Differential role of glucocorticoid receptor based on its cell type specific expression on tumor cells and infiltrating lymphocytes

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tumor cells and infiltrating lymphocytes

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Abstract

Background: The glucocorticoid receptor (GR) is frequently expressed in breast cancer (BC), and its prognostic implications are contingent on estrogen receptor (ER) status. To address conflicting reports and explore therapeutic potential, a GR signature (GRsig) independent of ER status was developed. We also investigated cell type-specific GR protein expression in BC tumor epithelial cells and infiltrating lymphocytes.

Methods: GRsig was derived from Dexamethasone-treated cell lines through a bioinformatic pipeline. Immunohistochemistry assessed GR protein expression. Associations between GRsig and tumor phenotypes (proliferation, cytolytic activity (CYT), immune cell distribution, and epithelial-to-mesenchymal transition (EMT)) were explored in public datasets. Single-cell RNA sequencing data evaluated context-dependent GR roles, and a cell type-specific prognostic role was assessed in an independent BC cohort.

Results: High GRsig levels were associated with a favorable prognosis across BC subtypes. Tumor-specific high GRsig correlated with lower proliferation, increased CYT, and anti-tumorigenic immune cells. Single-cell data analysis revealed higher GRsig expression in immune cells, negatively correlating with EMT while a positive correlation was observed with EMT primarily in tumor and stromal cells. Univariate and multivariate analyses demonstrated the robust and independent predictive capability of GRsig for favorable prognosis. GR protein expression on immune cells in triple-negative tumors indicated a favorable prognosis.

Conclusion: This study underscores the cell type-specific role of GR, where its expression on tumor cells is associated with aggressive features like EMT, while in infiltrating lymphocytes, it predicts a better prognosis, particularly within TNBC tumors. The GRsig emerges as a promising independent prognostic indicator across diverse BC subtypes.

Key words: Glucocorticoid receptor, Gene signature, Proliferation, Tumor microenvironment, Epithelial to mesenchymal transition, Prognosis
Introduction
Breast cancer (BC) represents the prevailing malignancy globally, with an annual incidence of approximately 2.3 million cases\(^1,2\). Despite notable improvement in early diagnosis and treatment, around 30\% of cases experience recurrence or metastasis within the initial 5 years\(^3\). Immunophenotyping, specifically for estrogen and progesterone receptors (ER, PR), and human epidermal growth factor receptor 2 (HER2), is the predominant methodology employed for subtype categorization, classifying BC into Hormone Receptor positive (HR+), HER2-amplified, and Triple Negative Breast Cancer (TNBC)\(^4,5\).

In addition to ER and PR, BC, being a heterogeneous disease, expresses various other nuclear receptors, among which the androgen receptor (AR) and glucocorticoid receptor (GR) are extensively studied\(^6,7\). While these receptors can be individually targeted, the intricate crosstalk between multiple nuclear receptors has been demonstrated to significantly modify the signaling of individual receptors\(^8\). Consequently, this phenomenon exerts a notable influence on the emergence of alternative pathways in tumor progression and contributes to the development of therapeutic resistance\(^9\).

The GR, a member of the nuclear receptor superfamily and a corticosteroid receptor, functions as a transcription factor with diverse roles, including chromatin remodeling\(^7\). Numerous studies have shown its varied expression, underscoring its potential as a target for enhanced therapeutic interventions\(^10\). The association between GR expression and patient prognosis is intricately linked to the specific molecular subtype of BC\(^11,12\). Notably, in ER positive (ER+) tumors, high GR expression is correlated with favorable features and improved outcomes, whereas in ER-negative tumors, higher GR expression is associated with shorter relapse-free survival, highlighting the context-dependent nature of GR action\(^11,13\).

Glucocorticoids (GCs), serving as ligands for GR, are adrenal cortex-secreted hormones known to regulate circadian rhythm, homeostasis, cellular proliferation, and apoptosis\(^14\). Their well-established role in influencing immunity and inflammation reveals varied and opposing effects on the immune system. While GCs enhance innate immune responses, they concurrently suppress the adaptive immune pathway, potentially fostering a tumorigenic immune microenvironment\(^15,16\). In the context of BC, synthetic GCs are frequently employed as pre-medication alongside chemotherapy interventions\(^17\).

Despite numerous studies elucidating the prognostic implications of GR, its detection has proven challenging. The complexity in GR detection is attributed to the existence of at least two isoforms
of the protein (GRα and GRβ) and various spliced variants of the mRNA. This molecular diversity contributes to the ambiguity in GR detection.\(^\text{18}\)

Considering the context-dependent function of GR in BC and the controversial role of GCs in either promoting or inhibiting the tumor immune microenvironment, we aimed to develop a GR signature using a set of genes regulated by GR independent of the ER context. We further demonstrated the cell type specific expression and prognostic utility of derived GR signature.

**Materials and Methods**

**Collection of Datasets**

**Cell line data:** The datasets utilized in this study, GSE113571 (derived from MDA-MB-231) and GSE79761 (derived from MCF-7) were obtained from the Gene Expression Omnibus (GEO), comprises global gene expression profiles from ER- and ER+ BC cell lines\(^\text{11,19}\) were used to derive GR-regulated genes. GSE113571 features gene expression data from ER- cell lines treated with vehicle and 100nM Dex for 4 hours, and GSE79761 includes profiles from ER+ cell lines subjected to treatments such as vehicle, 100nM Dex, 1 nM estradiol, and Dex/E2 for 4 hours.

**Datasets accessed for analysis and validation:** Robust clinical data and comprehensive gene expression profiles sourced from repositories including the Molecular Taxonomy of BRCA International Consortium (METABRIC) project via cBioPortal\(^\text{20,21}\), The Cancer Genome Atlas (TCGA) through Genomic Data Commons Portal, and the Sweden Cancerome Analysis Network - Breast (SCAN-B) dataset, identified by the series GSE202203 in GEO, were major resources for downstream analysis and validation of our findings. Single-cell RNA sequencing (scRNA-seq) data, GSE75688, was utilized to examine the expression profiles of individual cell types, including tumor and immune cells\(^\text{22}\).

**Data analysis and prioritization of candidate genes**

Differential gene expression analysis of the cell line data was conducted using the R package limma\(^\text{23}\). Both GSE113571 and GSE79761 datasets were independently analyzed. In the MDA-MB-231 cell lines, a comparative analysis was performed between Dex treated cells and the control group (Vehicle). A similar analysis was carried out for the MCF-7 cell line. Differentially expressed genes (DEGs) between the control and treatment groups were filtered based on a significant p-value (p<0.05). Subsequently, the prioritization of genes followed a previously established methodology, incorporating features such as protein interaction network, graph parameters, and semantic similarity, to elucidate key candidates with potential functional significance\(^\text{24}\).
Next, GR regulated genes obtained from both the analysis (GSE113571 and GSE79761) were compared. The DEGs which were overlapping in both datasets were considered as GR regulated genes "ER-independent" status.

**Derivation of GR Gene Signature**

Genes that exhibited a significant correlation (p<0.01) with GR transcript expression were identified (using METABRIC dataset). Subsequently, these genes were compared against the reported target genes of GR as identified in chip-seq data. Genes located within ±1000 base pairs of the transcription start site (TSS) of GR were included in the analysis as GR gene signature (GRsig). GRsig was stratified into Low GR and High GR categories based on a quantile >0.33 and <0.66 cutoffs for the Mean/average expression of GRsig in the given dataset.

**Association of GRsig with patient and tumor characteristics**

Association of GR High and GR Low tumors with patient characteristics such as patient age, tumor grade, stage, menopausal status, and patient prognosis was studied. Additionally, associations with tumor characteristics such as proliferation, cytolytic activity (CYT), distribution of immune cells and epithelial to mesenchymal transition (EMT) were also explored.

**Glucocorticoid receptor expression in breast cancer: Immunohistochemistry (IHC)**

To examine the cell type specific expression of GR on different cells within the tumor, we accessed tumor samples from an independent cohort with a long term follow up available, median follow up being 72 months. Tumor tissues were obtained from a cohort of 175 patients who underwent surgery between 2008 and 2013, following the acquisition of informed consent and ethical approval. Information regarding various clinical parameters such as age, tumor size, lymph node status, stage, grade of the tumor, other histopathological parameters and treatment received was obtained from patient’s clinical records.

IHC for GR was carried out on formalin fixed paraffin embedded (FFPE) tissue blocks manually using standard protocol. Briefly, 5µm FFPE tissue sections were fixed on the glass slides, followed by deparaffinization in xylene, rehydration in graded alcohol, 3% H$_2$O$_2$ treatment for 30 minutes, antigen retrieval using citrate buffer (pH 6.00) for 15 minutes and blocking with 3% bovine serum albumin (BSA, Sigma) for 30 minutes. Sections were further incubated with primary antibody against GR (clone (D8H2) XP® Rabbit mAb, cat no. 3660, dilution 1:200) for 1 hour at room temperature. Incubation with secondary antibody (DAKO REAL™EnVision™) was done for 30 minutes. 3-3' Diaminobenzidine (DAB, DAKO REALTMEnVisionTM) for 10 minutes was used as chromogenic substrate and haematoxylin was used as a counterstain. Sections were dehydrated,
dried, mounted with DPX and visualized under microscope. All sections were examined by pathologists to score tumor cells and TILs separately. The evaluation of GR expression was conducted separately in invasive tumor cells, stromal cells, and tumor-infiltrating lymphocytes (TILs). A staining pattern was considered positive when more than 1% of the cells within each subpopulation displayed positive staining.

**Statistical Analysis**

A comprehensive descriptive analysis was undertaken to elucidate the distinctive characteristics of GR High and GR Low tumors. Spearman correlation analysis, with an adjusted p-value threshold of < 0.05, was employed to explore associations among variables. To distinguish differences in clinical variables between the two groups, independent Student's t-test or Mann-Whitney U test was used for continuous variables, and the chi-square test was applied for categorical variables. Kaplan-Meier survival curves, coupled with log-rank tests, were employed to compare overall survival (OS) and disease-free survival (DFS) rates between GRSig high and low tumors. Additionally, for the validation of the EMT phenomenon, single-sample Gene Set Enrichment Analysis (ssGSEA) was systematically conducted on each sample within the subgroups. Furthermore, univariate, and multivariate Cox regression hazard models were executed to establish GRSig as an independent predictor for survival prognosis. This multifaceted approach ensured a robust and comprehensive statistical evaluation of the dataset, providing insights into the characteristics, associations, and prognostic implications associated with GRSig. All statistical analyses were done using statistical software R version 4.2.2.

**RESULTS**

**Identification of GRSig independent of ER status and its correlation with GR transcript**

GR-regulated genes were systematically derived using the pipeline outlined in the methods, using publicly available gene expression profiles sourced from ER+GR+ BC cell line (MCF-7) and ER-GR+ BC cell line (MDA-MB-231) subjected to dexamethasone treatment. The identified genes were categorized according to the context of ER status, delineated in figure 1A as ER-dependent, ER-independent, or ER-absent. Within this classification, 21 genes were seen as being regulated by GR independently of ER status, with 16 of them exhibiting a consistent trend of regulatory pattern. Notably, 9 out of (FKBP5, KLF13, KLF3, KLF9, PAG1, PDK4, RGCC, RGS2 and SGK1) them demonstrated a significant correlation (p < 0.05) with GR transcript levels and were also identified as the target genes of GR within a ±1000 base pair range of TSS. Employing the mean expression values of these 9 genes, a GRSig was computed, and the quantiles of the gene score,
as elucidated in the methods, were employed to categorize tumor samples into distinct groups, thereby establishing high and low GRsig groups for subsequent analyses. We also looked at the transcript expression of GR and GRsig to see the difference between a single gene and gene score from multiple genes. The transcript expression GR had no significant difference between HR+ and TNBC samples whereas GRsig had higher expression in TNBC compared to HR+ tumor samples ($p<2\times10^{-16}$) (Figure 1B-C). We examined the differences in clinicopathological characteristics between high and low GRsig groups. High GRsig groups were associated with lower tumor grade, reduced tumor size ([Supplementary File, Table 1](#)).

![Figure 1](#). A). workflow for deriving GRsig independent of ER status. B). comparison of GR transcript and GRsig in hormone receptor positive (HRPOS) and triple negative breast cancer (TNBC).

**High GRsig is associated prognosis across different subtypes of the BC**

Survival analysis using Kaplan-Meier curves revealed a noteworthy prognostic association with GRsig in METABRIC cohort as visually depicted in Figure 2A-F. Patients exhibiting a high GRsig displayed a favorable prognosis, evident in both OS ($p = 0.0053$) and RFS ($p = 0.017$). The difference in median survival between GRsig high and low tumors was substantial, with a 10.3-month difference in OS and a notable 19-month difference in RFS. To validate this trend across different BC subtypes, the analysis extended to HR+ and TNBC. Strikingly, in both HR+ and TNBC subtypes, patients with a high GRsig exhibited significantly improved prognosis in terms of both OS (TNBC: $p < 0.002$, HR+: $p < 0.008$) and RFS (TNBC: $p < 0.008$, HR+: $p < 0.019$). The median survival differences in months were noteworthy, with high GRsig tumors demonstrating better outcomes. In the OS analysis, the differences were 39.3 months for TNBC, and 16.5 months for
HR+. Additionally, in the RFS analysis, the differences were 68.7 months for TNBC, and 33.3 months for HR+. Notably, a distinct separation in the survival curves was observed from the outset in TNBC tumors, emphasizing the prognostic significance of GRsig in this specific subtype. Overall, the above analysis has robustly confirmed that a high GRsig is consistently associated with a more favorable prognosis, regardless of BC subtypes. This also substantiates the fact that GRsig performs their function regardless of the ER status.

Figure 2. The upper panel shows the association of GR score with overall survival in all tumors, TNBC and HR+ tumors. Lower panel shows association of GR score with Relapse free survival in all tumors, TNBC and HR+ tumors.

High GRsig in tumor is associated with favorable tumor characteristics
We systematically evaluated various characteristics associated with tumor prognosis, focusing on proliferation, CYT, and immune cell enrichment, for their association with GRsig. Firstly, association of proliferation was performed using the average expression of MKI67, MCM6, and PCNA genes. Analysis revealed that tumor samples characterized by a high GRsig exhibited significantly lower proliferation in both TNBC (p=1.5e-15) and HR+ (p=0.012) samples, as evidenced in Figure 3A-C.
CYT was assessed based on the ratio of mRNA expression levels of GZMA and PRF1\textsuperscript{28}. The investigation into CYT demonstrated that samples with a high GRsig displayed a substantial increase in CYT across all tumors (p<2e-16), with specific trends observed in TNBC (p=3.8e-08) and HR+ (p<2e-16) samples, as illustrated in Figure 3D-F.

Figure 3. Association of GRsig with proliferation in A). All tumors B). Triple negative breast cancer (TNBC) C). Hormone receptor positive tumors (HR+); Association of GRsig with CYT in D). All tumors E). Triple negative breast cancer (TNBC) F). Hormone receptor positive tumors (HR+); G). Distribution of immune cells in GRsig high and low tumors based on CIBERSORT analysis.

Further to elucidate the immune cells within GRsig high and low tumor transcriptomes, the CIBERSORT algorithm was employed\textsuperscript{29}. Immune cell distribution analysis revealed a significant enrichment of anti-tumorigenic immune cells in high GRsig tumors. Notably, M1 Macrophage (p=1.3×10^{-12}), CD8 T Cell (p=1×10^{-9}), Gamma-delta T Cell (p<2×10^{-26}), CD4 T Cell (p=2.2×10^{-6}), Mast Cell (p=3.7×10^{-7}), B Cell (p=1.3×10^{-13}), and Natural Killer cell (p=8.6×10^{-14}) were all significantly enriched. Conversely, pro-tumorigenic immune cell types, including neutrophils (4.8×10^{-13}) and regulatory T cells (p<2×10^{-26}), were found to be enriched in low GRsig tumors (Figure 3G).
The robustness of these findings was further confirmed through validation in independent cohorts, such as TCGA and SCAN-B (Supplementary Figures S1-S4). Importantly, the observed properties of lower proliferation, higher CYT, and anti-tumorigenic immune cell enrichment collectively support the assertion that tumor samples with a high GRsig exhibit a more favorable survival prognosis.

**Phenotypes of GR driven tumors shows enhanced EMT irrespective of BC subtypes**

Furthermore, the association of EMT and GRsig was assessed using a pan-cancer EMT signature derived from 11 different cancer types. Additional EMT signatures from different studies were employed for validation purposes, ensuring the robustness of the findings\textsuperscript{30–36}. We identified a total of six signatures for inclusion in our study (Supplementary file, Table 1).

A persistent activation of the EMT process has consistently been observed in tumors with high GRsig across various EMT gene expression signatures. This phenomenon holds true not only across distinct EMT signatures but also exhibited robust consistency within different subtypes of BC, including TNBC and HR+ subtypes (Figure 4A-C). Notably, high GRsig tumors consistently demonstrated an enrichment of expression patterns indicative of enhanced mesenchymal characteristics, diminished epithelial features, and increased expression of EMT-inducing genes (Figure 4D-F).
Figure 4. Expression of epithelial and mesenchymal (EMT) signatures derived from published literature in GR high and GR low groups in A. All tumors B. Triple negative breast cancer (TNBC) C. Hormone receptor positive (HR+) tumors; Expression of EMT inducer signature, epithelial and mesenchymal signatures in D. All tumors, E. TNBC, F. HR+

**EMT Driven by Tumor Cells, Not Immune Cells: Insights from Single-Cell Data Analysis**

Building upon our earlier observations, which revealed a correlation between high GRsig tumors and its association with increased CYT, and enhanced immune cell enrichment, we were intrigued by positive correlation with the process of EMT. We hypothesized that GR might exert a differential role based on cell specific expression on tumor cells, infiltrating lymphocytes and stromal fibroblasts.

To probe deeper into our hypothesis, we conducted analysis using single-cell RNA sequencing data from 549 primary BC cells representing diverse molecular subtypes, including HR+, HER2+, and TNBC. The dataset comprised of 178 immune cells, 23 stromal cells, and 326 tumor cells, providing a holistic view of the cellular landscape within the breast tumor microenvironment (TME). Subsequently, we employed ssGSEA to assess the expression of selected EMT signatures at the single-cell level.
Our investigation revealed an interesting pattern, specifically, we observed higher expression of GRsig in immune cells compared to tumor (p<2.22e-26) and stromal (p=1.9e-08) cells (Figure 5A). Interestingly, a negative correlation between GRsig expression in immune cells and EMT was consistently observed across all EMT signatures. In contrast, EMT exhibited a positive correlation with stromal and tumor cells (Figure 5B-D). These findings strongly support the notion that the activation of EMT is primarily driven by GR in tumor or stromal cells, unraveling a previously unexplored dimension in the regulation of EMT dynamics within the BC microenvironment.
Figure 5. Single cell data analysis. A). Expression of GRsig in tumor, stromal and immune cell. B-D) represents the correlation of GRsig with EMT score in tumor, stromal and immune cells.

Interplay of GRsig with EMT, Immune cell activation and utility as a prognostic marker

Encouraged by the positive association of GRsig with better survival observed across public and independent cohorts, we set out to examine the interdependence of the observed phenomenon of proliferation, CYT with GRsig. Additionally, we examined whether GRsig could serve as an independent predictor for prognostic outcomes in the context of OS. Univariate and multivariate cox proportional hazards regression analyses were carried out to evaluate the independent prognostic potential of GRsig in predicting OS. The analysis included key features such as proliferation, CYT, EMT, and GRsig.

In the univariate analysis, proliferation demonstrated a significant positive association with survival (HR=1.2, p=0.011991), signifying that higher proliferation rates correlate with an elevated risk of the event. Conversely, CYT exhibited a negative association with survival (HR=0.88, p=0.0024994), indicating that higher CYT is associated with a lower risk of the event. In contrast, GRsig emerged as a robust and highly significant predictor of survival (HR=0.79, p=0.00052582), implying that higher levels of GRsig independently correspond to a lower risk of the event (Figure 6A).

The multivariate cox proportional hazards regression analysis reaffirmed the independent prognostic value of GRsig. Proliferation (HR=1.30, p<0.001) and CYT (HR=0.91, p=0.05) maintained their associations, further supporting their independent predictive roles. GRsig continued to demonstrate a significant negative association with the risk of the event (HR=0.80, p=0.008) in the multivariate analysis. Consequently, the results from both univariate and multivariate analyses highlight the robust and independent predictive capability of GRsig in determining survival prognosis, either in isolation or in conjunction with other features (Figure 6B).

In comprehensive analysis of survival prognosis, we examined the combinatorial effects of GRsig with key factors such as EMT, proliferation (PRO), and CYT. Our findings reveal that a high GRsig, when coupled with low EMT, significantly correlates with a lower hazard rate (HR= 0.67, p<0.001). Similarly, a high GRsig in conjunction with high EMT is associated with a lower hazard rate (HR=0.81, p = 0.02). Conversely, when GR is low and EMT is high, the hazard is not significant. Importantly, a high GRsig independently serves as an indicator of a lower hazard (Figure 6C). Examining the interplay with proliferation, we observed that a high GRsig coupled with low proliferation yields a significant reduction in hazard (HR=0.75, p = 0.005). Moreover, a high GRsig
paired with high proliferation also shows a lower hazard rate (HR=0.81, p = 0.044), while the hazard is not significant when GRsig is low, and proliferation is high (Figure 6 E).

Considering CYT, a high GRsig combined with high CYT significantly correlates with a lower hazard (HR=0.71, p < 0.001). However, the hazard is not significant when GR is low and CYT is high, or when GRsig is high and CYT is low. Notably, a high GRsig, particularly when accompanied by CYT, emerges as a robust indicator of lower hazard. Higher GRsig levels are associated with elevated CYT, leading to enhanced immune enrichment, and ultimately contributing to a lower hazard and favorable prognosis (Figure 6 D).
Figure 6. Cox proportional hazards regression analyses using GRsig, EMT, CYT and proliferation.
**Exploration of cell type specific expression of GR protein**

In the single-cell data analysis, we observed a compelling correlation between GR in immune cells and the negative regulation of EMT. Remarkably, the scenario takes a different turn when examining GR in stromal or tumor cells, as it appears to drive the EMT process. To delve deeper into the cell-specific actions of GR on distinct cellular contexts, we evaluated the presence of GR protein by immunohistochemistry on an independent cohort of tumor samples across various cell types within the tumor, specifically focusing on tumor cells and TILs.

The median age of the cohort is 56 years. Majority of the tumors were T2 size (63.2%), lymph node (LN) positive (63.4%) and grade II (45.1%) and grade III (40.6%) tumors. Approximately two-thirds of the patients were postmenopausal. Around 70% tumors were HR+ and 18% were HER2+. All the clinicopathological characteristics of the patient cohort are summarized in figure 7A.

**GR protein expression in Tumor Cells and TILs: A Comprehensive Analysis of Prognostic Significance in BC**

The investigation of GR protein expression in tumor tissues through IHC revealed that 74% (130/175) of tumors exhibited GR expression in invasive tumor cells, while a higher proportion, 84% (147/175), displayed GR expression in TILs, (Figure 7B). Notably, the distribution of GR in tumor cells and TILs did not exhibit a significant difference between ER+ and ER- tumors (Figure 7C), suggesting that GR expression patterns are independent of ER status.

The analysis of disease-free survival (DFS) based on GR status within tumor cells across all tumor types had no significant differences but a trend towards improved survival was noted in GR+ tumors compared to GR-. Expanding the analysis to incorporate GR expression in TILs, the analysis across all tumors failed to unveil any notable difference in DFS based on GR expression in TILs. However, within the specific subgroup of TNBC tumors exhibiting increased GR expression in immune cells were linked to significantly better survival outcomes, with a mean survival time of 74 months compared to 41 months for those with lower GR expression in TILs (log-rank test p=0.03) (Figure 7D). This observation highlights the potential immunomodulatory role of GR in influencing the prognosis of TNBC, indicating a more intricate interplay between GR expression and TME.
Figure 7. A) The clinicopathological characteristics of the patient cohort. B) GR expression in tumor cells, stroma and tumor-infiltrating lymphocytes. C) The distribution of GR in tumor cells and TILs between ER positive and ER negative tumors. D) Disease-free survival based on GR status in TILs within TNBC tumors.

DISCUSSION

BC is a complex and heterogeneous disease, characterized by diverse molecular profiles. Within this sophisticated landscape, the nuclear receptors play a pivotal role, including ER, PR, AR, and GR, influencing the disease's progression. The interplay between these receptors, known as cross-talks, adds another layer of intricacy to BC biology. Notably, a higher AR/ER ratio has been identified as a marker for a subgroup of patients with a worse prognosis and aggressive biological features, underscoring the significance of androgen receptor involvement in certain BC cases. Additionally, the expression levels of GR hold prognostic value, with high GR expression correlating with a poor prognosis in ER-negative BC, while it is associated with a favorable prognosis in ER-positive BC. These findings highlight the complex and context-dependent nature of nuclear receptor interactions and their implications for understanding and treating BC.

In this study we have attempted to decipher GR driven pattern independent of context of ER status. We have derived a distinctive GRsig which has the potential to be applicable across all subtypes. Many previous studies have attempted to derive GRsig using data derived from cell
lines or gene expression data from tumors. GRsig identifies the specific signature associated with therapy resistance in ER negative BC\textsuperscript{11,19}. Genome wide target identification was evaluated by Pan et al to identify the regulated genes after GR activation\textsuperscript{41}. Butz et al examined the positively and negatively correlating genes with GR expression\textsuperscript{18,42}. GRsig derived in our study is unique in that it uncovers the pattern driven by GR both in ER positive and ER negative BC. We demonstrated that the derived gene signature holds the potential to be relevant to both ER+ and ER- BC, bridging the gap between these distinct subtypes.

Our results showed GRsig is associated with favorable prognostic characteristics, when focusing on proliferation, cytolytic activity, and immune cell enrichment. Association of GR with favorable tumor characteristics was shown in the study by S Gandhi et al\textsuperscript{43}. They investigated the presence of GR in immune cells along with tumors from bulk tumor transcriptomic data. Using single cell RNA sequencing and immune cell identification algorithms they were the first to show GR high tumors were enriched for favorable immune cell infiltrates, increased cytolytic activity and decreased proliferation. They used the transcript of NR3C1 coding for GR to identify the tumors driven by GR, while we derived a GRsig using multiple genes. Unlike single genes that may have limited scope, a gene signature encompasses a set of genes that, when analyzed collectively, can provide a more comprehensive view of the biological processes at play. Gene signatures are particularly advantageous as they capture the intricacies of multifaceted diseases like BC, offering a more holistic and reliable approach to prognostication, classification, and treatment decisions\textsuperscript{44–48}. Our results are in agreement with their findings in reflecting tumors with higher expression of GR have better overall survival.

Despite GRsig being associated with better survival we observed a positive correlation with multiple EMT signatures. Association of GR with EMT was observed in previous studies (Shi et al, Pan et al, S Gandhi et al) but were without deciphering the cell type specific expression. Utilizing the single-cell RNA sequencing data, we investigated pivotal role of GR in the activation of the EMT process either in tumor or adjacent cells, we uncovered a noteworthy pattern: GRsig expression was notably higher in immune cells compared to tumor and stromal cells. Interestingly, a consistent negative correlation was observed between GRsig expression in immune cells and EMT across all EMT signatures. In contrast, EMT displayed a positive correlation with GRsig expression in both stromal and tumor cells. These findings suggest that the activation of EMT is primarily driven by GRsig expression in tumor or stromal cells, unveiling a previously unexplored dimension in the regulation of EMT dynamics within the BC microenvironment.
Expression of GR protein has been observed in nuclei, cytoplasm of luminal epithelial cells, adipocytes, myoepithelial cells in the normal breast\(^49\). GR expression has also been observed in the endothelial and stromal cells\(^50\) Human protein atlas using the CAB010435 (Cat#sc-8992, Santa Cruz Biotechnology) antibody has found cytoplasmic staining in most of tumor cells. While using another antibody (HPA004248: Cat#HPA004248, Atlas Antibodies) showed only nuclear staining. Butz et al. used GR total antibodies to detect both α & β isoforms of GR, (RRID:AB_1078976; and RRID:AB_2155784) and observed both nuclear and cytoplasmic stain. While nuclear positivity indicates GR activation and cytoplasmic positivity reflects the expression and non-genomic action of GR, the reliable detection of GR is considered crucial when correlating with clinicopathological parameters. We have used a monoclonal antibody (clone D8H2, Cell signaling) most used for GR detection by IHC. We observed staining was heterogenous irrespective of the subtypes. Our approach of evaluating the presence of protein both on the tumor cells and the TILs allowed us to examine the significance of GR expression in both components of TME. Interestingly, the results from the independent cohort analysis revealed a substantial survival association in TNBC tumors where GR was expressed specifically in TILs. The identification of a positive prognostic association in TNBC, a subtype known for its aggressive nature, underscores the potential clinical relevance of GR expression in immune cells as a prognostic biomarker and indicates its ability to identify differential immune microenvironment, which may play a crucial role in shaping the overall prognosis of TNBC patients.

Despite encouraging results derived to understand the cell specific role of GR in tumors, this study has limitations. We have derived the gene signatures from cell line data sets which were publicly deposited and evaluated the phenotypic characters in large open data. Though the gene score was derived from transcriptomic data, our findings were further substantiated in an independent cohort with GR protein detection. GR protein detection was done using a single antibody in small sample size. Multiple antibodies could not be tested due to the limitations of the tissue. Lack of large datasets with availability of long term follow up and human tissues for examination of GR protein expression is a significant drawback for testing our finding.

**CONCLUSION**

Our study contributes significant insights into the complex landscape of BC, specifically focusing on the association of the nuclear receptor, GR. We have observed that the relationship between GR expression and GRsig, irrespective of ER status, manifests differently. GRsig emerges as a powerful prognostic indicator, positively correlated with favorable tumor characteristics such as low proliferation, heightened cytolyltic activity, and an anti-tumorigenic immune microenvironment.
Furthermore, our investigation into the association between EMT and GR reveals a distinct correlation: EMT is positively linked with GR in tumor cells and inversely associated with GR in immune cells. These findings emphasize the pivotal role of GR in shaping the BC microenvironment, particularly in driving the activation of EMT in tumor or stromal cells. This study expands our understanding of the molecular dynamics within BC, providing valuable groundwork for future research and potential therapeutic interventions.
References


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