Atypical Chemokine Receptor 1 (DARC/ACKR1) in Breast Tumors Is Associated with Survival, Circulating Chemokines, Tumor-Infiltrating Immune Cells, and African Ancestry

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Abstract

Background: Tumor-specific immune response is an important aspect of disease prognosis and ultimately impacts treatment decisions for innovative immunotherapies. The atypical chemokine receptor 1 (ACKR1 or DARC) gene plays a pivotal role in immune regulation and harbors several single-nucleotide variants (SNV) that are specific to sub-Saharan African ancestry.

Methods: Using computational The Cancer Genome Atlas (TCGA) analysis, case–control clinical cohort Luminesx assays, and CIBERSORT deconvolution, we identified distinct immune cell profile–associated DARC/ACKR1 tumor expression and race with increased macrophage subtypes and regulatory T cells in DARC/ACKR1-high tumors.

Results: In this study, we report the clinical relevance of DARC/ACKR1 tumor expression in breast cancer, in the context of a tumor immune response that may be associated with sub-Saharan African ancestry. Briefly, we found that for infiltrating carcinomas, African Americans have a higher proportion of DARC/ACKR1-negative tumors compared with white Americans, and DARC/ACKR1 tumor expression is correlated with proinflammatory chemokines, CCL2/MCP-1 (P < 0.0001) and anticorrelated with CXCL8/IL8 (P < 0.0001). Sub-Saharan African-specific DARC/ACKR1 alleles likely drive these correlations. Relapse-free survival (RFS) and overall survival (OS) were significantly longer in individuals with DARC/ACKR1-high tumors (P < 1.0 × 10⁻¹⁶ and P < 2.2 × 10⁻⁶, respectively) across all molecular tumor subtypes.

Conclusions: DARC/ACKR1 regulates immune responses in tumors, and its expression is associated with sub-Saharan African-specific alleles. DARC/ACKR1-positive tumors will have a distinct immune response compared with DARC/ACKR1-negative tumors.

Impact: This study has high relevance in cancer management, as we introduce a functional regulator of inflammatory chemokines that can determine an infiltrating tumor immune cell landscape that is distinct among patients of African ancestry.

Introduction

Breast cancer disparities have been well-documented for over 50 years, indicating significant differences in rates of incidence and mortality among women of varying self-reported race and ethnicity (1, 2). While the focus of disparities research has traditionally been studied through a lens of health care and/or socioeconomic barriers (3), we and others have recently identified additional biological factors that are associated with disparate clinical outcomes, including disproportionate burdens of aggressive tumor subtypes (refs. 4–8; i.e., triple-negative basal-like) in women of west African descent (9–12). Overall, women of African descent are more likely to be diagnosed with the worst types of breast cancer, including triple-negative breast cancer (TNBC), at earlier ages with the poorest prognosis indications (8, 10, 13–15), despite having access to proper screening and standard treatments (16). There is a clear need to better understand how the molecular dynamics of tumor progression may differ among women of African descent, compared with women of European descent.
Some evidence of these differences in pathologic/histologic tumor variables indicate that breast cancer in women of African ancestry has a distinct genetic signature (4, 17) and distinct associations with immune responses (18–20) even within the TNBC subtype. These differences are the likely causes for increased prevalence of refractory tumors in certain patient groups. With the advent of genomic technologies and increased genetic diversity in multi-ethnic studies, differences in these tumor characteristics can now be investigated to fully comprehend dynamics of ancestry-related immunologic interactions and how immunotherapy may or may not work for certain groups (21). Recent studies have begun to directly address this question from the perspective of genetic risk (22); however, we sought to identify key biological pathways that are systemically altered in West African populations, which may drive immunologic differences in tumor biology in these patients. In terms of genetic risk, the typical focus of genetic background studies, these genetic drivers might not alter tumor etiology, per se, but rather tumor phenotypes. We utilized data from The Cancer Genome Atlas (TCGA) to uncover expression pattern differences in immunologic pathway genes that were associated with self-identified race and identified an enrichment in chemokine signaling. The study presented here describes our in-depth investigation of a key regulator of these chemokine signaling pathways, atypical chemokine receptor 1 (ACKR1, aka DARC), in a breast cancer case-control cohort.

The DARC/ACKR1 is a seven-transmembrane G-protein-coupled receptor that is found on the surface of red blood cells (RBC)/erythrocytes, and as such on endothelial cells lining postcapillary venules, and most recently has been shown to be expressed on lymphoblast cells (7). DARC/ACKR1 functions as a decoy receptor for a variety of CXC and CC chemokines, including those with promalignant and proinflammatory effects, such as CCL2 and CXCL8 (IL8; ref. 23). It is considered a decoy receptor because it binds and internalizes these chemokines, and targets them for lysosomal degradation, efficiently sequestering their signaling capabilities (24, 25). In addition, DARC/ACKR1 receptor has also been shown to regulate transcytosis of the bound ligands in endothelial cells (26), influencing leukocyte trafficking and the ability of the receptor to maintain homeostatic chemokine levels (27). Erythrocyte-specific DARC/ACKR1 expression plays a key role in inflammation by sequestering chemokines from circulation to recover homeostatic levels during inflammatory responses (26). A population-private DARC/ACKR1 gene mutation, defined as DARC/ACKR1<sup>es</sup> (erythrocyte silent), removes expression of DARC/ACKR1, specifically on erythrocytes, and is also known as the extensively characterized "Duffy-null blood group" (28, 29). This mutation is mostly restricted to populations of West African descent and remains fixed (100% allele frequency) in present-day populations within west and central sub-Saharan Africa. Our study defines a set of significant clinical associations of DARC/ACKR1 among patient demographics, population-private alleles, and tumor phenotype characteristics that provide greater insight to ancestry-specific differences in tumor biology, particularly immunologic responses that are associated with clinical outcomes and that may ultimately inform the decisions for oncologic use of immunotherapy treatments (19, 20, 30). We anticipate that this research will aid in decreasing the tumor-subtype disparities in incidence and mortality among race groups.

Materials and Methods

Ethics statement, study design, biospecimens, and cohort summary

This study is a human subjects study and all biospecimens used in this study were obtained after approval from one of two institutional review board (IRB)-approved protocols from either the University of Georgia (Athens, GA; IRB ID: MOD00003730) or Henry Ford Health System (Detroit, MI; IRB ID: 4825). All research was performed in accordance with the IRB guidelines, in accordance with a file assurance of the IRB by the U.S. Department of Health and Human Services. All participants were informed of the study purpose and procedures and have signed an informed consent prior to participation and donation of blood, saliva, and/or tissue for this study.

Gene expression and DARC/ACKR1 subtype analyses

Gene expression levels were obtained from RNA sequencing (RNAseq) data accessed through the web-portal of The Cancer Genome Atlas Breast Cancer cohort [n = 838, 167 African Americans (AA), 671 White Americans (WA); https://cancergenomics.nih.gov]. After filtering samples for tissue status (removing samples that were normal tissue and metastatic tumors) and histologic findings (removing samples that were annotated as “other,” “metaplastic, or noninfiltrating”), we conducted linear regression analyses for gene correlations, stratified as indicated for specific contexts of interactions (i.e., molecular subtypes/phenotypes) and nominal logistical regressions across derived tumor status. For DARC/ACKR1 subtype analyses, DARC/ACKR1 expression (Supplementary Fig. S1A) was stratified on the basis of quartile ranking and shown as high (upper 30th quartile), medium (intermediate quartile), and low (lower 30th) categories (Supplementary Fig. S1B). DARC/ACKR1 subtypes were then measured for associations with specific demographic or clinical variables by stratifying the population according to these variables (e.g., race and molecular breast cancer subtype; Supplementary Fig. S1C and S1D) and comparing distributions of DARC/ACKR1 subtypes within or among each category. We conducted multivariate modeling to assess effect estimates and adjust for demographic variables (i.e., race and age).

Cytokine analysis with DARC/ACKR1 subtypes

The UCSC Xena Browser (http://xenabrowser.net, accessed April 2018) was used to generate a heatmap of TCGA breast-invasive carcinoma RNAseq gene expression data (IlluminaHiSeq, n = 399) compared with a user-generated geneset of relevant cytokine genes (n = 67, Supplementary Table S1). Dichotomized DARC/ACKR1-positive (red) and -negative (blue) subgroups were determined according to the values in Supplementary Table S2. P values for select cytokines were determined using Welch’s t test.

Blood and serum specimens

Following informed consent, and at time of enrollment or time of surgery, approximately 4 mL of blood was collected in EDTA-treated vacuum-tube tubes (BD Biosciences) from each newly diagnosed patient pretreatment at University Cancer and Blood Center (Athens, GA; n = 41) and Henry Ford Health System (HFHS, Detroit, MI; n = 225). Blood samples from noncancer controls (n = 67) and breast cancer survivors (n = 17) were collected in a similar manner at the time of enrollment at the University of Georgia (UGA) Clinical and Translational Research Unit (CTRU).
Athens, GA; n = 84). All blood samples were processed within 24 hours of collection. Undiluted plasma was collected through centrifugation, and the remaining sample was separated via a Ficoll–Paque gradient (GE Healthcare) according to the manufacturer’s instructions. Serum control samples from premenopausal (n = 31) and postmenopausal women (n = 30) and breast cancer survivors (n = 11) were purchased from the Susan G. Komen Tissue Bank (n = 72). A flowchart detailing patient numbers and study exclusions can be found in Supplementary Fig. S2.

**Luminex human chemokine multiplex assay**

The multiplex assay was completed using patient undiluted plasma and serum. Plasma chemokine levels were quantified using the Bio-Plex Pro Human Chemokine Assay Kit (Bio-Rad) that was custom designed to measure the following chemokines and cytokines: CCL2/MCP-1, CXCL8/IL-8, CXCL9/MIG, CXCL11/Gro-α, and IL10. The assay was carried out following manufacturer’s instructions, and the results were analyzed using the Bio-Plex Manager Software version 6.1.1. Statistical multivariate pairwise correlation analyses with these analytes can be found in Supplementary Table S3.

**Red blood cell phenotyping by immunofluorescence**

RBCs isolated from Ficoll–Paque blood separation techniques are fixed in 4% paraformaldehyde and stained with DARC/AKCR1 goat anti-human (Novus Biologicals) primary antibody and Alexa Fluor 488 chicken anti-goat (Invitrogen) fluorescent secondary antibody using standard immunofluorescence techniques. Positive plasma membrane control stains were done using CellMask Plasma Membrane Stain (Life Technologies) according to the manufacturer’s instructions. RBCs were imaged using a Keyence BZ fluorescence microscope at 40× magnification.

**Duffy-null genotyping**

Genotyping for the Duffy-Null SNV, rs2814778, was performed using the TaqMan GTXpress Master Mix and variant-specific probes (Applied Biosystems) according to the manufacturer’s instructions. The assay was executed using an Applied Biosystems 7500 Fast Real-Time PCR System. Cohort allelic and genotypic frequencies compared with global populations from 1000 Genomes data via ENSEMBL (www.ensembl.org) are provided in Supplementary Table S4.

**Tumor-associated leukocytes' gene profiling**

The CIBERSORT online platform (31) was used to estimate the absolute fractions of 22 leukocyte populations in TCGA samples denoted as breast primary tumor samples. The analysis was run with 500 permutations, and quantile normalization disabled (as recommended by the tool for RNASeq data). Only those samples with a maximum significance value of P < 0.05 were included in the final analysis (n = 472). Survival analyses

Patient survival associations with gene expression data were calculated from 3,951 patients with breast cancer. The data (platforms: Affymetrix Microarrays: HG-U133A, HG-U133 Plus 2.0, and HG-U133A 2.0) were downloaded from Gene Expression Omnibus (GEO) and Array Express (AE) databases (ref. 32; Supplementary Table S5). Recurrence-free survival (RFS) and overall survival (OS) was assessed across all breast cancer subtypes and treatment types with dichotomized grouping for expression of DARC/AKCR1 (probe ID: 208335_s_at), CCL2 (216598_s_at), and CXCL8 (211506_s_at) using the Kaplan–Meier plot application (ref. 33; http://kmplot.com). Distributions for high and low cut-off values (Supplementary Fig. S3). P values, HRs, and confidence intervals for all survival analyses can be found in Supplementary Materials (Supplementary Table S6).

**Clinical tumor specimens**

Primary breast tumor specimens were acquired from the UCBC (Athens, GA; n = 8). UCBC patients were newly diagnosed (within ~1 month), and following informed consent at time of enrollment, primary tumor samples were collected at local Athens area hospitals. There were no exclusion criteria for this study cohort, which consisted of a racially diverse patient group having various molecular subtypes of breast cancer (Table 1). Tumor grade was determined using WHO guidelines, as well as the Ellis & Elston system of histologic grading. Clinical staging was evaluated using the TNM staging system maintained by American Joint Committee on Cancer and Union for International Cancer Control following the most current National Comprehensive Cancer Network guidelines at the time of staging. Molecular breast cancer subtypes were determined using IHC staining for estrogen receptor, progesterone receptor, and human epidermal growth factor receptor-2 (HER2) on the surface of the primary breast tumor.

**IHC**

Formalin-fixed paraffin-embedded tumor blocks were obtained from local Athens-area hospitals through UCBC (Athens, GA). Subsequent slide preparations were conducted through the University of Georgia’s Histology Core, using standard operating protocols (FFPE blocks used to cut 4 µm sections
onto glass slides). DARC/ACKR1 staining was done using a goat anti-human polyclonal antibody (Novus Biologicals, NB100-2421, isotype IgG). CCL2 and CXCL8 staining were both done using mouse anti-human mAbs (R&D Systems, MAB2791, isotype IgG2B and Lifespan Biosciences, LS-B6427, isotype IgG1, respectively).

Statistical analysis
Statistical analyses were done using appropriate functions in JMP (SAS) Pro v. 13.0.0. All Student t tests performed were two-tailed and the appropriate absolute P values are indicated in the figure legends of each statistical analysis. F values are reported for ANOVA analyses and P values are reported for linear regression or paired t test analyses. Numbers of included data points and degrees of freedom are also shown for the appropriate analyses.

Results
DARC/ACKR1 tumor expression is significantly different across race groups and tumor molecular subtypes
Our previous studies of genomic signatures in breast cancer subtypes indicated an enrichment in immune-related gene pathways (34). We identified a pattern of dichotomous variation, measured by first-generation microarray platforms from GEO (35, 36) in a chemokine receptor that regulates immune cell signaling molecules, the DARC/ACKR1 gene. To quantify these findings more robustly, we investigated DARC/ACKR1 expression differences at a higher resolution, using the TCGA breast-infiltrating carcinoma RNAseq dataset (n = 838; 167 AAs, 671 WAos), and found a broad range and distribution of DARC/ACKR1 expression across the cohort (Supplementary Fig. S1A and S1B). A statistical response screen across anthropomorphic and clinical variables suggested that DARC/ACKR1 tumor expression was distinct across race and tumor phenotypes (Supplementary Fig. S1C and S1D). Our analysis revealed that WAos have a nearly equal distribution of DARC/ACKR1 tumor expression subgroups, with the highest proportion being DARC/ACKR1-high (35.3%), and AAos have the highest proportion of DARC/ACKR1-low tumors (40.1%; Fig. 1A, top). These race-related trends indicate a distinct regulation of DARC/ACKR1 that is likely linked to geographic genetic ancestry, which is closely associated with race.

However, because DARC/ACKR1 expression occurs across all organ systems within endothelial cells of postcapillary venules (24, 37), we also considered the relative expression of an endothelial cell–type marker, Von-Wildbrand Factor (VWF) within the tumor samples. When tumors were stratified by DARC/ACKR1 status and VWF (Fig. 1A, bottom), the differences of DARC/ACKR1 status between race groups were more evident (P = 0.06); just above the threshold for significance. Interestingly, the correlation of DARC/ACKR1 to VWF was very significant (P < 0.0001, Supplementary Fig. S1F), which suggests that a significant portion of DARC/ACKR1 expression within these tumor samples might be derived from endothelial tissue. However, even within tumors with low VWF, we still find high expression of DARC/ACKR1, particularly in WA patients.

Figure 1. DARC/ACKR1 is significantly associated with VWF, breast cancer molecular subtypes, and pro-inflammatory chemokines in TCGA data. TCGA Breast Cancer RNAseq data (n = 838) were used to compare (A, top) the distribution of DARC/ACKR1 expression subgroups (high, n = 289, pink; medium, n = 268, yellow; low, n = 281, purple) and race (AA, n = 167; WA, n = 671). Bottom panel of A same as top but with VWF expression subgroups (VWF-High, n = 336; VWF-Mid, n = 309; VWF-High, n = 193, P = 0.06, ANOVA) by race. B, Distribution of DARC/ACKR1 expression subgroups compared with molecular breast cancer subtypes (Basal-like, n = 74; HER2+, n = 24; Luminal A, n = 196; Luminal B, n = 58, P < 0.0001). Bottom panel of B same as top but broken down by race. C, Heatmap (UCSC Xena Browser) of TCGA breast-invasive carcinoma RNAseq gene expression data (IlluminaHiSeq) shows cytokine expression after creating dichotomized DARC/ACKR1-positive (red) and -negative (blue) subgroups. Gene expression was assessed in 399 breast tumors against a panel of 67 genes associated with known cytokines (blue; low expression; red, high expression). Welch t test was performed on CCL2 (P = 0.0001, t = 10.28) and CXCL8 (P = 0.0001, t = 16.44). D, DARC/ACKR1 high and low categories compared with CCL2 (left, Student t test, P < 0.0001, DF = 438.05) and CXCL8 (right, Student t test, P < 0.0001; DF = 376.25) gene expression (mean ± SEM).
In the context of current standards of breast tumor molecular phenotypes, we also found that DARC/ACKR1 tumor status (e.g., DARC/ACKR1-high vs. DARC/ACKR1-low) was significantly distinct across the PAM50 (36) subtype categories ($P < 0.0001$), as defined by RNAseq gene expression profiles (Fig. 1B). Specifically, nearly 40% of the PAM50 Luminal A subtypes are DARC/ACKR1-high tumors, which is the largest proportion of DARC/ACKR1-high tumors across all subtypes. Contrarily, the highest proportion of DARC/ACKR1-low tumors is within the Basal-like subtype, making up over 60% of this category. When the PAM50 subtypes are also stratified for (self-reported) race (Fig. 1B, bottom), we observed clear distinctions in the distributions of DARC/ACKR1 tumor status between AA and WA, although these differences did not pass thresholds for statistical significance.

To investigate whether the status of DARC/ACKR1 tumor expression is connected to genes that function in DARC/ACKR1-related chemokine signaling pathways, we investigated over 60 chemokine genes (Supplementary Table S1) and found that there were significant differences in chemokine gene expression, correlated with DARC/ACKR1 status (Fig. 1C; Supplementary Table S2). Two of these genes, CCL2 and CXCL8, are high-affinity DARC/ACKR1-binding targets (38, 39) and have significant expression in breast tumors (Supplementary Fig. S4A) correlated with DARC/ACKR1 expression (Supplementary Fig. S4C and S4D). When compared across DARC/ACKR1 tumor types, we found that DARC/ACKR1-high tumors have significantly correlated high levels of CCL2 ($P < 0.0001$), but significantly anticorrelated lower levels of CXCL8 in primary tumors ($P < 0.0001$; Fig. 1D).

Circulating levels of DARC/ACKR1-related chemokines, CCL2 and CXCL8, are associated with disease status, Duffy-null phenotype, self-reported race, and African ancestry-specific alleles of DARC/ACKR1.

We conducted a Luminex analysis in an independent breast cancer case–control cohort (case, $n = 266$, controls, $n = 156$) to identify associations among DARC/ACKR1-related chemokines and histopathologic variables in our cohort. When comparing race between cases and controls overall, we identified a significant difference in circulating CCL2 levels between AA cases and WA cases, where AA had significantly lower CCL2 than WA ($P = 0.0031$, Fig. 2A, top). We did not observe a significant difference between cases and controls in AA, but CCL2 levels were decreased in AA cases only, a trend we did not observe in WA. We also observed a significant increase in circulating CXCL8 levels in AA cases ($P = 0.0013$, Fig. 2A, bottom) and WA cases ($P < 0.0001$, Fig. 2A, bottom) compared with their respective controls. These lower circulating CCL2 protein expression levels in AAs compared with WAs is a trend that indicates a potential race-specific circulatory inflammatory response; however, this same trend was not observed in the tumor gene expression data from TCGA (Supplementary Fig. S4B).

To determine the distribution and impact of the Duffy-null phenotype on cancer-related inflammatory response in our cohort, we tested our cases and controls for the erythrocyte-silent phenotype (Fig. 2B), as well as the Duffy-null gene mutation (rs2814778). As expected, we found both a higher frequency of the DARC/ACKR1*+/Duffy-null blood group and the Duffy-null/ACKR1*+ allele (rs2814778) frequency in AAs (Fig. 2C). These
distributions were in concordance with published global and population-specific allele frequencies (Supplementary Table S4). We then compared circulating levels of CCL2 and CXCL8 with the DARC/ACKR1 RBC phenotype and Duffy-null genotype for case and control groups (Fig. 2D and E). For phenotype, we found significant differences in CCL2 levels between those with positive DARC/ACKR1 erythrocyte expression and negative expression among the newly diagnosed breast cancer cases only (P = 0.0436; Fig. 2D, left). When considering genotype, we found significant differences in CCL2 levels across all newly diagnosed cases (P = 0.0002; Fig. 2E, left). We also observed individual associations between those that are homozygous for the reference allele (AA) and homozygous for the alternate Duffy-null allele (GG, P < 0.0001; Fig 2E, left), and those that are heterozygous and homozygous for the Duffy-null allele (P = 0.0176; Fig 2E, left) in newly diagnosed cases. This correlation was similar when the phenotype groups were broken down by race (Supplementary Fig. S5). Interestingly, the significant differences in CCL2-circulating levels increased among the DARC/ACKR1 allele carriers (Fig. 2E) indicating that the genetic allele is a more robust indicator of these circulating chemokine levels than the RBC phenotype, in newly diagnosed patients.

**DARC/ACKR1 tumor gene expression is associated with distinct tumor-associated leukocyte (TAL) gene expression profiles**

Because the chemokines under regulation of DARC/ACKR1 direct the infiltration of immune cells during inflammatory responses, we investigated the differences in immune cell responses, relative to DARC/ACKR1 tumor expression. Using TCGA RNAseq data, we employed a cell-type deconvolution algorithm, CIBERSORT, and determined that overall leukocyte infiltration (total TAL score) is greater in DARC/ACKR1-high tumors (Fig. 3A; ref. 31). These counts were directly correlated with quantified DARC/ACKR1 gene expression (Supplementary Fig. S6A), suggesting that DARC/ACKR1 could be a marker that predicts the level of tumor immunogenicity. The increase of total

Figure 3.

DARC/ACKR1 expression levels are positively and significantly associated with TAL abundance in breast tumors. Using CIBERSORT absolute mode, the absolute score totals the quantitative abundance of each individual leukocyte population among TCGA breast primary cases. Those cases with significant CIBERSORT results (P < 0.05) were included in this analysis (n = 472). A, The total TAL absolute score reported is a total of abundance scores across all 22 TAL populations. By plotting total TAL absolute score by DARC/ACKR1 expression status, we see significant increases in TAL abundance as DARC/ACKR1 expression is increasing between low, medium, and high categories. Looking at individual TAL populations, B shows the 12 leukocyte populations that are significantly different between DARC/ACKR1 expression levels. Student t test was performed comparing each DARC/ACKR1 expression group, and the P values for the associations are represented by the heatmap in C. Highly significant associations, dark red; less significant associations, dark blue.
immune cell infiltrates was also correlated with increasing CCL2, but to a lesser extent (Supplementary Fig. S6B), indicating that CCL2 has some influence on, but is not sufficient for, the total observed immune cell profiles.

We found distinctions in both the proportions and combinations of specific infiltrating immune cell subtypes of DARC/ACKR1-high tumors compared with DARC/ACKR1-low tumors. Specifically, there is an increase in numbers of B cells, T cells (CD8 and CD4), T-regulatory cells, and activated macrophages (M1 and M2; Fig. 3B and C). Conversely, DARC/ACKR1-low tumors appear to lack dendritic and B (memory) cells. Differences in the DARC/ACKR1-associated immune cell--type profiles are also present among race groups (*P* = 0.08). Specifically, AA patients with the DARC/ACKR1-low tumor subtype have more infiltrating M0 macrophages and fewer B cells compared with WA patients (Supplementary Fig. S6C).

Higher DARC/ACKR1 tumor gene expression is associated with longer survival

To determine the clinical significance of DARC/ACKR1 gene expression in breast tumors, we investigated publicly available datasets for associations of DARC/ACKR1 tumor gene expression with survival (cohorts are summarized in Supplementary Table S6). We found that when patients have higher DARC/ACKR1 tumor expression, they have significantly longer OS and RFS (Fig. 3A and B; OS *P* < 2.2 × 10^{-6}, *n* = 1,402; RFS *P* < 1 × 10^{-16}, *n* = 3,951). This survival association remained significant for RFS across all molecular subtypes (including Luminal A, Luminal B, Basal-like, and HER2^+^) (Supplementary Fig. S6C). We found distinctions in both the proportions and combinations of specific infiltrating immune cell subtypes of DARC/ACKR1-high tumors compared with DARC/ACKR1-low tumors. Specifically, there is an increase in numbers of B cells, T cells (CD8 and CD4), T-regulatory cells, and activated macrophages (M1 and M2; Fig. 3B and C). Conversely, DARC/ACKR1-low tumors appear to lack dendritic and B (memory) cells. Differences in the DARC/ACKR1-associated immune cell--type profiles are also present among race groups (*P* = 0.08). Specifically, AA patients with the DARC/ACKR1-low tumor subtype have more infiltrating M0 macrophages and fewer B cells compared with WA patients (Supplementary Fig. S6C).

Similarly, the <5-year survival outcome in TCGA is also linked to DARC/ACKR1 tumor expression (Supplementary Fig. S1 G). The impact of CCL2 (Fig. 4G–L) and CXCL8 (Fig. 4M–R) can also be seen within certain molecular subtypes of breast cancer, but often to a lesser degree than DARC/ACKR1 associations. In all subtypes, it is clear that inflammatory markers related to DARC/ACKR1 tumor expression have a significant impact on survival. Specifically, CCL2 has the most significant survival impact on basal-like and HER2^+^ cases (Fig. 4K and L) where higher CCL2 is associated with longer survival. Conversely, CXCL8 also has a significant impact in basal-like cases (Fig. 4Q), but in the opposite direction, where low expression of CXCL8 leads to longer survival. This opposing trend of CCL2 and CXCL8 is directly associated with DARC/ACKR1 status, as shown previously in Fig. 1D.

In tumor epithelial cells, DARC/ACKR1 is correlated with higher CCL2 and lower CXCL8 by IHC data

DARC/ACKR1 has been shown to be expressed on endothelial postcapillary venules, which are present in normal breast tissue (Supplementary Fig. S7A) and tumors as well. However, our preliminary data in breast cell lines indicated DARC/ACKR1 was expressed in tumor cells (Supplementary Fig. S7B). To confirm the genomic and cell line data, we conducted IHC on a small cohort of primary tumor specimens (n = 8) to validate the spatial expression of DARC/ACKR1 in the tumor microenvironment. Our initial results indicate that when DARC/ACKR1 is expressed, it was not limited to endothelial cells, but was uniformly expressed within the tumor-specific epithelial cells. This finding validates the VWF-associated DARC expression trends shown in Fig. 1A and
Supplementary Fig. S1F. Specifically, our IHC subset suggests that the DARC/ACKR1 gene product is also expressed over the full spectrum of scoring (0–4) in our cohort (Fig. 5A), similar to the RNAseq distributions (Supplementary Fig. S1A and S1B). Despite the small size of the pilot IHC cohort (n = 8), we observed a similar trend as the RNAseq findings, where tumors with high DARC/ACKR1 IHC scores showed correlated high CCL2 expression and low CXCL8 expression (Fig. 5A).

Anticipating that the role of DARC/ACKR1 on epithelial cells would be similar to that on endothelial cells, we investigated whether circulating chemokine levels were associated with DARC/ACKR1 tumor scores. We integrated the Luminex chemokine assays from our clinical cohort with a pilot IHC study with a subset of cases and found a significant positive correlation between DARC/ACKR1 tumor scores and circulating levels of CCL2 (P = 0.0061) and CXCL8 (P = 0.0291; Fig. 5B; Supplementary Table S3). This finding was also aligned with our RNAseq findings for CCL2, CXCL8, and DARC/ACKR1 levels.

Discussion
Distinct tumor immune responses among ancestry groups with cancer disparities
Disparities in cancer outcomes among race groups parallel distributions of tumor phenotypes, including distinct signatures of tumor-specific gene expression signatures. These findings have shifted the lens of clinical disparities research to now encompass molecular investigations of tumor phenotypes that drive poor prognoses, which can be now be linked to genetic ancestry, particularly West African Ancestry.

Work from our group and others uncovers molecular mechanisms of cancer that parallels the mortality and tumor phenotype incidence among groups of African and AA women (12). Genomic studies conducted in large AA and/or multi-ethnic cohorts have begun to utilize modified genomic tools in search of population-specific risk alleles. SNVs, structural, chromosomal, and epigenetic variations are being discovered and associated with a still theoretical genetic predisposition for the distinct tumor biology observed among women of West African descent (40–43). While more risk alleles are being detected through increased diversity of multi-ethnic genome-wide association study (GWAS), the specific risk alleles transmitted into non-white populations through African genetic ancestry have not yet been definitively shown (44). Our current study deepens our understanding of a repeated finding among gene expression studies, which describe differences in disease biology rather than risk of disease incidence/predisposition among race groups. Multiple groups have reported AA (race-specific) expression profiles that implicate immune response pathways in a distinct category of disease progression (45–47), but again without definitive links to African genetic ancestry.

In this report, we have shown that a West African–specific allele of DARC/ACKR1, a gene that regulates immune and inflammatory response, is strongly associated with both the epithelial expression and circulating levels of CCL2 and CXCL8, which are proinflammatory chemokines previously shown to be related to cancer progression. We have thus implicated the epithelial tumor expression of DARC/ACKR1 to be a key modulating factor of chemokines in tumors, putatively confirmed in small IHC study within our clinical cohort. These findings suggest that DARC/ACKR1 has a dual role in controlling chemokine gradients that determine tumor immune cell response, specifically by regulating both the levels of chemokines in tumors and in circulation. Interestingly, tumor expression of CXCL8 is negatively correlated with DARC/ACKR1 tumor expression while circulating levels of CXCL8 are positively correlated with DARC/ACKR1 tumor expression. These opposing trends are recapitulated in our survival analyses where high levels of DARC/ACKR1 in tumors and/or low levels of CXCL8 yield longer survival. We hypothesize this is an unmasking of a distinction in DARC/ACKR1 control of CXCL8 compared with CCL2 that may be related to DARC/ACKR1 tumor activity involving clearance of CXCL8 from the tumor and/or DARC/ACKR1 isoform functionality, which we are currently investigating.
Previous studies have already shown that the spatial expression of DARC/ACKR1 is governed, at least in part, by the Duffy-null allele (rs2814778). Given that AA cohorts have over 70% allele frequency of this allele (48, 49), we anticipate that most AA’s would carry the allele and therefore have distinct regulation of the gene in certain tissues. On the basis of our findings, this allele would predispose patients with breast cancer, who are Duffy-null carriers, to having a specific tumor phenotype, defined by the tumor immune cell landscape, creating a tumor immune response that is directed by the DARC/ACKR1 expression in tumors. Our case–control cohort indicates that the Duffy-null genotypes are a stronger indicator of circulating chemokines, compared with correlations that use self-identified race. The differences in chemokine correlations among race groups, compared with strict heterozygote/homozygote allele groups, corresponds to what would be expected within an admixed population where alleles related to genetic ancestry are convoluted within the proxy of race, where a portion of the individuals that may self-identify with African ancestry are harboring a non-African allele. Therefore, the associations of DARC/ACKR1 and chemokine levels in breast cancer cases can be rationally interpreted as ancestry-associated.

While we have found this association in our study, we believe this allele has not been identified in multi-ethnic GWAS that investigate cancer risk among race groups (22, 50) because this gene would not impact tumor etiology, but rather disease progression, and therefore does not qualify as a “disease (onset) risk” allele. However, when prevention fails, survival becomes imperative. This allele is associated strongly with both OS and RFS across all breast cancer subtypes and is linked to race through genetic ancestry. Therefore, this important work begins to unravel how African ancestry directly impacts the tumor biology and microenvironment of tumor progression, even systemically. We suspect that the trends of DARC/ACKR1 tumor expression were not quite significant in the TCGA data simply due to lack of true African ancestry measurements in lieu of self-identified race proxies. The genetic admixture of European genetic inheritance within the AA group has likely confounded DARC/ACKR1 tumor expression race associations. We will seek to follow-up with genetic ancestry estimates in these cases in a subsequent validation cohort.

The cancer-related mechanisms that derive aggressive tumor phenotypes, such as TNBC, have yet to provide clear and actionable markers for therapeutic decisions in clinic. Studies that seek to identify the factors that lead to treatment-refractory tumor conditions have often implicated the tumor microenvironment (TME; ref. 51). A significant component of the TME includes immune cells that are either associated with or infiltrating into the tumor and tumor-associated stroma (21). The assortment of immune cells that respond to tumor formation can determine the course of tumor progression (52). Tumor immunology studies suggest that TALs can lead to either good or bad prognoses, such as with higher proportions of B cells versus T cells, respectively (53–57). While our ability to discern immune cell types in the tumor space has great prognostic value, it is a tedious task to quantify each TAL cell type to establish adverse versus favorable immune profiles and is not feasible on a case-by-case basis in most clinics. Our discovery of a single genetic marker that could definitively regulate the course of immune response in tumors and therefore could forecast TAL profiles would be an invaluable biomarker to identify patients suitable for immunotherapies. In our report, we have introduced such a potential biomarker, showing that tumor expression of an atypical chemokine receptor, DARC/ACKR1, is associated with immune response. This association is aligned with the gene’s extensively defined regulation of chemokine levels in circulation, which may now also include the regulation of these levels in tumors as well. This suggests that DARC/ACKR1 can modulate the chemoattractants in patients with breast cancer, ultimately directing the actions of immune cells within the tumors.

With further investigations that characterize and quantify DARC/ACKR1-dependent TAL infiltration, in the context of African ancestry, we will develop a systematic approach to determine whether DARC/ACKR1 status may predict immune response profiles in breast tumors and ultimately predict immunotherapy treatment responses. This current study has established the feasibility of DARC/ACKR1 as a marker to define a novel tumor subtyping that defines its immune status. A subset of our cohort study, reported here, indicates the feasibility of integrating single-marker tumor IHC with a multi-marker peripheral blood assay. In combination, our data have shown that differences in tumor immune cell profiles, which are relevant to race, are associated with the tumor expression of DARC/ACKR1. Specifically, the African-specific Duffy-null variant of DARC/ACKR1, which is already associated with the gene’s spatial expression, determines a unique biological scenario for distinct TAL infiltrates. This landmark finding has high relevance in cancer management, as a functional regulator of proinflammatory and chemotactic cytokines that can specify an infiltrating tumor immune cell–type profile and specify differences between patients of distinct ancestry. This further implicates DARC/ACKR1 in tumor phenotype distinctions that could significantly drive treatment outcomes that lead to cancer mortality disparities. Indeed, DARC/ACKR1 may also begin to unravel the racial disparities of treatment outcomes within tumor phenotype categories that have effective targeted therapies and the overall outcomes in TNBC (57).

As with any epidemiologic study, limitations of our study include confounding factors within cohorts that are inseparable from variables of interest. Specifically, differences in DARC/ACKR1 gene expression between race groups could also be a function of a well-documented bias in occurrence of specific molecular tumor phenotypes across these populations (58). For instance, women of African descent have a higher relative incidence of TNBC, and we see this same trend in our cohort and included supplementary analyses in Supplementary Data. Interestingly, the Duffy-null allele has previously been implicated in other immune-related diseases, such as neuropenia (59), and therefore the distinct chemokine levels we observe in AA patients, associated with the mutation, now implicates a novel role of DARC/ACKR1 in a breast cancer disparities context. These correlations in chemokine levels were found primarily in newly diagnosed cases, suggesting that DARC/ACKR1 plays a similar role in tumors as what has been shown in endothelial cells—facilitating the entry of tumor-associated chemokines into circulation (i.e., transcytosis; ref. 26)—and so it may be regulating increase in circulating chemokine levels by facilitating chemokine transfer from epithelial tumor cells. Therefore, despite our limitations, these compelling findings indicate that the African-specific allele of DARC/ACKR1 modulates cancer-driven circulating chemokine levels and therefore may regulate the infiltration of tumor-associated immune cells we observed in tumors expressing DARC/ACKR1 at high levels compared with the lower numbers of tumor-associated immune cells when DARC/ACKR1 is expressed at low levels, if at all. Our ongoing
investigations in larger cohorts with higher numbers of non-white cases and better representation of all intrinsic tumor subtypes can further solidify the race/ancestry findings we have uncovered here.

Disclosure of Potential Conflicts of Interest

C. Yates is a consultant and has ownership interest at Riptide Biosciences. L. Newman is an attending physician at Henry Ford Health System. No potential conflicts of interest were disclosed by the other authors.

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Atypical Chemokine Receptor 1 (\textit{DARC}/\textit{ACKR1}) in Breast Tumors Is Associated with Survival, Circulating Chemokines, Tumor-Infiltrating Immune Cells, and African Ancestry

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