms. Together, our study outcomes could enhance our understanding of the vital role of GR transcriptional activity in the immune microenvironment and aggressiveness of ACC, thus facilitating the development of strategies to improve immunotherapy efficacy.

MATERIALS AND METHODS

Patients and samples

Seventy-eight primary tumor tissues from a retrospective cohort (78 patients with non-metastatic ACC) were used for this study. Patients who underwent radical adrenalenalectomy for primary tumors were identified from the records of West China Hospital (WCH), Sichuan University, between 2009 and 2019. Details on cohort construction, patient selection, characteristics, treatment, and follow-up protocol have been described in our previous report. The GR status has been previously reviewed. Briefly, an anti-GR monoclonal antibody (#12041, 1:400, Cell Signaling Technology (CST)) was employed to assess the expression level of GR in primary tumors using immunohistochemistry. The immunoreactivity score from 0 to 12 was evaluated based on the modified “quick-score” protocol, as previously described. This study was approved by the ethical committee of our institution.

Fluorescence immunohistochemistry and image analysis

Immunofluorescence staining of marker molecules on immune cells was performed on 5 µm formalin-fixed and paraffin-embedded (FFPE) tissue sections. After being baked at 60°C for 2 hours, the sections were immersed
in 100% xylene for deparaffinization, rehydrated with an ethanol gradient, and washed with phosphate-buffered saline (PBS). Antigen retrieval was performed with 0.01 M sodium citrate buffer using microwave heating. After cooling to ambient temperature, the slices were washed with PBS and then incubated with blocking buffer. The primary antibody was diluted in 1% bovine serum albumin (BSA) and subsequently incubated at 4°C overnight. Mouse anti-CD8a (#372902, 1:100, BioLegend), mouse anti-CD4 (#48705, 1:1600, CST), rabbit anti-CD16 (16559–1-AP, 1:100, Proteintech), rabbit anti-CD68 (#76437, 1:400, CST) and mouse anti-CD11c (#62824, 1:400, CST) antibodies were used for immunofluorescence staining. On the next day, the slices were washed twice with PBS, followed by incubation with appropriate secondary antibodies (anti-rabbit or mouse conjugated Alexa Fluor 488 or 555; #4412, #4413 and #4409, CST) for 1 hour at room temperature. After washing, the slices were sealed using ProLong Gold Antifade Reagent with DAPI (#8961, CST).

All fluorescent images were digitized with a pathology slide scanner (NanoZoomer Digital Pathology, Japan). For each tissue section, 10 high-power fields (HPF, 40X) were randomly selected by two independent observers to count positive tumor-infiltrating immune cells. Only cells with specific membrane/nuclear staining were considered as positive. The positive immune cell staining was reported as the number per HPF.

Gene expression data

The RNA sequencing expression (level 3) profiles and the corresponding clinical data sets of ACC were downloaded from The Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo). TCGA data was obtained to evaluate the immune landscape and GR target genes in ACC. The GEO data set was obtained to explore the gene expression pattern between cortisol-secreting ACC and non-functioning ACC. GSE19775 contains the gene expression profiling of 6 cortisol-secreting tumors and 5 non-functioning tumors. GSE19776 contains 8 cortisol-secreting tumors and 12 non-functioning tumors. Additionally, GSE10927 (n=24) and GSE33371 (n=23) data sets comprise the messenger RNA (mRNA) expression data, clinical annotations and survival information of 47 patients with ACC for external validation. The raw MINIml files downloaded from the GEO database were normalized using the normalize quantiles function of the preprocessCore package in R software (V.3.4.1). The probes were converted into gene symbols according to the corresponding platform annotation information. The mean expression value of the corresponding gene measured by multiple probes was regarded as the final expression level of the gene, and the probes matching multiple genes were removed from the data set.

Immune landscape analysis

To obtain reliable results of the immune score evaluation, xCell and CIBERSORT bioinformatics algorithms were used to estimate the tumor-infiltrating immune cell composition in TCGA-ACC samples. Additionally, we extracted the expression values of immune checkpoint-relevant molecules, major histocompatibility complexes, common immunotherapeutic biomarkers, and T-cell stimulators. Subsequently, we compared the expression values of these genes among different groups.

Construction and evaluation of the GR activity signature

GR is a corticosteroid receptor and a widely active transcription factor. To identify GR-mediated genes involved in ACC progression, we initially performed a differential gene expression analysis between cortisol-secreting ACC and non-functioning ACC from the GEO data set. Next, we examined the global gene expression differences between GR-high versus GR-low tumors among TCGA-ACC. Finally, we identified the intersection of the above differential genes and further screened out the genes associated with patient survival. A GRsig was defined as those genes with OS-associated p<0.05 and an HR>1.5 or HR<0.5.

The “Limma” R package was applied to explore the differentially expressed mRNAs. An adjusted p<0.05 and at least ±1.5-fold change was set as the threshold for the differentially expressed genes. Univariate Cox regression analysis was applied to identify GR-regulated genes associated with patients’ OS, expressed as HRs with 95% CIs. Subsequently, the least absolute shrinkage and selection operator (LASSO) Cox regression model was applied to identify the most predictive markers among the OS-related genes, using the “glmnet” and “survival” R packages. Finally, the GR signature-related risk score model was constructed with a linear combination of the optimal set of genes expression level, weighted by the LASSO Cox regression coefficients using cross-validation. After estimating the risk score of each patient, all patients were further divided into high-risk and low-risk groups based on the median risk score. Kaplan-Meier survival analysis was used to compare the differences in survival outcomes between the high-risk and low-risk groups. TimeROC (V.0.4) analysis was implemented to draw the receiver operating characteristic curve and examine the predictive accuracy of the GRsig.

Consensus clustering analysis of the GR signature

Consensus clustering analysis was performed to determine distinct molecular subtypes based on the GRsig. This consistency analysis was executed by using the “ConsensusClusterPlus” R package, and the optimal number of clusters was determined using the cumulative distribution function (CDF) and relative change in the CDF Delta area. The “pheatmap” R package was used for clustering heatmaps. Kaplan-Meier survival analysis was adopted to examine the survival outcome differences among different clusters. Furthermore, we explored the differences in clinicopathological features and immune status between these subtypes.
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Statistical analyses
The contingent variables were described as percentages and analyzed with the χ² or Fisher’s exact test. The distributed variables were expressed as medians with ranges or IQRs and assessed using the Mann-Whitney U test. X² test’s Phi coefficient was conducted to assess the correlation between different investigated markers. Heatmaps were created to display the immune cell abundances and gene expression of individual tumors in different groups. Event-free survival among subgroups was estimated by the Kaplan-Meier method and compared by the log-rank test. For survival comparisons involving multiple groups, Bonferroni correction was used as p values. Cox regression modeling was performed to identify independently relevant prognostic factors for patients with ACC, and HRs with 95% CIs were calculated. All statistical analyses were implemented by GraphPad Prism V.5.0 or R V.4.0.3 software, and a p value<0.05 was considered significant.

RESULTS
Patient characteristics and GR expression
Demographic data and clinicopathological characteristics of a cohort of 78 patients with ACC are shown in table 1. The patient cohort included 33 men and 45 women, with a median age at diagnosis of 44 years (IQR: 36–53), who presented hormonal hypersecretion features (46/78, 59%). The majority of tumors were pathologically staged as pT1-2 (54/78, 69%), with a median size of 9 cm (range: 2–27). GR was expressed in 90% of primary ACC tumors. The immunohistochemical staining and immunoreactivity score of GR expression per tumor are shown in figure 1. The median immunoreactivity score for GR was 6 (IQR: 4–12). As a result, we used 6 as the cut-off value and divided all patients into a low GR group (n=42) and a high GR group (n=36).

We examined the correlations between GR expression and clinicalopathological features. As shown in table 1, no correlations were found with most variables, including age at diagnosis, sex, tumor stage, tumor size, Weiss score, and Ki67 index, except for hormone secretion status. The “GR-high” group tended to be associated with hormonal excess (p<0.001). Therefore, we further investigated the association between the type of hormonal secretion and GR expression, and observed that GR protein expression was lower in cortisol-producing ACC than in non-cortisol-secreting tumors (p<0.001, figure 1D). However, there was no significant difference in GR expression between non-functional and other hormone-secreting tumors (including androgen, estrogen, or mineralocorticoid) (p=0.15, figure 1D).

Infiltrating immune cells in the primary tumor
To perform a comprehensive analysis of immune cell infiltration levels in ACC, we selected several common immune markers, CD8α (cytotoxic T cells), CD4 (CD4+ T cells), CD16 (cytotoxic NK cells), CD11c (dendritic cells) and CD68 (macrophages), for immunofluorescence

Table 1  Patient characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All patients (n=78)</th>
<th>GR expression</th>
<th>P value</th>
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<tr>
<td></td>
<td></td>
<td>Low (n=42)</td>
<td>High (n=36)</td>
</tr>
<tr>
<td>Age (years, median, IQR)</td>
<td>44 (36–53)</td>
<td>44 (33–54)</td>
<td>45 (37–51)</td>
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<td>Sex (male/female)</td>
<td>33/45</td>
<td>18/24</td>
<td>15/21</td>
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<tr>
<td>Tumor size (cm)</td>
<td>9 (2–27)</td>
<td>10 (2–25)</td>
<td>8.5 (3–27)</td>
</tr>
<tr>
<td>T stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1–2</td>
<td>54 (69%)</td>
<td>27 (64%)</td>
<td>27 (75%)</td>
</tr>
<tr>
<td>T3–4</td>
<td>24 (31%)</td>
<td>15 (36%)</td>
<td>9 (25%)</td>
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<td>Hormone secretion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>32 (41%)</td>
<td>5 (12%)</td>
<td>27 (75%)</td>
</tr>
<tr>
<td>Yes</td>
<td>46 (59%)</td>
<td>37 (88%)</td>
<td>9 (25%)</td>
</tr>
<tr>
<td>Weiss grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>15 (19%)</td>
<td>7 (17%)</td>
<td>8 (22%)</td>
</tr>
<tr>
<td>High</td>
<td>63 (81%)</td>
<td>35 (83%)</td>
<td>28 (78%)</td>
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<tr>
<td>Ki67 index</td>
<td>10 (2–70)</td>
<td>10 (3–70)</td>
<td>10 (2–50)</td>
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<td>CD8+ T cell</td>
<td>8.6 (0.1–309)</td>
<td>3.2 (0.1–286)</td>
<td>12.3 (0.3–309)</td>
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<td>CD4+ T cell</td>
<td>7.0 (0.1–120)</td>
<td>2.8 (0.1–40)</td>
<td>8.9 (0.1–120)</td>
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<tr>
<td>Natural killer cell</td>
<td>2.5 (0.1–20)</td>
<td>1.8 (0.1–15)</td>
<td>3.2 (0.6–20)</td>
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<tr>
<td>Macrophages</td>
<td>2.1 (0.1–34)</td>
<td>1.1 (0.1–20)</td>
<td>3.2 (0.5–34)</td>
</tr>
<tr>
<td>Dendritic cell</td>
<td>0.8 (0.1–19)</td>
<td>0.5 (0.1–12)</td>
<td>1.5 (0.4–19)</td>
</tr>
</tbody>
</table>

GR, glucocorticoid receptor.
staining on matched FFPE sections of ACC tissue (figure 2). Immunofluorescence microscopy showed that CD8+ cytotoxic T lymphocytes were the most common infiltrating cell type, detectable in 88% of the 78 ACC specimens (8.6 cells/HPF). CD4+ T cells were visualized in 80% of ACC samples (7.0 cells/HPF), NK cells were detectable in 78% of primary tumors (2.5 cells/HPF), and macrophages were observed in 76% of ACC samples (2.1 cells/HPF). However, only 65% of the tumors were positive for CD11c+ dendritic cells (0.8 cells/HPF).

Overall, our findings indicated relatively sparse immune infiltration in ACC through the scanning and analysis of fluorescence images.

**Relationship between GR expression and immune cell infiltration in ACC**

The distribution of infiltrating immune cells between GR-high and GR-low tumors is shown in table 1 and figure 3. Notably, the number of all immune cell subtypes was significantly higher in GR-high ACC than...
in GR-low ACC. We further investigated the correlation between immune cell infiltration and GR expression in primary ACC samples using the Pearson correlation coefficient (figure 3F). Tumor GR expression was positively correlated with CD8⁺ T-cell (Phi=0.342, p<0.001), CD4⁺ T-cell (Phi=0.280, p<0.001), NK cell (Phi=0.280, p<0.001), macrophage (Phi=0.285, p<0.001), and dendritic cell (Phi=0.397, p<0.001) infiltration with the tumor. A heatmap of GR and immune cell marker expression in primary ACC also showed a strong enrichment in GR-high versus GR-low tumors for immune cell infiltration (figure 3G). Additionally consistent with previous findings, cortisol excess showed a significant negative correlation with immune cell infiltration in primary ACC, and low immune cell infiltration was observed in cortisol-secreting ACC compared with non-functioning tumors (online supplemental figure S1A–F).

We subsequently used TCGA-ACC data with the CIBERSORT algorithm to dissect the potential correlation between GR expression, cortisol excess, and immune cell infiltration in GR-high versus GR-low tumors for immune cell infiltration (figure 3G). Additionally consistent with previous findings, cortisol excess showed a significant negative correlation with immune cell infiltration in primary ACC, and low immune cell infiltration was observed in cortisol-secreting ACC compared with non-functioning tumors (online supplemental figure S1A–F).

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Figure 3  GR expression correlates with immune cell infiltration of primary ACC tumors. (A–E): CD8+ T cell (A), CD4+ T cell (B), NK cell (C), macrophage (D) and dendritic cell (E) infiltrated in GR-high versus GR-low tumor samples. (F) Analysis of the correlation between immune cell infiltration and GR expression in primary ACC samples using the Pearson correlation coefficient. (G) Heatmap of GR expression and immune cell composition in individual tumors. ACC, adrenocortical carcinoma; GR, glucocorticoid receptor; HPF, high-power field; NK, natural killer.
cell infiltration (online supplemental figure S1G). As expected, GR-high tumors had significantly higher immune cell infiltration; conversely, ACC with excess glucocorticoids showed lower immune cell presence.

Impact of GR expression and immune cell infiltration on the prognosis of patients with ACC

We first evaluated the prognostic significance of GR expression and immune cell infiltration using univariate Cox regression analyses. High GR expression and increased immune cell infiltration were significantly associated with better survival in patients with ACC (online supplemental figure S2A,B). More precisely, the GR-high group had a significantly lower risk of disease progression and better OS compared with the GR-low group (disease-free survival (DFS): HR=0.51, 95% CI: 0.29 to 0.88, p=0.032; OS: HR=0.55, 95% CI: 0.32 to 0.95, p=0.043). In line with GR, elevated infiltration of CD8+ T, CD4+ T, NK, and dendritic cells was significantly correlated with better DFS and OS in patients with ACC. Conversely, in our series, there was no significant association between macrophage infiltration and patient outcome. Furthermore, we supported these results by performing survival analysis in the TCGA-ACC cohort based on the median expression of GR and each type of immune cell (online supplemental figure S2C,D). Additionally, in multivariate analyses of OS, encompassing other potential prognostic factors including tumor stage, glucocorticoid excess, Weiss score, and Ki67 index, we confirmed that strong GR protein expression, high CD8+ T, and high CD4+ T cell infiltration remained predictors of prolonged OS (online supplemental table S2).

Finally, we divided all patients into four subgroups according to the heatmap results for GR expression and immune cell infiltration in individual tumors, resulting in four more refined prognostic subgroups (online supplemental figure S2E,F, online supplemental table 3). Tumors with high GR expression and elevated immune cell infiltration (n=23) exhibited the most favorable survival, followed by ACC tumors with high infiltration of immune cells and low GR expression (n=15). With low immune cell infiltration in tumors, the prognosis was clearly poor and again GR expression dependent.

Construction of the GR activity signature in ACC

Figure 4 depicts the identification schema and process of the GRsig. First, 1,744 differentially expressed genes were identified from non-functioning tumors and cortisol-secreting ACC in the GEO data set (GSE19775 and GSE19776). Second, differentially expressed genes from the GEO data set that were modulated by cortisol were cross-evaluated with the list of differentially expressed genes among GR-high versus GR-low primary ACC in the TCGA cohort (n=1569) to obtain a subset of GR target genes (n=67). Next, the prognostic relationship between the mRNA expression of these genes and OS was estimated in the TCGA-ACC cohort to identify clinically valuable GR-regulated genes using univariate Cox regression analysis (online supplemental table S1 and figure S3).

Finally, we constructed a putative GRsig by selecting 34 shared genes that were significantly associated with OS (HR>1.5 or HR<0.5, and p<0.05). Numerous genes (n=30) from the GRsig were associated with worse OS (HR>1.5) and were GR-downregulated, while four genes were associated with better OS (HR<0.5) and were GR-upregulated (figure 4E). We then compared the survival outcomes between patients with ACC patients with above-median GRsig expression and those with below-median GRsig expression in the TCGA cohort. As expected, patients with above-median GRsig expression experienced poor DFS (HR=3.9, p<0.001, figure 4F) and OS (HR=2.5, p=0.024, figure 4G) compared with those with below-median GRsig expression. To assess the clinical applicability and prognostic significance of the GRsig genes, we conducted further validation by examining the protein expression of EFNB1 and BMP4 in the WCH cohort comprising 78 ACC FFPE tissues, using immunohistochemistry (online supplemental figure S4). As expected, patients with strong EFNB1 or BMP4 expression exhibited a significant reduction in DFS, and worse OS.

Identification of GRsig-related subtypes in ACC

Two GRsig-related subtypes were defined using the unsupervised clustering method based on the expression data of the GRsig in the TCGA cohort. The Consensus Cluster Plus analysis determined the optimal k value of 2 in the TCGA cohort based on the consensus matrix, CDF curve and CDF Delta area (figure 5A–C). As a result, all patients with ACC are classified into two distinct subtypes. The heatmap of the transcriptomic profile further confirmed significantly dissimilar gene expression patterns between these two subgroups (figure 5D). We subsequently performed Kaplan-Meier survival analysis to compare the survival differences between the two subtypes. We found that patients in cluster 1 had significantly shorter progression-free survival (HR=5.09, p<0.001, figure 5E) and OS (HR=3.63, p=0.009, figure 5F) compared with those in cluster 2. Additionally, we investigated the relationship between clinical features and the two subtypes, as displayed in table 2. Patients in cluster 1 had a higher proportion of women, tumor stage III/IV, glucocorticoid excess, and high Ki67 index than those in cluster 2.

To better understand the difference in underlying functional mechanisms between the two subtypes, we carried out pathway enrichment analysis and discovered that the cluster-related differentially expressed genes significantly participated in immune and inflammatory pathways, including the proliferation, migration, differentiation, and activation of immune cells, cytokine receptor interactions, and antigen processing and presentation (figure 5G,H).
Figure 4  Discovery of the GR activity signature (GRsig) in adrenocortical carcinoma. (A,B) Volcano plot showing the differentially expressed genes from non-functioning tumors and cortisol-secreting ACC in the GSE19775 (A) and GSE19776 (B). (C) The volcano plot of the differential gene expression in GR-high versus GR-low primary ACC in the TCGA data set. (D) Venn diagram showing differential gene intersection of the GSE19775, GSE19776 and TCGA. (E) Identification schema for the GRsig. (F,G) Kaplan-Meier estimates showing that the above-median GRsig expression (vs all others) is associated with disease free survival (F) and overall survival (G) in the TCGA cohort. ACC, adrenocortical carcinoma; GEO, Gene Expression Omnibus; GR, glucocorticoid receptor; TCGA, The Cancer Genome Atlas.
The immune landscape of GRsig-related subtypes

Considering the close correlation between GRsig-related subtypes and immune status, we explored the tumor microenvironment of two subtypes based on the TCGA data set using the xCell algorithm. The cluster 1 subtype displayed high infiltration of common lymphoid
progenitors, B-cell plasma, and gamma delta T cells, whereas cluster 2 exhibited elevated infiltration of activated myeloid dendritic cells, common myeloid progenitors, endothelial cells, NK T cells, regulatory T cells, CD4+ effector memory T cells, CD4+ memory T cells, CD4+ naïve T cells, CD8+ T cells, CD8+ memory T cells, M2 macrophages, monocytes, and myeloid dendritic cells (figure 6A). Additionally, we calculated stromal score, immune score, and microenvironment score for each sample in the two clusters based on the TCGA expression profiles. As shown in figure 6A, cluster 2 also had significantly higher stromal scores, immune scores, and microenvironment scores than cluster 1. Then, we investigated the differences in the expression levels of major histocompatibility complexes and T-cell stimulators between the two subtypes. Overall, the expression of most genes tended to be higher in samples of cluster 2, except for HLA-G, HLA-DMβ, HLA-C, and TNFRSF (4, 8, 14, 25) (figure 6B,C).

Furthermore, we examined the association of the two subtypes with the expression levels of immune checkpoint molecules and immunotherapy-related biomarkers. As shown in figure 6D,E, cluster 2 was characterized by high expression levels of most of the immune checkpoint molecules and immunotherapy-related genes, except for LAG3, SIGIEC1 and TGFβ signaling-related genes (TGFBI, TGFBR2, ACVR1). These findings implied that GRsig-related subtypes may have a relationship with the immunotherapy effectiveness.

**GR activity signatures for the prognostic prediction of ACC**

To assess the prognostic significance of the GRsigs, we used LASSO Cox regression analysis to construct a prognostic model for patients with ACC based on TCGA cohorts. We screened six genes (NR4A3, EFNB1, MRPL41, IFI27, RPP40, SGPP2) from GR signatures with non-zero coefficients according to 10-fold cross-validation and the minimized lambda=0.1463 determined by the LASSO algorithm. Then, we constructed the GRsig-related risk score model based on these optimal prognostic biomarkers (online supplemental figure S5). The relationships between the expression levels of six GR-regulated genes and OS are visualized in the forest plot (online supplemental figure S5C). The GRsig-related risk score was further calculated with the following formula: Risk score=(0.109*NR4A3)+(0.147*EFNB1)+(0.0721*MRPL41)+(0.0093*IFI27)+(0.0333*RPP40)+(−0.0449*SGPP2). Subsequently, we classified patients with TCGA-ACC into high-risk and low-risk subgroups based on the median risk score cutoff. The distribution of risk scores, survival time and status in the TCGA-ACC data set is illustrated in online supplemental figure S5D. Patients with high risk scores were more likely to have advanced stage, glucocorticoid excess, and deadly outcome compared with those with low risk scores (online supplemental figure S5E). Kaplan-Meier survival analyses revealed that patients with high-risk ACC exhibited a worse OS than their counterparts (HR=6.05, p<0.001, online supplemental figure S5F). The average Area under
curve (AUC) values of 1-year, 3-year and 5-year survival predictions in patients with TCGA-ACC reached 0.86 (95% CI: 0.74 to 0.97), 0.84 (95% CI: 0.76 to 0.93) and 0.83 (95% CI: 0.74 to 0.93), respectively (online supplemental figure S5G).

Furthermore, to validate the reliability and reproducibility of the model, we computed the risk score for each patient with ACC in two independent GEO data sets (GSE10927 and GSE33371) using the same risk formula. As expected, Kaplan-Meier survival analysis also

Figure 6  Immune landscape of GRsig-related subtypes in the TCGA cohort. (A) Immune cell score heatmap shows the expression distribution of immune score in two GRsig-related subtypes. (B–E) Gene expression of major histocompatibility complex, T-cell stimulator, immune checkpoint molecule and immunotherapy-related biomarker gene sets between two distinct clusters. GRsig, GR activity signature; NK, natural killer; TCGA, The Cancer Genome Atlas.
demonstrated that patients in the high-risk group had a notable trend towards increased mortality risk compared with cases in the low-risk group (online supplemental figure S5H and I).

**DISCUSSION**

Of all ACCs, approximately 40% were considered overt hypercortisolism, excluding cases with high cortisol levels without associated symptoms, which could still impact tumor–immune interactions and the microenvironment. A comprehensive immune-genomic analysis of over 10,000 RNA sequencing samples from the TCGA database found that ACC had the second-lowest percentage of leukocytes among all 33 diverse cancer types, labeled as a classic “immune desert” lacking tumor-infiltrating immune cells. Recently, researchers further determined that hypercortisolism and a high steroid phenotype are associated with an immunosuppressed profile and poor survival in patients with ACC.

In this study, we first demonstrated the positive correlation of GR expression with cytotoxic T cell, CD4+ T cell, NK cell, dendritic cell, and macrophage infiltration in primary ACC tumors. Interestingly, this novel finding in ACC tumors was also observed in breast cancer. GR-high tumors are enriched with CD8+ T cells, CD4+ T cells, B cells, dendritic cells, macrophages, and neutrophils but have fewer immunosuppressive regulatory T cells. This information combined with our findings in the WCH and TCGA-ACC cohorts indicates that the GR signaling pathway may be involved in cancer immune evasion and immunotherapy resistance in ACC.

The immune microenvironment was deemed to play an important role in cancer development. Tumor-infiltrating immune cells, especially cytotoxic T cells and tumor-associated macrophages, have been extensively researched and have shown associations with patient outcome across various cancers. Similarly, in our study, we also found that patients with high immune cell infiltration had a lower risk of recurrent metastasis and mortality. Moreover, increasing evidence has proven that high T lymphocyte infiltration is associated with immunotherapy responsiveness, defined as the “hot” tumor immunophenotype. Therefore, numerous ongoing studies are currently underway to elucidate the mechanisms behind tumor immune escape, hoping to provide potential strategies for turning “cold” tumors (“immune-desert” phenotype) into “hot” tumors.

For ACC, our study reveals that cortisol excess can cause a state of immunosuppression in ACC, which is supported by prior studies. More importantly, we found in the exploratory analysis a higher immune cell infiltration and higher expression of immune modulators in patients with high GR expression than in patients with low GR expression. This suggests that targeting GR signaling is an actionable strategy to convert the immunologically “cold” microenvironment into a “hot” tumor microenvironment, signifying an effective immune infiltrate.

Glucocorticoids and GR signaling have long been thought to control tumor growth by indirectly influencing the proliferation, differentiation and apoptosis of immune cells in the tumor microenvironment. In a preclinical study, Acharya et al revealed that glucocorticoids are locally produced by tumor monocyte-macrophage lineage cells, and the presence of active GR signaling could promote CD8+ T cell dysfunction by transactivating the expression of multiple checkpoint receptors, resulting in tumor growth and a poor response to checkpoint blockade therapies. Recently, Deng and colleagues discovered a novel tumor cell-intrinsic role for GR in pancreatic cancer. GR could directly transcriptionally regulate programmed cell death ligand 1 (PD-L1) and MHC-I expression. Tumor cell-specific depletion of GR or pharmacological inhibition of GR increased the abundance of tumor-infiltrating cytotoxic T cells, thereby enhancing the antitumor immune effects, and overcoming immunotherapy resistance. Our findings further corroborate that GR signaling affect the expression of multiple immune checkpoint molecules and MHC surface levels. Taken together, these results support a link between GR signaling activation and the immunosuppressive microenvironment in ACC. Pharmacologic GR signaling inhibition as well as glucocorticoid biosynthesis could increase T lymphocyte infiltration, thus improving the immunotherapeutic response and controlling tumor growth. Beyond the classic GR-mediated effects, glucocorticoids can also exert regulatory influences through non-GR mechanisms (eg, NF-κB pathway modulation, epigenetic regulation, microRNA modulation, and direct protein–protein interactions). These mechanisms provide additional layers of complexity to the actions of glucocorticoids and contribute to their diverse physiological effects. Understanding both GR-mediated and non–GR mechanisms is essential for comprehensively grasping the multifaceted roles of glucocorticoids in cancer.

The GR signaling pathway can affect tumor growth through various mechanisms. Besides its indirect influence on tumor growth by acting on immune cells and other microenvironment cells, it can also directly modulate the biological behavior of tumor cells by controlling their survival pathways. Existing evidence suggests that GR signaling has a complex functional role in solid tumors, highly dependent on tumor type and microenvironment, exhibiting dual roles: as a tumor suppressor or oncogene. For example, numerous studies have proposed that GR plays a role as a tumor suppressor gene in various solid tumors, such as non-small cell lung, liver, and bladder cancers. In contrast, other studies suggest that GR may function as a tumor driver gene in other tumors, including endometrial cancer, colon cancer, and ovarian cancer. Interestingly, in breast and prostate cancers, GR can exhibit completely opposite functional roles in different subtypes and disease stages. Here, we found that GR expression was not only associated with ACC immune status but also closely correlated with tumor recurrence, distant metastasis and survival outcomes of
Competing interests

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Data availability statement

Data are available upon reasonable request. All data generated or analyzed in this study are available upon reasonable request.

Supplemental material

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Contributors

KL and XL conceived and designed the experiments. KW, ZL and JL analyzed the data and contributed reagents/materials/analysis tools. KW, ZL, YZ and JL sample collection and preparation by Yongquan Tang and Fuxun Zhang.

Conclusion

GR expression correlates with tumor-infiltrating immune cells in ACC. Furthermore, the GRsig discovered herein was obviously associated with the tumor immune microenvironment and patient with ACC survival and could serve as a robust prognostic signature for ACC, helping predict patient prognosis and response to immunotherapy. Consequently, targeting the GR signaling pathway might improve clinical outcomes and immune efficacy in ACC. However, this hypothesis requires testing through clinical studies.

Patient consent for publication

Not applicable.

Ethics approval

This study was conducted in accordance with the Declaration of Helsinki, and all samples were collected from West China Hospital, Sichuan University, with the informed consent of the patients, and approved by the Medical Ethics Committee of West China Hospital, Sichuan University (20211068A).

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