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Molecular Influences of Racial Disparities in Pancreatic Neuroendocrine Tumors

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MOLECULAR INFLUENCES OF RACIAL DISPARITIES IN PANCREATIC NEUROENDOCRINE TUMORS

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BIOMEDICAL SCIENCES

ABSTRACT

Pancreatic Neuroendocrine Tumors (PNETs) are highly heterogeneous neoplasms arising from the hormone-secreting cells of the endocrine pancreas. PNETs are broadly categorized as functional and non-functional based on the presence of symptoms associated with hormone secretion, which occurs in approximately 30% of cases. While functional tumors are all considered for resection because of these symptoms and their associated sequelae, non-functional tumors > 2cm are currently resected based on increased risk of developing metastatic disease. However, this criterion is based on data from predominantly White patients. Recent studies have shown that Black patients have much higher rates of lymph node metastatic disease at tumor sizes under 2cm, indicating that interracial differences in tumor biology may be responsible.

Upon evaluating the diversity of genomic studies in PNETs to investigate this further, we found little representation of Black patients and other ethnoracial minority groups. We then discovered differences in the mutation of key genes in PNETs between Black and White patients; among which was MEN1, the most frequently mutated gene in PNETs. Subsequently, we investigated the transcriptome, tumor microenvironment, and prognostic protein biomarkers for differences between Black and White patients. We discovered that numerous genes were differentially expressed between these cohorts, and that multiple differentially expressed genes were associated with clinical outcomes,

including tumor progression to metastasis and progression-free survival. Furthermore, we found differences in the infiltration of immune cells into tumors between Black and White patients, alongside differences in the expression of multiple previously established prognostic protein biomarkers. These data indicate that a host of molecular influences may indeed be driving racial disparities in PNET clinical outcomes.

Keywords: Pancreatic Neuroendocrine Tumors, Transcriptomics, Racial Disparities, Clinical Outcomes

DEDICATION

This work is dedicated to my Mother, Marilyn McLane, who died of breast cancer 17 years before she was able to see me write this. It is because of you that I came all this way.

This work is dedicated to my Grandmother, Anne Glidewell, who sacrificed her retirement to raise me after my mother's passing and died of lung cancer before she was able to see me get into medical school.

This work is dedicated to my Aunt, Tracie Eldridge, who finished raising me and got to see me start my journey to become an Oncologist before she died of colorectal cancer.

I will be the doctor you needed.

This work is dedicated to my Sister, Rachel Herring, who has supported me endlessly.

I think we did it, Rachel.

I think we made Them proud.

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My mentor Bart Rose pushed me when I needed a push and was both kind and understanding when I needed compassion. He also makes a mean Michelada. I couldn't have asked to have a better scientist, clinician, and person in my corner.

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MOLECULAR INFLUENCES OF RACIAL DISPARITIES IN PANCREATIC NEUROENDOCRINE TUMORS

INTRODUCTION

Pancreatic Neuroendocrine tumors (PNETs) are a heterogeneous group of neoplasms originating from the cells of the endocrine pancreas. PNETs are the second most prevalent pancreatic malignancy with an estimated 4,032 new cases in 2020[1]. PNETs are broadly categorized into two subsets- the hormonally active (termed functional), which occur in up to 30% of cases[2-4], and non-functional tumors, which do not secrete sufficient hormone to elicit symptoms. Functional PNETs often cause severe symptoms such as debilitating diarrhea, life-threatening glucose imbalances, bleeding ulcers, and heart failure as a result of this aberrant hormonal secretion [5]. While all functional tumors should be considered for resection, up to 80% of pNETs are non-functional and the recommendation for resection is instead based on the risk of malignancy. Chiefly, these guidelines use tumor size as a prognostic surrogate for lymph node metastasis (LNM)[6]. According to these guidelines, tumors <1cm are observed (6% risk LNM), 1-2cm are resected based on clinical judgement (10% risk LNM), and those ≥2cm are resected (40% risk LNM)[7]. Estimates of 5-year survival for patients with metastatic pNETs vary widely; rates >50% are frequently reported for well-differentiated tumors[8,9], but as low as 10% is reported for the more aggressive, poorly-differentiated tumors[10]. Therefore, clinicians need balance the potential for favorable survival outcomes with the risks of death or severe complications associated with pancreatic resection, occurring in 3% and 50% of cases respectively [10]. Given the weight and difficulty of this decision, it is critical to identify non-functional pNETs at risk for LNM early and offer

appropriate resection.

Racial Disparities in Pancreatic Neuroendocrine Tumors

Recent work has uncovered alarming disparities in the clinical outcomes between White patients (individuals of primary and/or proximate European ancestry) with PNETs, and other ethnoracial groups with pNETs. These disparities are of particularly great when contrasted with Black patients (individuals of primary/proximate African ancestry). Black patients with pNETs are more likely to present with metastatic disease, are less likely to undergo curative surgery, and have a 20% worse overall survival rate when compared to White patients[11]. However, if Black patients have their tumors resected they have the same overall survival as White patients[11]. At first glance, this would suggest that the worse survival of Black patients with pNETs is likely due to a myriad of socioeconomic factors and access to healthcare, rather than any particular biological differences among racial groups. However, given that the only published clinical outcomes data specifically studying at disparities in PNET care is from a single analysis of the Surveillance, Epidemiology, and End Results (SEER) dataset, there is nuance to this picture that is unexplored. Another plausible explanation is that guidelines recommending resection based on tumor size are simply not accurate for Black patients; and that smaller tumors need to be resected to prevent the onset of metastatic disease in this population. Indeed, the current size-based resection criterion may not be predictive of outcomes for all patients. Our lab recently conducted a retrospective analysis of resected pNETs at our institution to evaluate clinicopathologic factors that differentially influence disease-free survival (DFS) between Black and White patients with resected pNETs[12]. 151 patients

underwent surgical resection of pathology-confirmed pNETs between 2010 and 2019. Clinicopathologic variables and DFS were compared between 37 Black and 114 White patients. Black patients presented with larger median tumor size (3cm vs 2cm; p=0.02) than White patients. Furthermore, on Cox regression analysis, increasing tumor size was found to significantly impact DFS (p<0.01, HR 1.02, 95% CI 1.01-1.04). We concluded that there may be variance in clinical presentation warranting further investigation and noted that because current resection guidelines draw from datasets predominating in White patients, the size threshold for resection may not be accurate across diverse groups.

Given the foundation of these current size-based resection criterion in the heightened risk of developing lymph node metastasis (LNM), our lab subsequently conducted a multi-institutional analysis of resected pNETs to evaluate if tumor size correlated with LNM risk in Black patients as it did in White patients. Significantly, this size to LNM risk correlation had never been investigated in a racial context. Analysis of the multi-institutional United States NET Study Group (USNETSG) consortium dataset included 454 (389 White & 65 Black) patients that underwent surgical resection of pNETs from 1998-2019. While size >2cm correlated to LNM in White patients by logistic regression, it did not in Black patients (OR 8.0 [3.9 - 16.4];p< 0.01 vs. OR 1.5 [0.5 - 4.3];p= 0.49). This was then validated in a larger dataset from the National Cancer Database (NCDB) containing 5532 patients (4472 White & 760 Black). Comparing the incidence of LNM across racial groups by size category (<2cm, 2-3cm, >3cm) in both datasets revealed that Black patients have a statistically higher rate of LNM in <2cm tumors in both the USNETSG (5% vs 23%;p=<0.01) and NCDB datasets (12% vs 21%;p=<0.01) than White patients. While prior data found tumors <2cm may be safe to

surveil due to a lower risk of LNM, these data show this is likely not the case for Black patients. Furthermore, this observation suggests biological differences between these racial cohorts that promote earlier development of LNM in Black patients. This prompts the question: what might these biological differences be?

The Argument for Interracial Variation in PNET Biology

Most data on the (epi)genetic aberrations in pNETs are from well-differentiated, grade 1 or 2 tumors. These data suggest pNETs have a relatively low mutational burden compared to other neoplasms, even among locally advanced and metastatic lesions[13]. Among these, most pNETs bear mutations in MEN1, DAXX, & ATRX (~40%, 25%, 17%). Additional mutations in TSC1/2 (6%), PTEN (7%), homologous recombination repair and base excision repair genes (CHEK2, BRCA2; MUTYH; <5%), SWI/SNF chromatin remodeling (<5%), and histone methylases (SETD2, MLL3; <5%) have also been implicated. DAXX/ATRX functions to deposit histone 3.3 across the genome, with mutated pNETs displaying the alternative lengthening of telomeres phenotype portending chromosomal instability. Notably, MEN1, DAXX, and ATRX all profoundly influence the epigenome by interacting with transcription factors (TFs), mobile genetic elements, and chromatin remodeling, indicating that confounding epigenetic states may influence the effects of their mutation.

Epigenetic gene regulation occurs in response to a host of environmental and physiologic stimuli. DNA and histone methylation, TF binding, and noncoding RNA(ncRNA)-based transcriptional modulation are among the most common forms of epigenetic regulation. DNA methylation occurs most often at CpG dinucleotides

concentrated in clusters (CpG islands) proximate to promoter regions, where alterations are known to influence cancer phenotypes by multiple mechanisms, to include the inactivation of tumor-suppressor genes. TF binding is dynamically regulated by chromatin architecture, and complex networks regulate expression of ncRNAs. Robust data convey the presence of differentially expressed and regulated genes in tumors among ethnoracial groups, representing potentially targetable differences in tumor biology[14-23]. Given the high prevalence of epigenetic dysregulation in pNETs, baseline differences in DNA methylation between racial/ethnic groups, and close association with epigenetic states and race in other cancers, differences in the epigenetics and gene expression profiles of pNETs between White and Black patients are highly likely[19,20,24-26]. Of utmost importance is the improved prognostication, clinical decision making, and therapeutic targeting that understanding these differences may confer. Supporting this assertion are findings in sporadic insulinomas (the most common functional pNET) that mutations in the YY1 chromatin remodeling gene are heavily enriched in Asian patient populations compared to White patients (30% vs 10%). YY1 is a direct target of mTORC1, inhibitors of which are among the few approved therapies for pNETs, prompting suggestion that this population may uniquely benefit from mTOR inhibitors[27]. Notably, MGMT hypermethylation (present in 17-50% of pNETs) has been shown to predict response to alkylating agents such as temozolamide, wherein understanding differences in this hypermethylation in Black patients might have immediate clinical implications[28,29].

It is well established that Black patients are rarely represented and/or race is not reported in genomic studies[30]. No published data exists looking at differential (epi)genetic expression profiles between Black and White patients with pNETs. This lack

of representation in sequencing datasets can have significant clinical impact, as numerous studies have concluded that polygenic risk scores generated with mostly White genomic data woefully underperform when applied to subjects of African descent[31-33].

It is well-known that epigenetic gene regulation can be influenced by the environment. However, in recent years the relationship between epigenetic changes, macroeconomics, environmental exposures, and stress have become recognized as factors implicated in health disparities among varied ethnoracial groups[24,34,35]. While much of this research has focused on the impacts of epigenetic changes in cardiovascular and metabolic diseases, a rapidly growing body of research is forming around the discovery of racially distinct epigenetic changes in malignancy and the implications of these changes on therapeutic targets and decision making[19,20,24-26,34,36]. Recent studies have demonstrated across multiple tumor types that there is an inflammatory, immune, and metabolic genes signature prevalent in tumors from Black patients. However, there is a dearth of information with respect to PNETs, and how genetic ancestry and race may be influencing disparities in clinical outcomes.

UNDER-REPRESENTATION OF RACIAL GROUPS IN GENOMICS STUDIES OF GASTROENTEROPANCREATIC NEUROENDOCRINE NEOPLASMS

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Abstract

Not all populations are poised to benefit from advancing genomics in gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs), as genomics have focused on White patients. This study aimed to evaluate racial populations represented in genomic studies of GEP-NENs and to provide evidence of differential genomic findings between racial groups in GEP-NENs. Manuscripts analyzing DNA, RNA, or DNA methylation in GEP-NENs were queried using PUBMED and EMBASE. NIH race/ethnicity term frequency was then determined by Natural Language Processing, followed by manual evaluation of tumor types and subjects by racial group. Immunohistochemistry of institutional tissue micro-arrays and analysis of AACR GENIE data analyzed was performed to determine mutational differences between Black and White pancreatic NEN (pNEN) patients.

313 manuscripts conducted the requisite genomic analyses, 16 of which included subject race data. Race data were included in 13/184 DNA, 4/107 RNA, and 1/54 DNA Methylation analyses. These studies included 89% White subjects (n=2032), 5.8% Asian subjects (n=132), 4.0% "Other" subjects (n=93), and 1.2% Black subjects (n=27). No Native American/Alaska Native, Native Hawaiian/Pacific Islander, or ethnically Hispanic/Latinx subjects were represented. There were significant differences in *MEN1* mutations among Black and White patients in immunohistochemical (13:40) and GENIE data (24:268 patients per group, respectively), with 9 additional genes differentially mutated in the GENIE dataset.

Genomic sequencing data for GEP-NENs is almost racially homogenous. Differences in pNEN genomics may exist between racial groups, highlighting a need for diversity in future genomic analyses of GEP-NENs to understand the putative influence of interracial genomic variation on GEP-NEN prevention, diagnosis, and therapy.

Introduction

Gastroenteropancreatic Neuroendocrine Neoplasms (GEP-NENs) are a heterogeneous group of tumors arising from the enteroendocrine secretory cells of the gastrointestinal tract and endocrine pancreas. Pancreatic (pNENs) and small intestinal (siNENs) NENs are the most common types of GEP-NENs, accounting for 3-5% and 35–42% of pancreatic and small intestinal malignancies, respectively.[1] Some GEP-NENs secrete bioactive substances (functional tumors) that cause severe symptoms such as debilitating diarrhea, life-threatening glucose imbalances, bleeding ulcers, and heart failure. Surgery is the only curative therapy, but many patients with GEP-NENs have metastases at presentation, making curative resection unlikely.[2] Most GEP-NENs are diagnosed between the ages of 55 and 69, although colonic NENs peak after age 70.[3] However, across all GEP-NENs, White patients are diagnosed at significantly older ages compared to all other ethnoracial groups. Notably, an analysis of the SEER database found that the incidence of GEP-NEN varies significantly among ethnoracial groups, occurring more frequently in Black patients (5.19 cases per 100,000 individuals) compared to other ethnoracial groups (White 3.05; ethnically Hispanic/Latinx 2.46; others 2.39).[4] Additional analyses have found significant differences in the rate of metastatic disease among racial groups, with Black patients having the highest rate of advanced stage/metastatic disease in pancreatic, gastric, and appendiceal, NENs (36.3%, 20.9%, and 13.5%), followed by White patients (34% 16.7%, 12.1%), Hispanic/Latinx patients (28.2%, 15.1%, 5.65%), and Asian patients (26.7%, 16.7%, 19.8%).[3]

Interestingly, White patients presented with significantly more advanced stage disease in small intestine, colon, and rectal NENs (25%, 40%, 11.3%) than ethnically Hispanic/Latinx patients (25.6%, 27.5%, 6.5%), Black (18.1%, 35.3%, 7.2%), and Asian patients (18.8%, 35.3%, 6.5%), raising questions as to the etiology of these differences in GEP-NENs of various primary sites.[3]

Regarding clinical outcomes, recent work has uncovered alarming disparities between White and minority GEP-NEN patients- particularly Black patients.[3,5,6] Black patients with pNENs are more likely to be diagnosed with late-stage disease, undergo curative surgery less frequently, and have a 20% worse overall survival than White patients.[6] However, if Black patients have their tumors resected they have the same overall survival as White patients.[6] It is also well described that larger pNEN size directly correlates to increased risk of lymph node metastasis (LNM) and that Black patients often present with larger tumors.[5,7] Alongside these findings, Black patients have been found to have higher rates of LNM in both siNENs and pNENs.[8] Notably, Black patients with pNENs have a 360% higher rate of LNM even at small tumor sizes (< 2cm) compared to White patients (23% vs. 5%).[9] These data, particularly the large disparity in metastasis of pNENs at smaller tumor sizes, suggest that clinically relevant biologic differences in GEP-NENs may exist between these populations.

There is robust precedent demonstrating differentially mutated, expressed, and regulated genes in various cancers among racial groups, representing potentially targetable differences in tumor biology.[10-14] DNA methylation is the most stable and best characterized epigenetic modification, although epigenetic modulation of gene expression can occur through histone modification, regulation by noncoding RNA, and a host of

other processes. Interestingly, DNA methylation is known to differ among racial populations at CpG loci throughout the genome in healthy tissue.[15] Many of these differences are present even at birth, with significant enrichment for these differences at loci associated with cancers that include lung, prostate, and pancreatic among others.[16] Relative to other cancers, pNENs and siNENs are mutationally silent, with epigenetic dysregulation as a prevailing hallmark of these neoplasms (occurring in approximately 75-80% of pNENs & 70-80% of siNENs).[17-22] Given this high prevalence of epigenetic dysregulation in GEP-NENs and the known baseline differences in DNA methylation between racial groups, differences in the genetic, transcriptomic, and epigenetic ((epi)genetic) profiles of GEP-NENs between these patient populations are highly likely.[11,15] However, there are extensive disparities in the representation of diverse racial groups across all of genomics, extending into the realm of cancer biology.[23] This not only diminishes our ability to specifically study (epi)genetic differences between various racial groups in cancer, but it also prevents minority populations from benefiting from advances in precision medicine, genomic screening, and prognostication. Furthermore, equitably assessing (epi)genetic data across more diverse groups may well help the scientific community to further understand the oncogenesis and progression of various cancers. Despite increasing research aimed at expanding minority representation in (epi)genetic studies and characterizing racially distinct (epi)genetic states in other cancers, there is no work characterizing the state of diversity in GEP-NEN sequencing efforts. Furthermore, there are no studies specifically evaluating the interracial (epi)genomic variation in GEP-NENs that may be present. Accordingly, this study aims to evaluate the representation of racial groups in studies of GEP-NEN (epi)genomics, and to highlight the importance of

improved diversity in such studies by providing evidence that indicates the presence of differential (epi)genetic features among racial groups with these cancers.

Methods

Literature Search and Data Selection

Literature search was carried out in the PubMed and EMBASE ((https://www.ncbi.nlm.nih.gov/pubmed/; https://www.embase.com/) databases (**Fig. 1**). Database searches were time-delimited, selecting for publications between the year 2000 until June 2021. Structured search queries included terms pertaining to (epi)genetic analyses of GEP-NENs and are described in the supplementary data. Articles were included if they met the following selection criteria: published in the English language; conducted in humans; n > 1; conducting -omics analysis of DNA, RNA, or epigenetic states (i.e. DNA methylation) in GEP-NENs; conducting array-based or massively parallel next-generation sequencing analysis. Articles analyzing single gene expression, PCR arrays of < 5 genes (not including housekeeping genes), or point mutations at singular base loci (i.e. base 3, *KRAS* G12D/G12C/G12V alone) were excluded. Articles that included only pre-established cell line-based analysis were excluded. Articles such as editorials, letters, commentaries, reviews, clinical practice guidelines, and abstracts from conferences without associated published manuscripts were excluded. Meta-analyses or studies that exclusively studied previously published data, where one would not expect data on subject race to be uniquely presented, were excluded. Cases in which studies included multiple types of neuroendocrine neoplasms (e.g. lung, pituitary, adrenal) were included only if the GEP-NEN component of the study met the inclusion criteria with other NEN types excluded. Furthermore, only the GEP-NEN cases in these studies were included in the analysis of racial/ethnic representation. Studies of mixed neuroendocrine and non-neuroendocrine neoplasms were excluded. Manuscripts focused on pediatric

diagnoses were also excluded, as the incidence of all pediatric GEP-NENs is extremely low (under 0.1 per million, excepting appendiceal NENs at 0.5 per million) and these cases may be less likely to conform to the wider distribution of representation across all GEP-NENs.[24] Reference lists of all articles meeting criterion were also reviewed for any additional studies meeting criterion. GEP-NENs were classified into 5 groups: pNENs, siNENs, colorectal NENs (including anal NENs), other gastrointestinal NEN (GI-NEN; e.g. appendiceal, gastric, gallbladder, esophageal NENs), and GEP-NENs not otherwise specified (GEP-NEN NOS). Two researchers independently reviewed titles and abstracts manually and selected full manuscripts for inclusion. Disagreements were resolved by discussion and consensus. Manuscripts were reviewed in their entirety, including supplemental materials where present.

Natural Language Processing

Natural Language Processing (NLP) using the python packages NLTK (v. 3.6.2) and PDFMiner.six (release 20201018) was used to determine the frequency of the words "Race," "Ethnicity," "African American," "Black," "Hispanic," "Latino," "Latina," "Latinx," "Asian," "Native American," "American Indian," "Alaska Native," "Native Hawaiian," "Pacific Islander," "Caucasian," and "White," in published original research manuscripts performing sequencing on GEP-NENs gathered by a systematic review of the literature as described above. Specifically, PDF files were read using the extract_text function from PDFMiner.six. Multi-word tokens were then generated using the above race/ethnicity terms using the MWETokenizer function from NLTK. Tokenization and keyword searching was then performed using MWETokenizer.tokenize and

text.concordance functions. Natural language processing included supplemental materials where present. Subject numbers by racial group were then determined via manual review following NLP. All manuscripts that were negative for the race/ethnicity terms in our NLP search were likewise manually reviewed to verify that subject race/ethnicity data were not reported.

Immunohistochemical Analysis

Protein expression of the *DAXX*, *ATRX*, and *MEN1* genes, which is altered by most mutations, was determined by immunohistochemistry performed on pNEN tissue microarrays (TMAs).[25,26] Following surgical resection, tumor specimens were fixed, embedded in paraffin and TMAs generated and sectioned by the UAB Pathology Core Research Lab. Slides were rehydrated using xylene and ethanol. Antigen retrieval was accomplished by immersing slides in citrate buffer (pH 6) and placing them in a pressure cooker for 10 min. Antibodies to Daxx (Sigma, HPA008736), Atrx (Abcam, ab97508), and Menin (Abcam, ab92443) were diluted at a 1:200, 1:700, and 1:100 respectively in PBS augmented with 0.3% Tween 20 and 5% goat serum. TMA sections were incubated in primary antibodies overnight at 4°C. Following biotin and peroxidase blocking, sections were incubated with an anti-rabbit biotin labeled secondary antibody (Pierce goat anti-rabbit IgG, #31820) for 1 hr at room temperature. Slides were then stained with DAB chromogen (Dako Liquid DAB+ substrate) and counter-stained with hematoxylin. TMA stains were then evaluated in a blinded manner by a board-certified pathologist specializing in GEP-NENs. All studies of patient-derived tissues were approved by the University of Alabama at Birmingham Institutional Review Board (IRB-300006067).

Mutational Analysis

Mutational panel data on pNENs were obtained from the American Association for Cancer Research's (AACR's) project GENIE database using cBioPortal.[27] Patient-level enrichments were determined for protein-altering mutations (nonsense, frameshift, non-start, non-stop, splice-site, and structural variants/fusions including copy number deletions). Due to the low numbers of non-White subjects included in the AACR GENIE data and the lack of patient samples from other racial groups in our institutional TMAs, only AACR GENIE data from Black or White patients were used in this analysis. Additionally, because of potentially confounding differences among sequencing assays used in the GENIE dataset, only sequencing assays that included data for both Black and White patients were included.

Protein-Protein Interaction Network Analysis

Differentially mutated epigenetic regulatory genes from AACR GENIE (*MEN1*, *KMT2D*, *EP300*, and *SMARCB1*) were used to generate a PPI-enrichment network using STRING version 11.5.[28] Interaction sources used in PPI network generation were curated databases, experimental determination, text mining, gene co-expression, and gene neighborhoods. Only PPIs with a confidence score > 0.7 based on interaction sources were considered for network generation, and first-shell interactors were limited to 10. Network clusters were determined by k-means clustering. Gene Ontology (GO) functional enrichment of biological processes and molecular functions was also performed using STRING. GO Functional enrichment strength was determined as $\log_{10}$

(observed enrichment/expected enrichment), with expected enrichment derived from randomly generated whole-genome background networks of similar size.

Statistical Analysis

Differences in representation among racial groups relative to their proportions in the United States population (US) and cancer genomics as a whole were evaluated by Fisher's exact test. Population data for US adults were obtained from the 2020 census,[29] while data on the representation of racial groups in cancer genomics was obtained from a recent study of four major cancer genomic studies (TCGA, TARGET, cancer-related GWAS and the OncoArray Consortium).[23] Mutation frequency and staining were likewise compared between Black and White patients using Fisher's exact test. Given the low numbers of samples available from Black patients in the mutational analysis, and the confirmatory nature of the AACR GENIE mutational analysis for our immunohistochemistry (IHC) findings, we report significance based on p-values unadjusted for multiple comparisons. False discovery rate (FDR) adjusted p-values are reported alongside p-values in the mutational analysis as q-values. The mutation comparisons made are reported in **Supplementary Data S1**. PPI network significance was determined in comparison to expected interactions within randomly generated whole-genome background networks, and p-values corrected using the FDR.[28] Significance of GO functional enrichment was likewise determined. Statistical analyses were performed in R version 4.02 and GraphPad Prism version 8.

Data Availability Statement

The data generated in this study are available within the article and its supplementary data files. Other data used are available within the AACR Project GENIE Database (https://GENIE.cbioportal.org/login.jsp). Further inquiries may be directed to the corresponding author.

Results

Manuscript Characteristics

Using structured queries as described in the Methods, 3,329 manuscript records were identified (**Fig. 1**). 205 manuscripts were duplicated across database queries and their duplicates removed from further analysis. 3,124 manuscripts were reviewed for inclusion criterion. Most articles were excluded for not meeting article type criterion (reviews, editorial/opinions, clinical practice guidelines, meta-analyses; n=1438), not performing a requisite (epi)genetic analysis (n=691), or for analyzing only pre-established cell lines (n=212). In total, 313 manuscripts met all inclusion criterion. PNENs were included in most studies (n=220), followed by siNENs (n=98) and colorectal NENs (n=44) (**Fig. 2A**). The total number of GEP-NENs that underwent (epi)genetic analysis in these studies was 14,845. PNENs were the most abundant GEP-NENs in these 313 studies (n=10,309), followed by siNENs (n=3,089) and colorectal NENs (n=794; **Fig 2B**). These manuscripts were further subjected to the NLP search strategy, resulting in 72 manuscripts containing our race/ethnicity terms of interest.

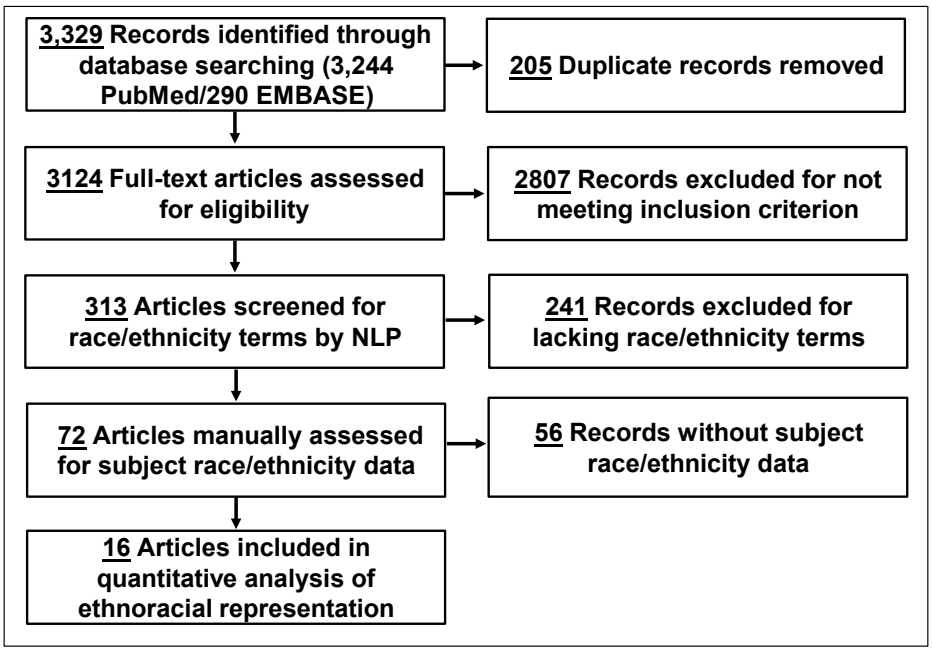

Figure 1. Systematic Review Process. Studies meeting inclusion criterion for NLP analysis were screened independently by two investigators before screening by NLP and manual review to determine subject number by race.

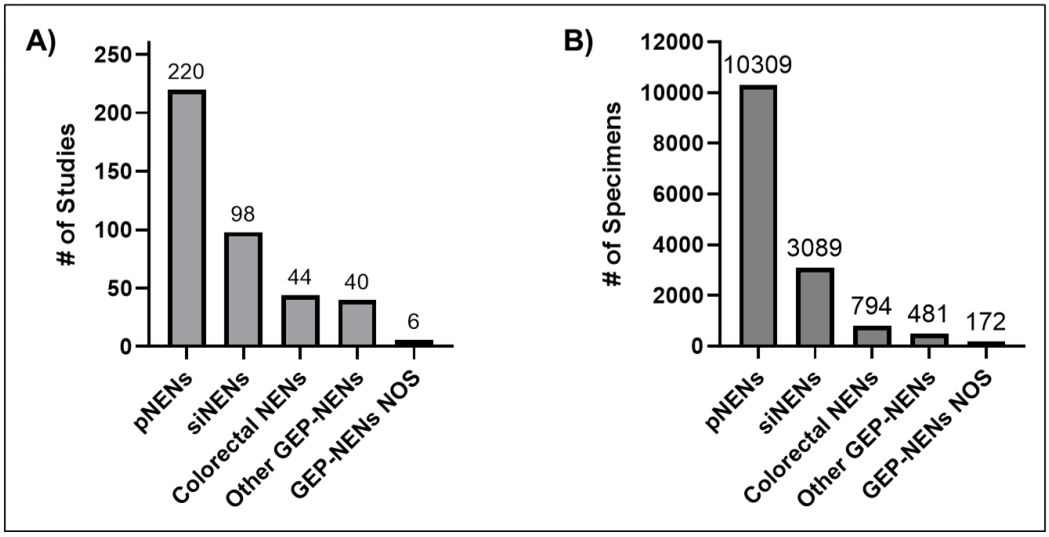

Figure 2. Manuscripts Analyzing GEP-NEN Types. **A)** Number of the 313 total manuscripts meeting criterion that analyzed each GEP-NEN type. **B)** Number of specimens from each GEP-NEN type included in all 313 manuscripts that met inclusion criterion.

Representation of Racial Groups

In total, 16/313 manuscripts included information on the race of the subjects included in their (epi)genetic analyses. 13/184 studies analyzing DNA included data on the race of their subjects, while 4/107 analyzing RNA, and 1/54 studies analyzing Methylation included such data (**Fig. 3**). The analyses performed in the 16 studies reporting subject race included: SNP and mutational analyses (including genome-wide association studies), targeted NGS/mutational panels, miRNA sequencing, PCR array, methylation-specific PCR, whole-genome sequencing, and gene copy number analysis (**Table S1**). In these studies, siNENs were the most abundant (n=697), followed by pNENs (n=695) and colorectal NENs (n=46; **Fig. 4A**). These studies included 89% White subjects (n=2032; s=16, where s = number of studies including White subjects), 5.8% Asian subjects (n=132, s=8), 4.0% "Other" subjects (n=93, s=11), and 1.2% Black subjects (n=27, s=6). No study reported race/ethnicity specific data for Hispanic/Latinx, Native American/Alaska Native, or Native Hawaiian/Pacific Islander subjects (**Fig. 4B**). The single methylation study that reported patient race included 90% White subjects (n=43) and 10% "Other" subjects (n=5). It should be noted that the discrepancy between total GEP-NENs analyzed in these studies by tumor subtype (n=1,457, **Fig. 4A**) and the total number of patients by racial group in these studies (n=2,284, **Fig. 4B**) is primarily the result of studies reporting the race of their entire cohort, while not specifying race information for the subset of their cohort included in (epi)genetic analysis. Hence, although the race representation results in **Fig. 4B** are as accurate as can be obtained by our methods, they are necessary extrapolations from these whole-cohort demographics and may differ somewhat from the real distribution. Overall, there was a significant

difference in the representation of race groups in GEP-NEN genomic studies, relative to their proportion of the United States population in the 2020 Census ($p<0.001$; **Fig. S1**).[29] White subjects were overrepresented (89.0% vs. 61.6%), Black subjects were underrepresented (1.0% vs. 12%), and subjects from "Other" racial groups were underrepresented (4.0% vs. 9%). Asian subjects appeared to be accurately represented with respect to the 2020 Census data (5.8% vs. 6.1%), but may be underestimated in GEP-NEN genomics due to the use of "Asian" without specific nationality terms in the NLP search strategy. Representation of racial groups in studies of GEP-NENs did not differ significantly from that across cancer genomics as a whole ($p = 0.27$).[23] While the publication dates of studies/databases used in the referenced studies do not differ widely (GEP-NENs 2003 & 2010-2020; aggregate cancer genomics 2007-2016), the aggregate cancer genomics data included pediatric data which may influence this result. Regarding the regional populations studied, most of the manuscripts that reported subject race data studied populations in the US (6/16, 5/16 exclusively in the Northeast US), 3/16 studied European populations, 3/16 studied populations in East Asia, 2/16 studied populations in both Europe and the US, and 2/16 studied worldwide populations.

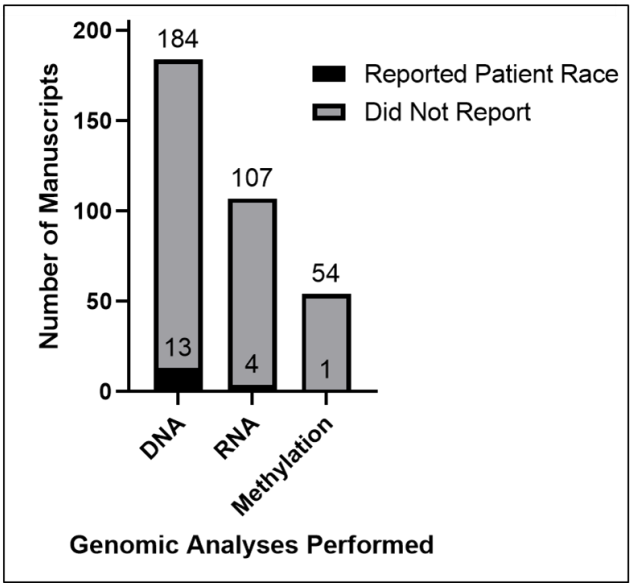

Figure 3. Biomolecules Analyzed in Manuscripts Reporting Patient Race. Number of the 313 total manuscripts that analyzed each type of biomolecule, depicted by the inclusion of patient race information.

Table S1) Assays Used in 16 Manuscripts Reporting Patient Race

Analysis Type	Assay
DNA	SNP/Mutational Array
DNA	Targeted NGS Panels
RNA	miRNA-Sequencing
RNA	PCR Array
Methylation	Methylation-Specific PCR
DNA	Whole-Genome Sequencing
DNA	Copy Number Analysis

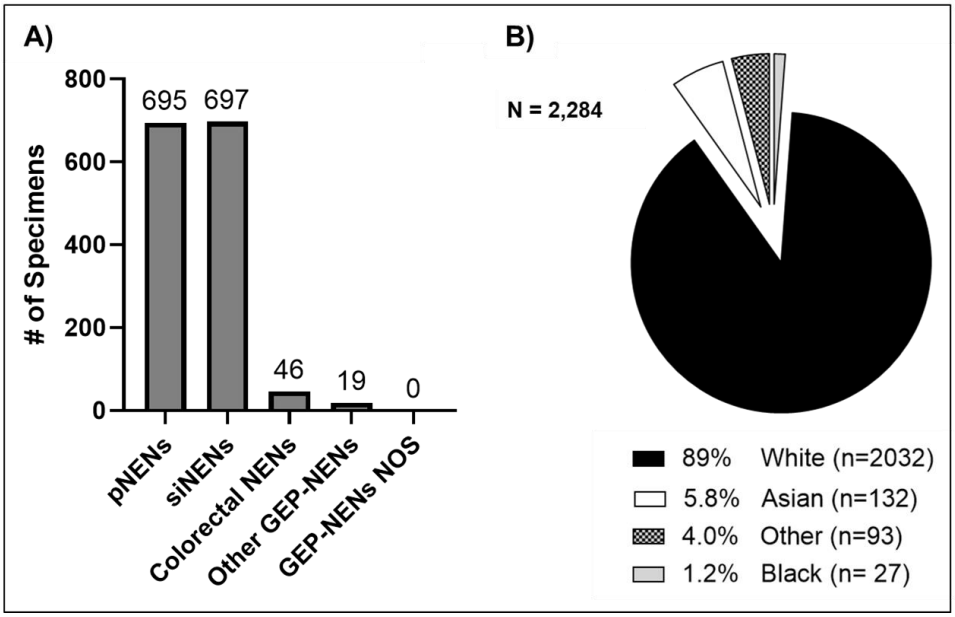

Figure 4. GEP-NEN Specimens Analyzed in Manuscripts Reporting Patient Race**. A)** Number of each type of GEP-NEN specimen analyzed in the 16 studies that reported patient race data. **B)** Number of patients from each ethnoracial group that were included in (epi)genetic studies of GEP-NENs that reported patient race.

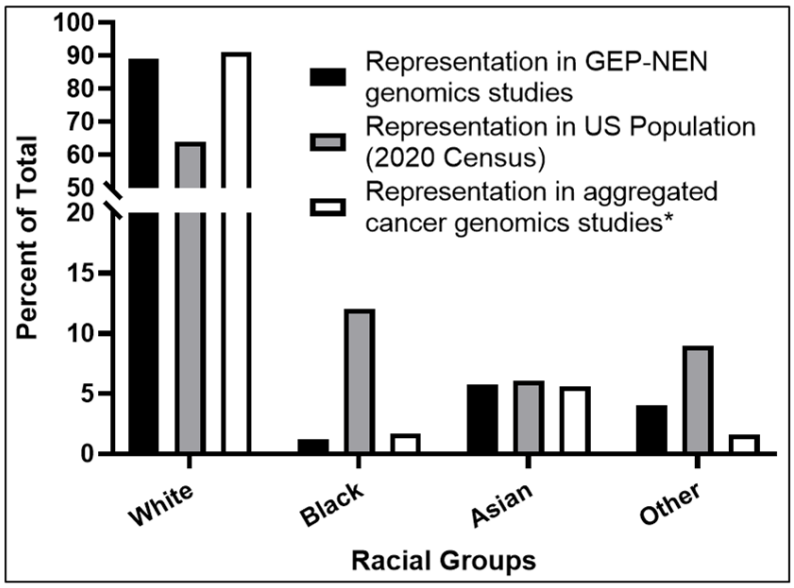

Figure S1. Comparison of racial group representation in GEP-NEN genomics, cancer genomics, and in the US population per 2020 census data. *Includes pediatric subjects

Immunohistochemical Analysis

Given the low representation of non-White racial groups in GEP-NEN (epi)genomics, we began to question the applicability of previous findings to these groups. As pNENs were the most analyzed tumor in GEP-NEN (epi)genomics, and the genes *MEN1*, *DAXX*, and *ATRX* are the most frequently mutated in pNENs, we investigated their mutational status across racial groups via IHC analyses. Negative staining on IHC indicates a protein-altering mutation, and has been shown to be highly concordant with mutation in these genes.[25,26] Pre-existing institutional TMAs containing samples from 40 White and 13 Black patients with primary, well-differentiated grade 1 and 2 pNENs were evaluated (**Fig. 5**). 9/13 (69.2%) Black and 22/40 (55%) White patients were female. Median age at resection (range) were 64 (35-93) and 64.5 (31-82) years for Black and White patients, respectively. Likewise, 6/13 (46.1%) and 24/40 (60%) tumors were grade 1 for Black and White patients. Regarding the IHC analysis, 9/40 (23%) White and 2/13 (15%) Black patients were negative for Daxx expression (p=0.711), 2/40 (5%) White and 1/13 (7%) Black patients were negative for Atrx expression (p>0.999), and 11/40 (28%) White and 0/13 Black patients were negative for Menin expression (p=0.047). Previous studies have found loss of Daxx, Atrx, and Menin expression by IHC in pNENs to occur in 59%, 25-85%, and 18-72% of cases, respectively.[30-32] The retention of normal Menin staining in specimens from Black patients supports the hypothesis that differential epigenetic modulation may be present in this population.[32]

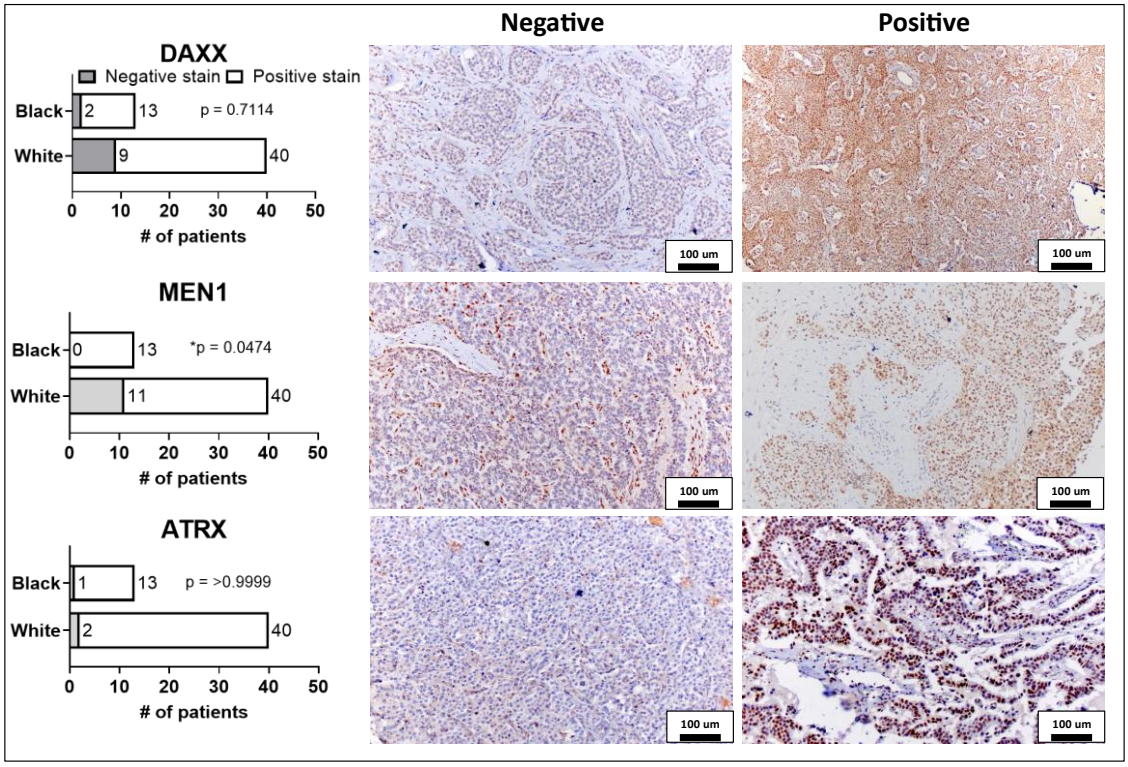

Figure 5. Immunohistochemical analysis of pNEN tissue microarrays containing tumor specimens from 13 Black and 40 White patients. The frequently mutated genes *DAXX*, *ATRX*, and *MEN1* were assessed for differential rates of mutation among groups.

Mutational Analysis

To provide further rationale for the inclusion of diverse subjects in genomic studies of GEP-NENs, we investigated suspected mutational differences between racial populations by querying the AACR GENIE database, which contains publicly accessible mutational data from 28 different institutional sequencing panels conducted on over 400 well-differentiated pNENs from Black and White patients (n=24 and n=399).[27] PNENs were chosen for follow-on studies, as they were the most prevalent neoplasms in the earlier reviewed sequencing analyses. Data from both Black and White patients together were available from nine sequencing assays (**Fig. S3**), consisting of 24 Black and 268

White patients that were included in further analysis. Median age (range) at sequencing were 55 (31-79) and 60 (19-85) years for Black and White patients, respectively. Samples were confirmed metastases in 45.8% of Black and 42% of White patients. Tumor grade was not available. We discovered 10 significant differentially mutated genes in pNENs between Black and White patient groups (**Fig. 6 & Table 1**), including *CHEK2* and *MUTYH* (DNA repair), *NF2* and *TP53BP1* (tumor suppression [TS]), *KMT2D* and *EP300* (histone methyl- and acetyl- transferases [HMT, HAT] respectively), the *CRKL* and *MAPK1* oncogenes, and *SMARCB1* (part of the n/npBAF SWI-SNF chromatin-remodeling complexes). Most notably, we found profound differences in the rate of *MEN1* mutations (TS, HMT), with White patients having a much higher rate of *MEN1* mutation compared to Black patients (37.3% v. 16.7%, p= 0.031). Importantly, 4/10 genes found to be differentially mutated have direct roles in epigenetic regulation (*KMT2D, EP300, SMARCB1, MEN1*), supporting previously described differences in epigenetic aberrations between racial groups in cancer.[33-37]

Table 1. Differentially Mutated Genes in pNENs among Black & White Patients in AACR Genie Dataset

Gene	Black	White	P-val[a]	Q-val[b]	Relevant Function
TP53BP1	2 (12.5%)	0 (0.00%)	0.005	0.35	Tumor Suppressor
CRKL	2 (8.33%)	0 (0.00%)	0.006	0.35	Proto-Oncogene
MAPK1	2 (8.33%)	0 (0.00%)	0.005	0.35	Proto-Oncogene
CHEK2	2 (8.33%)	0 (0.00%)	0.005	0.35	DNA Repair
NF2	2 (8.33%)	0 (0.00%)	0.005	0.35	Tumor Suppressor
MUTYH	2 (8.33%)	1 (0.37%)	0.019	0.75	DNA Repair
SMARCB1	2 (8.33%)	0 (0.00%)	0.006	0.35	Chromatin Remodeling
KMT2D	3 (13.04%)	4 (1.49%)	0.014	0.63	HMT[c]
MEN1	4 (17.39%)	140 (40.35%)	0.031	0.95	Tumor Suppressor, HMT
EP300	2 (8.33%)	2 (0.75%)	0.035	0.95	HAT[d]

a. Fisher's exact test, unadjusted for multiple comparisons; P-val = p-value

b. Q-val = FDR-adjusted p-value

c. Histone Methyltransferase

d. Histone Acetyltransferase

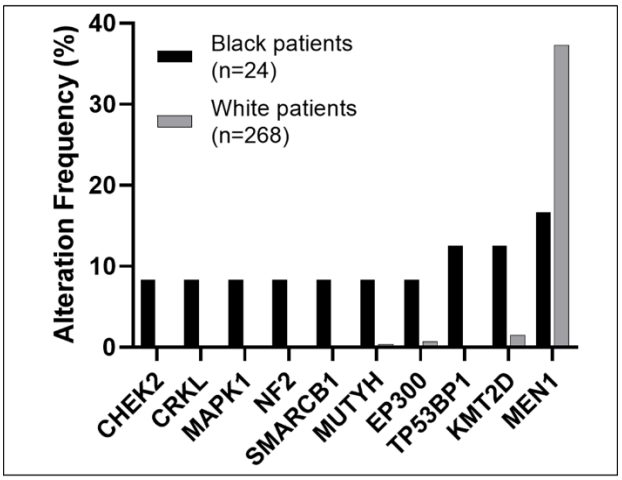

Figure 6. Analysis of mutational panel data from the AACR Project GENIE, comparing the incidence of significant differential protein-altering mutations between Black and White patients with pNENs.

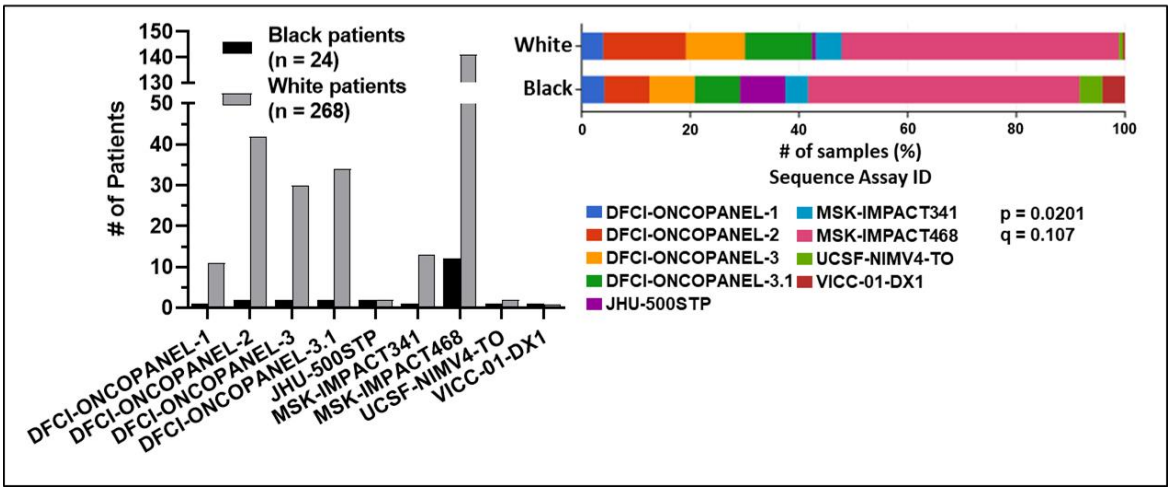

Figure S2. Composition of Black and White patient cohorts from the AACR GENIE dataset by sequencing assay ID's, depicted by raw subject numbers (Left) and by % composition (Right). Only sequencing assays that included both Black and White patients were considered.

Protein-Protein Interaction Network Analysis

Given known differences in epigenetics between racial groups, a PPI-enrichment network of the differentially mutated epigenetic regulatory genes *MEN1*, *KMT2D*, *EP300*, and *SMARCB1* was generated using STRING (**Fig. 7A**).[28] The network was highly interconnected and significantly enriched for PPI's ($p=2.11e^{-15}$), with a total of 132 PPI's discovered within the network and a mean of 8.25 interactions per node (**Supplementary data S2**). Notably, most PPI's fell within the *very high* (0.9-1.0) and *high* (0.7-0.9) confidence score categories (48.5% & 18.2%), with fewer in the *medium* confidence score category (33.3%; 0.4-0.7). *EP300* had the highest number of interacting partners (n=14), followed by *KAT2B* (n=13) and *KDM6A/YY1* (n=11 each). *KMT2D*, *SMARCB1*, and *MEN1* each had 9, 8, and 6 interacting partners, respectively. Gene ontology (GO) enrichment analysis was then performed for biological processes, molecular functions, and cellular components, discovering 196 significantly enriched GO terms in our PPI network (**Supplementary data S3**). Notable enriched GO terms

included histone H3-k4 methylation, histone acetylation, beta-catenin-TCF complex assembly, type b pancreatic cell differentiation, epigenetic regulation of gene expression, chromatin remodeling, MLL3/4 complex, and the nBAF/npBAF complex.

According to these data, we propose an interaction model of the epigenetic regulatory genes found to be differentially mutated between Black and White patients with pNENs in our analysis (**Fig. 7B**). KMT2D is targeted by the Menin protein (encoded by the *MEN1* gene), forming the MLL3/4 histone methyltransferase complex that regulates H3K4me3 deposition in promoter regions throughout the genome, as well as interacts with the p53 pathway through many of its target loci.[38] The histone H3K27 acetyltransferase EP300 requires KMT2D for its binding to a variety of enhancer regions and the facilitation of enhancer-promoter looping, tying mutations in these genes to one another functionally.[39] Furthermore, KMT2D has also been shown to associate with the SWI/SNF chromatin remodeling complex, acting as a coactivator for nuclear hormone receptor driven transcriptional activation.[40] This function further involves a core subunit of the SWI-SNF p/npBAF complexes SMARCB1. Of additional note is that SMARCB1 facilitates the effective activation of cell-type specific enhancers by KMT2D and CBP, and CBP closely associates with EP300 for H3K27 acetylation. The BAF complexes/SMARCB1, Menin, and KMT2D all share additional roles in their inhibition of canonical Wnt signaling, thereby acting in a tumor-suppressive manner.[41,42]

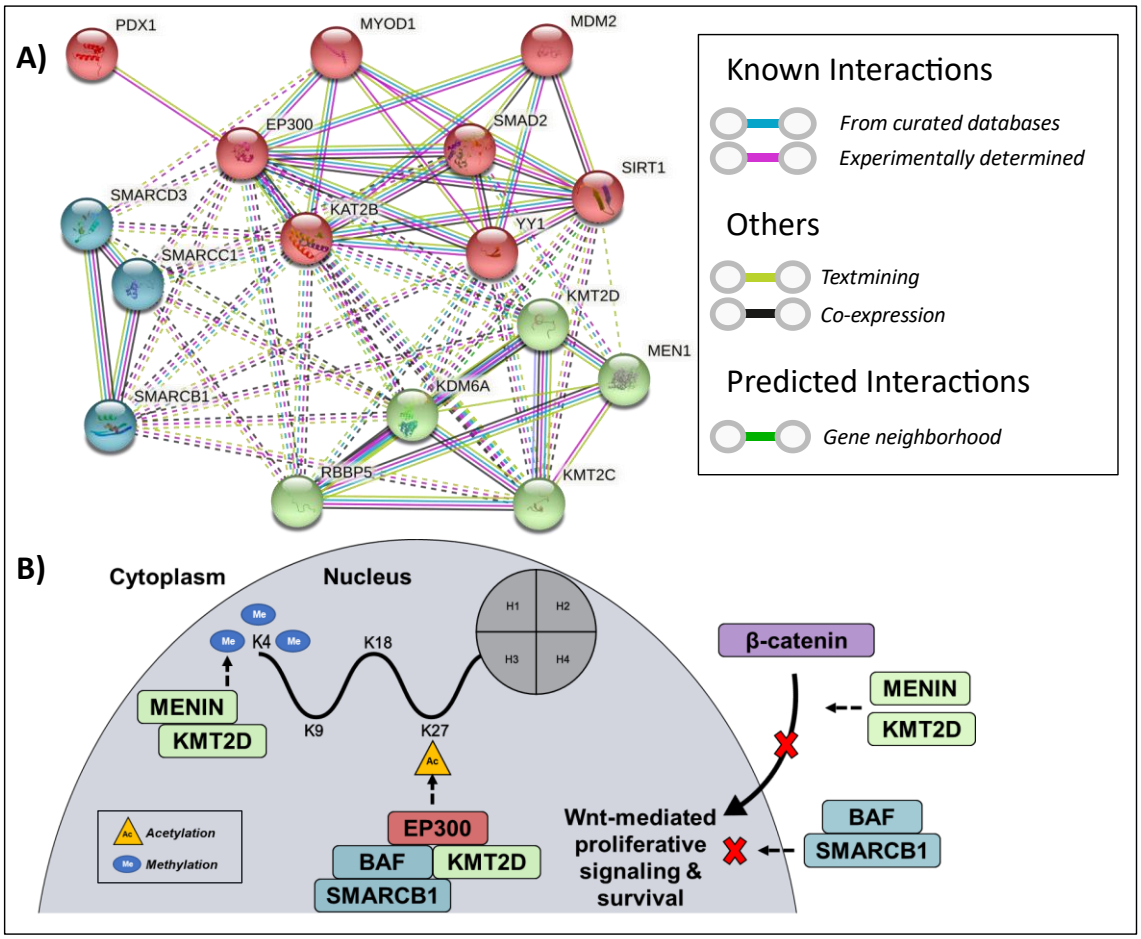

Figure 7. A) STRING interaction network of Menin, KMT2D, SMARCB1, and EP300. Between-cluster interactions are denoted by dotted lines, with intra-cluster interactions denoted by solid lines. **B)** A potential model of functional interdependencies and convergences of Menin, KMT2D, EP300, and SMARCB1 based on network interactions and literature review.

Discussion

We demonstrate herein that few studies on the (epi)genetics of GEP-NENs include data on the race of their subjects. We find that pNENs and siNENs make up the greatest proportion of manuscripts' topics and tumor specimens analyzed in the literature, as would be expected given their incidence compared to other GEP-NENs. We also find that GEP-NEN (epi)genomics overrepresents White subjects relative to the proportion of

the US population that they comprise (**Fig. S1**).[23,29] Indeed, based on the US population, the expected representation of White subjects is ~62%, exceeded herein by ~29%. In contrast, Black subjects are represented at ~10% of their expected proportion (1.2% vs. 12%). While these findings align with those from other studies characterizing the representation of racial groups in cancer genomics, no Native American/Alaska Native, Native Hawaiian, Pacific Islander, or Hispanic/Latinx subjects are explicitly represented in current studies of GEP-NEN (epi)genomics.[23,43] From these data, we can conclude that there is little representation of racial minorities in (epi)genetic studies of GEP-NENs. Following this, we discovered differences in the mutation of the *MEN1* gene between Black and White patients by IHC. We then conducted a pilot study of differentially mutated genes between these groups using AACR GENIE data wherein we affirmed this finding and discovered 9 other differentially mutated genes. We then conducted PPI enrichment network analysis on a subset of epigenetic regulators within those genes, finding them to be functionally interconnected, and propose a model of their key interrelated functions. According to the current genetic epidemiology of pNENs, *MEN1* is the most frequently mutated gene, followed by *DAXX* and *ATRX*. The data herein indicate that this may not be the case for different racial groups, warranting more comprehensive analyses of GEP-NEN (epi)genomics among racial groups to identify genomic aberrations that may be enriched in or unique to these populations.

The inclusion of diverse patient populations in (epi)genetic studies of cancer is crucial to understanding and rectifying cancer health disparities, as well as further understanding the biology of various cancers. Therapeutic susceptibility is one important translational aspect of inter-racial (epi)genetic variation that highlights the need for

diverse patient representation in sequencing analyses. For example, a phase III trial of the tyrosine-kinase inhibitor (TKI) gefitinib for non-small-cell lung cancer (NSCLC) found no benefit for any population other than those of Asian descent. Epidermal growth factor receptor (EGFR) mutations were then discovered to be far more prevalent in Asian patients with NSCLC compared to those of European descent (47% vs. 15%).[44] This led to increased use of TKI's targeting EGFR in this population as first-line therapy, in addition to the discovery that certain TKI's demonstrated increased efficacy in this population.[45,46] There is similar potential for targetable racial differences in sporadic insulinomas (the most common functional pNEN) as well. For example, mutations in the *YY1* chromatin remodeling gene are heavily enriched in Asian patient populations (30% vs 13%).[47] YY1 is a direct target of mTORC1, inhibitors of which are among the few approved therapies for pNENs, prompting suggestion that this population may uniquely benefit from mTOR inhibitors such as everolimus.[48] Furthermore, *MGMT* hypermethylation (present in 17-50% of pNENs) has been shown to predict response to alkylating agents such as temozolamide, wherein understanding differences in this hypermethylation in Black patients might have immediate clinical implications.[49,50] These points considered, the ability to identify such genomic differences across populations is currently limited by the lack of diverse groups in genomic studies of GEP-NENs, as depicted herein.

The establishment of prognostic genomic features, which allow clinicians to inform treatment selection and conduct prognostication, is an important element of cancer genomic analysis wherein a lack of diverse subjects may exacerbate racial disparities in clinical outcomes. Numerous prognostic genomic features have been characterized as

predictors of survival for pNENs, including mutations in *DAXX*/*ATRX* and *MEN1*, the expression levels of somatostatin receptors 2 and 5, elements of the tumor immune microenvironment, and enzymes involved in hormone metabolism.[51,52] However, it is likely that studies characterizing these various features as predictors of disease outcomes reflect the populations included in the (epi)genomic studies reviewed in the present study. This is highlighted by our findings that the *MEN1* gene was among those that were differentially mutated between Black and White patients with pNENs. However, while it may be that well-known genes such as *MEN1* for pNENs are not altered in diverse populations, it is possible that "non-canonical" genes representing key nodes of frequently altered pathways are preferentially affected in different racial populations. For example, oncogenic dysfunction of the Menin/MLL4 histone methyltransferase complex in pNENs may occur more frequently via *KMT2D* mutations as opposed to *MEN1* mutations in a given population. This and similar scenarios would entail that such aberrations are identified and included in future studies of association with patient outcomes- as well as clinical screens- to serve a more diverse population's needs adequately and equitably.

Another relevant issue in the diversification of cancer genomics is the exclusive use of self-reported race (SRR) as opposed to the inclusion of genetic ancestry when characterizing racially enriched genomic features. SRR is relatively easy to obtain while also being correlated with genetic ancestry; and its use has persisted in large-scale genomic analyses as a result. Furthermore, SRR acts as a surrogate metric for a highly complex array of behavioral, cultural, environmental, and social variables that are themselves influential in disease.[53] However, when SRR data are available they can be

inaccurate and incomplete, potentially leading to spurious associations between racial groups and genomic features.[54] Although few studies of genomic differences in cancer have incorporated genetic ancestry, broadening knowledge of their influence on tumor biology is driving wider adoption of the practice.[55,56] Numerous tools have been developed that allow for the incorporation of genetic ancestry into genomics analyses, such as Admixture[57] and STRUCTURE.[58] These tools use distinct methods to provide maximum-likelihood estimation of individual ancestries from multi-locus single nucleotide variants, or use Bayesian techniques to assign individuals to a pre-defined *k* number of racial groups based on their genetic features, respectively.[54,59-61] Genetic ancestry may be particularly important to consider for disaggregating racial groups that can become "invisible" in studies of genomics and racial disparities due to relatively low subject numbers, population admixture, discrepancies between SRR and genetic ancestry, or emphasis on studying disparities in certain minority groups over others.[62] Additionally, the use of genetic ancestry in the conduct of (epi)genomic analyses among racial groups, while not optimal, provides a method for circumventing the remiss practice of aggregating subjects from minority racial groups into an "Other" category without any additional data. Given the complexity of factors associated with SRR, there is ongoing discourse as to if and how genetic ancestry should be incorporated with SRR, rather than considered separately.[53,63,64] However, it is clear that genetic ancestry should be considered in genomic analyses of diverse populations.

Numerous obstacles have likely led to the current state of diversity in GEP-NEN genomics. Approximately 60% of patients with pNENs have metastasis upon presentation, precluding a curative resection that would generate tissue for biobanking.

Alongside this, Black and other non-white patients are less likely to undergo surgery for GEP-NENs, even when they meet societal guidelines for surgical resection and are good surgical candidates.[3,65] Understandably, this results in fewer patient tumor samples from these racial groups being banked for use in sequencing analyses. Alternatively, given that tumor biobanking generally takes place at academic institutions with high surgical volume *and* that racial minorities are less likely to undergo cancer surgeries at such high-volume centers, these differences likely influence the biobank composition where GEP-NEN (epi)genetic research is conducted.[66] A recent analysis of the SEER-Medicare database found that most patients with GEP-NENs receive surgery at medium or high-volume centers.[67] However, they also found no significant difference in the racial composition of those treated across hospital volumes. Unfortunately, this study included a relatively small sample size of patients with mixed surgically and medically treated patients (n=899) that was constrained by the availability of completed insurance claim data. As this is the only study analyzing the impacts of treatment center volume on GEP-NEN outcomes to the authors' knowledge, how the catchment populations of high-volume GEP-NEN research hospitals might be affecting the racial representation in GEP-NEN genomics remains unclear.

Various initiatives and strategies have been aimed at improving the diversity and representativeness of cancer genomics, with focuses on research infrastructure, clinical trial design, community engagement, and researchers themselves. Arguably, the most impactful strategy is the prioritization of inclusive research by institutions, as this manifests as the former through specific goals and increased investment. Increasing the diversity of the cancer genomics workforce is the goal of the AACR's Minorities in

Cancer Research Council and the National Cancer Institute's Partnerships to Advance Cancer Health Equity program, which aim to provide training, career development, and research funding for early-stage investigators and trainees from under-represented groups. Improving the diversity of the cancer research workforce may also improve the involvement of diverse groups in clinical trials and genomics studies, as researchers are motivated to address cancer health disparities in their communities and better understand the systemic, logistical, and cultural barriers they face.[68] Clinical trials are a key source of (epi)genomic data, as they often involve the collection of patient blood or tumor samples for (epi)genomic analyses alongside therapeutic regimens. Diversity in these clinical trials suffers immensely from the barriers faced by racial minority populations, as they are often confined to large research institutions that may be unreachable, require multiple visits, have restrictive inclusion criterion, have hidden costs of participation, or are distrusted by ethnoracial minority groups. In 2020, the US Food and Drug Administration released guidance to facilitate the involvement of underrepresented ethnoracial groups in clinical trials through Project Equity, which included recommendations for decentralizing clinical trial procedures into community facilities, relaxing inclusion criterion, and setting specific goals for enrollment of subjects from ethnoracial minority groups. In tandem with clinical trials, biobanking is a critical aspect of diversity in cancer genomics studies. However, in addition to lacking ethnoracial diversity, data show that biobank donors tend to be both healthier and wealthier than the populations they ostensibly represent.[69] Even small improvements in the numbers of subjects from ethnoracial minority groups have been able to improve the detection of genomic variants associated with disease processes, emphasizing the benefits of improving biobank diversity.[70] This might be accomplished

by using online consent models to facilitate the ease of consenting to biobank participation.[71] Such systems also allow institutions to provide information as to how patients' samples will or are actively being used, which has been demonstrated to be a key factor in decision-making for biobank participation across racial groups.[72] Decentralization, or the establishment of multi-institutional regional biobanks that source from community institutions, may be of particular benefit to ethnoracial diversity in GEP-NEN genomics, due to their relatively rare nature.[73] While decentralization may also benefit ethnoracial diversity in biobanking similarly to clinical trial enrollment, consistency and standardization of procedures must be carefully considered to ensure specimen and data quality.[74]

There are several limitations of the present study to be considered. First is the lack of precise data on the number of patient samples by racial group that underwent (epi)genetic analysis, highlighted by the discrepancy between the total numbers reported in **Fig. 4A** (n=1,457) and **Fig. 4B** (n=2,284). Because race representation was reported for whole cohorts rather than for cohort subsets that were included in (epi)genetic analyses, the results in **Fig. 4B** are an inexact depiction of the unobtainable real distribution. A minor limitation of this study lies in the lack of a specific distribution of subject ages in the comparisons of racial representation in GEP-NEN studies, aggregate cancer genomic studies, and the US population data from the 2020 census. Notably, only data from aggregate cancer genomics studies included pediatric subjects, but specific age distribution data were not readily available in these data and were both variably reported in the GEP-NEN studies that reported patient race data. Another limitation lies in the relatively low number of Black patients compared to White patients in our analysis of

pNENs in the AACR GENIE cohort (n=24 and n=268, respectively) and our immunohistochemical data (n=13 and n=40, respectively). While this disparity in representation illustrates the points made within and the statistical approach aimed to mitigate differences in sample size, these factors must be considered in the interpretation of our results. Accordingly, it is noted that unadjusted p-values are used herein to determine the number of significantly differentially mutated genes. The small number of subjects in the Black AACR GENIE cohort and the number of genes analyzed (n=323; **Supplementary Data S1**) rendered all analyses non-significant with traditional multiplicity adjustments (**Table 1**). However, while caution should be exercised in interpreting these data, the lack of MEN1 mutations in the Black AACR GENIE cohort parallels our observations in an independent cohort and strengthens this conclusion. Furthermore, as evidenced by the literature review conducted herein, the AACR GENIE dataset remains the only available repository of data upon which such analyses can currently be conducted, further demonstrating the need for additional studies of underrepresented racial populations in GEP-NEN genomics.

Our understanding of (epi)genetic variation among racial groups has important implications for our understanding of GEP-NENs, and may highlight genomic differences among racial groups influencing oncogenesis and tumor progression similarly to those described in other cancers. In conclusion, careful inclusion of diverse populations in (epi)genetic studies is integral for further understanding GEP-NEN biology, generalizing findings to diverse patient populations, and improving therapy for all.

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MOLECULAR INFLUENCES OF RACIAL DISPARITIES IN PANCREATIC NEUROENDOCRINE TUMORS

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Abstract

There are known disparities in outcomes between Black and White patients with pancreatic neuroendocrine tumors (pNETs). Recently, Black patients have been shown to have higher rates of lymph node metastasis in smaller tumors than White patients, indicating possible differences in tumor biology. Numerous prognostic gene expression differences between racial groups have been reported in other cancers, but no such analysis has been conducted in pNETs. This study aims to evaluate pNET transcriptomes and previously established prognostic protein biomarkers for differential expression, as well as differential elements of the tumor microenvironment that may be influencing racially disparate outcomes.

RNA-seq was conducted on RNA isolated from well-differentiated Grade 1 and 2 archival pancreatic NETs (PNETs) from 21 Black and 18 White patients. Following QC and alignment, ancestry estimates were generated from RNA-seq derived SNPs to estimate African ancestry. Differential gene expression was performed based on self-reported race and African ancestry. Differentially expressed genes (DEGs) were then evaluated for their relationship with progression-free survival and a composite endpoint of lymph node metastasis, distant metastasis, and progression. Tissue microarrays of PNETs were then stained for PNET markers, immune cell markers, and prognostic proteins via multiplexed immunofluorescence. Immune cell infiltration, PD-L1 positivity, and prognostic protein expression were then evaluated between racial groups.

Of 414 DEGs, 14 were significantly associated with clinical outcomes. T cell

infiltration and PD-L1 positivity varied between racial groups, along with expression of all prognostic biomarkers.

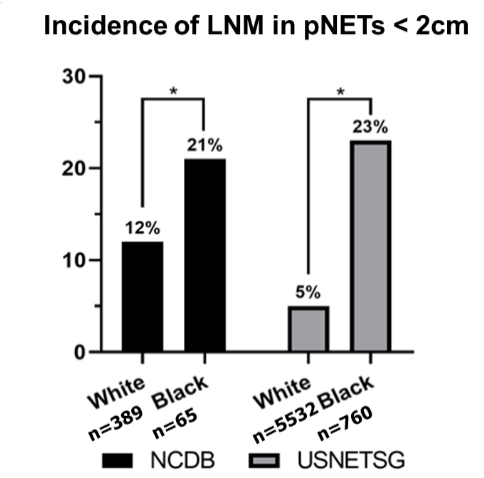
There are differences in gene expression between Black and White patients with PNETs that have a relationship with tumor progression to metastatic disease, as well as in the expression of previously established prognostic protein biomarkers and immune cell infiltration. These differences warrant further investigation into interracial variation in PNETs.

Introduction

Pancreatic Neuroendocrine tumors (PNETs) are a heterogeneous group of neoplasms originating from the cells of the endocrine pancreas. PNETs are the second most prevalent pancreatic malignancy with an estimated 4,032 new cases in 2020[1]. PNETs are broadly categorized into two subsets- the hormonally active (termed functional), which occur in up to 30% of cases[2-4], and non-functional tumors, which do not secrete sufficient hormone to elicit symptoms. Functional PNETs often cause severe symptoms such as debilitating diarrhea, life-threatening glucose imbalances, bleeding ulcers, and heart failure as a result of this aberrant hormonal secretion [5]. While all functional tumors are considered for resection, up to 80% of pNETs are non-functional and the recommendation for resection is instead based on the risk of malignancy. Chiefly, these guidelines use tumor size as a prognostic surrogate for lymph node metastasis (LNM)[6]. According to these guidelines, tumors <1cm are observed (6% risk LNM), 1-2cm are resected based on clinical judgement (10% risk LNM), and those ≥2cm are resected (40% risk LNM)[7]. Estimates of 5-year survival for patients with metastatic pNETs vary widely; rates >50% are frequently reported for well-differentiated tumors[8,9], but as low as 10% is reported for the more aggressive, poorly-differentiated tumors[10]. Therefore, clinicians need balance the potential for favorable survival outcomes with the risks of death or severe complications associated with pancreatic resection, occurring in 3% and 50% of cases respectively [10]. Given the weight and difficulty of this decision, it is critical to identify non-functional pNETs at risk for LNM early and offer appropriate

resection. However, the aforementioned size-based resection criteria rely heavily on analyses of European patient cohorts; where Black patients range from 4% of the population in the UK to 0.1% in Poland.[11,12] Indeed, according to a recent analysis of the National Cancer Database (NCDB) and United States Neuroendocrine Tumor Study Group (USNETSG), Black patients in particular have a much higher rate of LNM in tumors < 2cm compared to White patients (**Fig. 1**). Racial disparities have long been known to exist in PNET outcomes and therapy. Black patients are more likely to be diagnosed with advanced disease, receive surgery less often despite meeting criterion, and have worse overall survival when compared to White patients. [13-15] However, the high incidence of metastatic disease at small tumor sizes in Black patients prompts questions as to the etiology of this disparity.

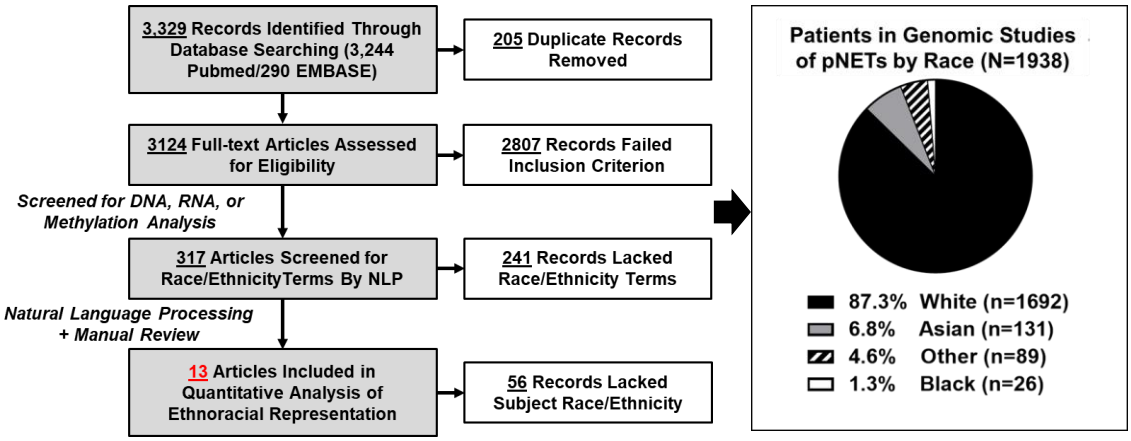
Figure 1. Black patients had higher rates of LNM in <2cm PNET tumors than White patients in the initial USNETSG dataset (23% vs 5%) and the validation NCDB dataset (21% vs 12). * p<0.01



Data have shown that a wide array of molecular features differ among racial groups in various cancers, some of which may be clinically actionable. Indeed, both intrinsic and extrinsic properties of cancer cells, including the genome, transcriptome, epigenome, and the tumor immune microenvironment have been demonstrated to differ

among racial groups. [16-21] However, identifying potentially actionable molecular features that are enriched in certain racial populations relies upon the inclusion of diverse racial groups in analyses that seek to study these molecular features. Previous studies have shown that the representation of racial groups in genomic studies of gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) is highly racially homogenous, with White patients making up an overwhelming majority of those represented in the literature.[22] A similar trend emerges when these data are subset for pNETs specifically, with White patients comprising 87% of patients in such studies while Black patients make up only 1.3% (**Fig. 2**). Furthermore, while this disparity specifically pertains to studies that conducted DNA, RNA, or DNA methylation sequencing analyses, it is not presumptuous to assert that previous studies to identify and characterize other clinically relevant PNET biomarkers (i.e immunohistochemical) were similarly diverse.

Figure 2. Representation of Racial Groups in Genomic Studies of PNETs. Adapted with permission from Cancer Research Communications.



Relative to other tumors, PNETs have a low mutational burden and are rather characterized by high levels of epigenetic dysregulation. Highlighting this is the fact that the three most frequently mutated genes in PNETs- *MEN1*, *DAXX*, and *ATRX*- are all key epigenetic regulatory genes.[23-28] Interestingly, epigenetic features are known to vary

widely between different racial groups in healthy states. Many of these variable features specifically overlap regions associated with various cancers, including those of the pancreas.[29,30] A recent study from our group also found variation in the mutation of multiple epigenetic regulatory genes between Black and White patients, which included *MEN1, KMT2D, SMARCB1,* and *EP300*. Notably, the lower rate of *MEN1* mutations in Black patients was also confirmed in an external cohort. This emphasizes not only differences in mutational dynamics among racial groups, but indicates resultant second-order epigenetic differences through the vast regulatory networks of these genes.

Altogether, there is a robust difference in metastatic propensity among Black and White PNET patients, possibly representing a difference in tumor biology that portends worse clinical outcomes for Black patients. There is little available to evaluate this hypothesis, as Black patients are underrepresented in PNET molecular analyses. However, this notion is substantiated by known genomic, epigenomic, and transcriptomic variation among racial groups in cancer, and has been further affirmed by findings at the genomic level in PNETs specifically. Therefore, the present study aims to evaluate additional molecular features including the transcriptome, tumor microenvironment, and previously established biomarkers for their potential influence on clinical outcomes and for their utility as biomarkers in the Black patient population.

Methods

Patient Selection and Clinical Data Collection

All studies of patient-derived tissues were performed in accordance with a protocol approved by the University of Alabama at Birmingham (UAB) Institutional Review Board (IRB-300006067). Patients with self-reported race (SRR) of Black or White were selected via chart review of pancreatic resections at the and/or query of UAB's Institutional Tissue Biorepository (TBR). To minimize noise due to confounding factors, patients were selected in matching pairs based on tumor grade, sex, and age in order of priority. Given the possibility for extensive RNA degradation despite proper block storage and nucleotide recovery methods, only cases occurring after January of 2010 were selected. After confirmation of the diagnosis with a board-certified pathologist, chart review was conducted to generate a database of patient clinical factors, including patient demographic characteristics, tumor characteristics, surgical and surgical pathology data, comorbidities, substance use habits, progression/recurrence status, survival status, and treatment information. Patients without follow-up in the past 12 months were surveyed by UAB Cancer Registry personnel to update clinical information.

Laser Microdissection

H&E sections were generated from resected pNET FFPE blocks and evaluated by a board-certified pathologist with neuroendocrine expertise to demarcate nests of tumor cells for laser microdissection. Immunostains for Chromogranin A (CgA) were likewise used to facilitate identification of tumor nests. Following strict decontamination to prevent RNAse exposure, 10uM sections were cut and mounted onto RNAse-free 2 uM

PEN membrane slides (Leica). Sections were dried overnight at 4C with desiccant. Following deparaffinization, sections were rehydrated in sequential dilutions of molecular-grade EtOH in RNAse-free water to 70% EtOH and stained with a 2% cresyl violet solution in 100% EtOH. Slides were then dehydrated in increasing concentrations EtOH to 100% and placed on ice until microdissected. Using the previously identified tumor nests from H&E and CgA staining as reference, microdissection was performed with a Leica LMD 6 using a laser at X power with an aperture of X to yield sufficient tissues for DNA & RNA isolation.

RNA Isolation and Sequencing

RNA were isolated using the Allprep DNA/RNA FFPE kit (Qiagen) according to manufacturer's specifications. Library preparation and sequencing were then performed by the UAB Heflin Genomics Core. Samples were screened for quality via Bioanalyzer (Agilent) and those with a RIN value < 3 were excluded. RNA-Sequencing (RNA-Seq) was performed using the SEQuoia Complete Stranded kit (Bio-Rad) with ribo-reduction. Sequencing was performed on an Illumina NextSeq550 at 30 million reads/sample for RNA-seq.

Bioinformatic Analyses

RNA-sequencing. Bioinformatic analyses were performed on an institutional Linux-based computing cluster. Illumina universal adaptor sequences and poly-(A) tails were trimmed from RNA-seq reads using cutadapt [31]. Reads were discarded if they were below 15 bases in length or had a quality score < 28. Reads were then mapped to a full-

genome decoy-aware GRCh38 transcriptome using Salmon. Differentially expressed genes (DEG's) were then determined using DESeq2. Covariates used in the design matrix included patient sex, tumor grade, sequencing batch (n=4), race (either self-reported or genetic ancestry, where denoted), and the age of the tumor block from which DNA/RNA was isolated. Numerical covariates were centered and scaled. Likelihood ratio tests as employed in *DESeq2* were used to evaluate covariates in a stepwise manner to confirm their contribution to differential gene expression by racial group. Differential gene expression was determined by false discovery rate (FDR) adjusted p-value (q-value; qv) < 0.05 and $\log_2$ fold-change ($\log_2$FC) ≥ 1. Significant DEG's were then funneled into gene set enrichment analysis (GSEA), which was performed using clusterProfiler and the Gene Ontology (GO) consortium gene sets. Enriched Gene Sets were likewise determined by q-value.

Ancestry estimation. Genetic ancestry was determined using *Admixture* (version 1.3.0), which provides a maximum likelihood estimation of individual ancestries from multi-locus SNVs. Prior to admixture analysis, GATK best practices were used to identify SNVs from the RNAseq reads. Specifically, we aligned our RNAseq reads to hg19 using STAR (version 2.5.2b). Variants were called using GATK HaplotypeCaller (version 3.8) and subsequently filtered to exclude rare variants (i.e., <5% across all phase 3,1000 genomes), all INDELs, and any SNPs that were not biallelic. Ancestral reference populations were based on the 1000 Genomes Project phase 3 superpopulations. [32]

Survival analysis. Rlog-normalized transcript counts for each patient were

generated using *DESeq2*. Gene expression was scaled into Z scores and Cox proportional hazards model generated for each gene using the *RegParallel* R package. *CutoffFinder* was then used to establish expression cutpoints for optimized prediction of PFS and Kaplan-Meier plots analysis conducted. Clinical endpoints used were progression, progression-free survival, recurrence, and the presence of either distant metastatic disease or LNM at the time of resection. Due to the relatively small number of events in this cohort, composite metrics of these outcomes are used where denoted in the *Results*.

Multiplexed Immunofluorescence

Slides containing 5-µm of FFPE tissue sections were generated and stored at 4°C until use. Prior to staining, slides were baked for 30 min to 1 h at 55 °C. According to Akoya Biosciences protocol, FFPE tissues were dewaxed, deparaffinized in xylene then rehydrated in descending ethanol concentrations (100% twice, 90%, 70%, 50%, and 30%, respectively) and washed in ddH_2O twice, each step for 5 min. Heat-induced epitope retrieval with antigen retrieval solution, pH 6, was performed using the pressure cooker at high-pressure protocol (80 °C) for 20 min. After cooling at room temperature (RT) for 30 min to 1 h, the coverslips were washed in ddH2O twice for 2 min. Then the sample coverslips were immersed in the hydration buffer six times before being placed in the staining buffer for 20–30 min. The antibody cocktail solution was prepared to contain 1–2 µL: 200 of the antibody/sample and then added to CODEX blocking buffer (staining buffer, N blocker, G blocker, J blocker, and S blocker) to block nonspecific binding of the antibody. For each coverslip, 190 µL of the antibody cocktail solution was added and incubated in a sealed humidity chamber for 3 h at RT or overnight at 4 °C. After staining,

sample coverslips were placed in the staining buffer twice for 2 min to rinse any unbound antibodies and then fixed in 1.6% paraformaldehyde diluted in the storage buffer (post-staining fixing solution) for 10 min, followed by a total of 9 quick washes in 1x PBS. After washing, the sample coverslips were incubated in 100% cold methanol for 5 min, followed by a total of nine dunks in 1x PBS. A fresh final fixative solution was prepared by diluting 20 µL of the CODEX fixative reagent in 1 mL of 1x PBS. The final fixative solution (190 µL) was added to the sample and incubated in a sealed humidity chamber at RT for 20 min, followed by nine quick washes in 1x PBS to remove the fixative reagent. Thereafter, sample coverslips were placed in a storage buffer at 4 ℃ for up to two weeks or further processed for imaging. At imaging time, the reporters' plate was prepared for the corresponding antibodies (one well/cycle), maintaining one dye type per cycle. The reporter stock solution was prepared for the total number of cycles. Each reporter was added (5 µL) to the corresponding cycle to create a reporter master mix per cycle, then gently mixed by pipetting before 245 µL of the mix was added into the corresponding well on the 96-well plate.

Multiplexed Immunofluorescence Analysis

Images were collected using a KEYENCE BZ-X800 fluorescent microscope config-ured with 3 fluorescent channels (TxRed, Cy7, Cy5) and DAPI with 20×. Whole-slide scanning was conducted with a 20× oil immersion objective in a 5 × 5 tiled acquisition at 9 z-planes per tile. Images were subjected to deconvolution to remove out-of-focus light. Then the raw experiment data were transferred using CODEX Instrument Management version (CIM v1.29) software and processed using CODEX Processor version 1.7. High-resolution whole-slide images of TMAs were scanned and uploaded

into the *Qupath* image analysis software (cite; version). Cell detection was performed using DAPI as input for the native cell detection function in Qupath.[33] Training images were then generated for each antibody, and thresholds for antibody positivity established. Random-forest classifiers for cell populations were then trained using approximately 30% of the cells for a given cell population per core, including tumor cells, macrophages, and CD4+/CD8+ T-cells. Stained normal tissue cores from various organs (spleen, liver, lymph node, tonsil, placenta, skin, skeletal muscle, cardiac muscle, and normal pancreas) were used as positive or negative references where appropriate. Once cell populations were classified, density maps were generated to establish tumor bed boundaries. Distance metrics for individual cells relative to tumor bed boundaries were then generated to identify cell populations infiltrating into tumor beds.

Statistical Analysis

Differences in patient and tumor characteristics were evaluated using either Fisher's exact test for categorical/ordinal characteristics, or t-test for numerical characteristics. Numerical characteristics were evaluated for normality and homoscedasticity using the Shapiro-Wilk and Levene's tests, respectively. The relationship between African ancestry estimates and rlog-normalized transcript values from RNA-seq were evaluated using Pearson's correlations and logistic regression implemented with *RegParallel*. Linear regression was used to evaluate the relationship between infiltrating immune cell populations into tumor cores with the number of tumor cells within tumor beds as a covariate within the model. The methods used to evaluate differences in the representation of racial groups in PNET genomic studies are described

elsewhere. Statistical analyses were performed in R version 4.02 and GraphPad Prism version 8.

Data Availability Statement

The data generated in this study are available within the article or upon reasonable request to the corresponding author.

Results

Patient Demographics and Characteristics

While 23 tumor specimens each from Black and White patients were selected, matched, and sequenced, two Black patients and five White patients were excluded from RNA-sequencing downstream analyses due to poor quality of their respective sequencing libraries. Hence, 21 Black and 18 White patients were included in RNA sequencing analyses. Demographics and characteristics of these 39 patients are reported in **Table 1**. Neither mean age nor BMI were significantly different among the Black and White patient cohorts (55.1 ± 8.4 and 58.8 ± 8.5, p= 0.18; 29.9 ± 9.3 and 34.5 ± 11.1, p=0.17). Both Black and White patient cohorts were predominately Female (61.9% and 55.6%, p=0.75). Due to the uneven dropout of White patients from sequencing analyses, the Black patient cohort had more grade 1 tumors than the White patient cohort (61.9% and 44.4%, p=0.34), although this difference was not statistically significant. There was no significant difference in tumor size between the Black and White patient cohorts (3.8 ± 2.5cm and 4.5 ± 3.2cm, p=0.47). Likewise, there was no difference in the presence of lymph node metastases (23.8% and 16.7%; p=0.7) or distant metastases (14.3% and 16.7%, p=1) at the time of resection in the Black and White patient cohorts, respectively. Diabetes and pancreatitis are known risk factors for the development of PNETs, although the influence that these conditions have on the specific phenotype of the disease is yet uncertain. Hence, the incidence of these comorbidities was also compared between cohorts. An established history of diabetes before PNET diagnosis and resection was present in 38.1% of Black and 44.4% of White patients (p=0.75). A history of chronic pancreatitis was present in one Black and two White patients, among whom one White

patient also had a history of acute pancreatitis (p=0.59). Smoking status was also evaluated among cohorts due to its potential to influence DNA methylation, as well as the increased risk of pancreatitis associated with smoking. [34,35] Most Black and White patients were never smokers (57.1% and 77.8%, p=0.48), and few patients were current smokers at the time of resection (14.3% and 5.5%). With respect to treatment, one Black patient received preoperative somatostatin analogue while one White patient received neoadjuvant chemotherapy for isolated metastatic disease to the liver (6 cycles of cisplatin + etoposide). Three Black and White patients each experienced progression of their cancer following resection (p=1). Progression-free survival did not differ significantly between the Black and White patient cohorts (median= 41.7 and 45.5 months; p=0.48). Three Black patients and One White patient were deceased at the time of sequencing- of which all but one Black patient died of cancer-related complications (p=0.61).

Table 1) Patient Demographics and Characteristics

	Black (n=21)	**White** (n=18)	**Total** (n=39)	**P**
Patient Characteristics				
Age (Mean +/- SD*)	55.1 ± 8.4	58.8 ± 8.5	56.8 ± 8.5	0.18
Sex (Female)	13 (61.9%)	10 (55.6%)	23 (59%)	0.75
BMI	29.9 ± 9.3	34.5 ± 11.1	32 ± 10.3	0.17
Tumor Characteristics				
Tumor Grade				0.34
G1	13 (61.9%)	8 (44.4%)	21 (53.8%)	
G2	8 (38.1%)	10 (55.6%)	18 (46.2%)	
Tumor Size (cm)	3.8 ± 2.5	4.5 ± 3.2	4.1 ± 2.9	0.47
Lymph Node Metastases	5 (23.8%)	3 (16.7%)	8 (20.5%)	0.7
Distant Metastases	3 (14.3%)	3 (16.7%)	6 (15.4%)	1
Comorbidities				
History of Diabetes	8 (38.1%)	8 (44.4%)	16 (41%)	0.75
History of Pancreatitis**	1 (4.8%)	2 (11.1%)	3 (7.7%)	0.59
Acute	0 (0%)	1 (5.6%)	1 (2.6%)	
Chronic	1 (4.8%)	2 (11.1%)	3 (7.7%)	
Habits				
Smoking Status				0.48
Never	12 (57.1%)	14 (77.8%)	26 (66.7%)	
Current/Former	9 (42.9%)	4 (22.2%)	13 (33.3%)	
Alcohol Use				0.52
Yes	11 (52.4%)	7 (38.9%)	18 (46.1%)	
No	10 (47.6%)	11 (61.1%)	21 (53.9%)	
*Outcome Status****				
Progression	3 (14.3%)	3 (16.7%)	6 (15.4%)	1
PFS (months; median [IQR*])	41.7 [67.9]	45.5 [41]	45 [48.1]	0.48
Malignancy*****	7 (33.3%)	5 (27.8%)	12 (30.8)	0.77
Deceased (%)	3 (14.3%)	1 (5.6%)	4 (10.3%)	0.61

* SD=standard deviation, IQR= interquartile range

** One White patient had a history of both acute and chronic pancreatitis

***** 1 Black patient received preop SSA, 1 White patient received neoadjuvant chemotherapy**

****** Malignancy denotes a composite endpoint of metastases (distant or LNM) or progression**

Differential Gene Expression Among Racial Groups

Self-Reported Race. Following laser microdissection to isolate tumor cells, DNA and RNA were isolated and sequenced. When evaluating differential gene expression based on SRR, 414 genes (of 26,162 tested) met cutoffs for differential gene expression (qv < 0.05 & absolute L2FC > 1; **Fig 3**, **Fig 4**). Significant DEGs were then funneled into GSEA, yielding 8 significantly enriched gene sets from the Gene Ontology's Biological Processes (**Fig. 5**). Downregulated in the Black patient cohort were gene sets relating to

neurogenesis, cell development, and nervous system development. Upregulated in the Black patient cohort were gene sets relating to the humoral immune response, antimicrobial humoral response, proteolysis, and epithelial cell differentiation. Further analysis of these gene sets revealed 79 leading edge genes most responsible for their enrichment (**Table 2**). Interestingly, leading edge genes in the upregulation of gene sets relating to the humoral immune response were comprised predominantly of proteolytic enzymes and other key pancreatic proteins expressed in states of pancreatic inflammation. Many of these genes have also been linked to increased severity of disease or poor clinical outcomes in pancreatic adenocarcinoma and other cancers.

Table 2) Leading Edge Genes in GSEA, Ranked by Number of Appearances in Enriched Gene Sets

HGNC Symbol	L2FC ± SE*	Gene Description	Gene Set Clusters**
CRYGD	21.4 ± 4.2	crystallin gamma D	ECD
HOXB13	14.1 ± 4.2	homeobox B13	ECD
PGC	15.6 ± 2.1	progastricsin	HIR, P
REG3G	12.5 ± 2.2	regenerating family member 3 gamma	HIR, ECD
BPIFB2	11.1 ± 2.7	BPI fold containing family B member 2	HIR
REG3A	10.7 ± 2.6	regenerating family member 3 alpha	HIR, ECD
GSTA2	9.3 ± 1.9	glutathione S-transferase alpha 2	ECD
REG1B	8 ± 2.3	regenerating family member 1 beta	HIR
CTRB1	8.4 ± 1.4	chymotrypsinogen B1	P
CELA3B	7.9 ± 1.5	chymotrypsin like elastase 3B	P
PLA2G1B	7.8 ± 1.5	phospholipase A2 group IB	HIR
CPA1	7.4 ± 1.7	carboxypeptidase A1	P
EMX1	7.3 ± 1.7	empty spiracles homeobox 1	ECD
CPA2	7.1 ± 1.9	carboxypeptidase A2	P
PRSS2	7.1 ± 1.8	serine protease 2	HIR, P
CELA3A	7.1 ± 1.8	chymotrypsin like elastase 3A	P
PRSS1	7 ± 1.6	serine protease 1	P
KLK1	6.7 ± 1.7	kallikrein 1	P
CELA2B	6.4 ± 1.9	chymotrypsin like elastase 2B	P
CTRB2	6.5 ± 1.7	chymotrypsinogen B2	P
AGR2	6.2 ± 1.5	anterior gradient 2, protein disulphide isomerase family member	ECD
GSTA1	6 ± 1.6	glutathione S-transferase alpha 1	ECD
FOXA1	6.2 ± 1.3	forkhead box A1	ECD
SIX2	6.1 ± 1.3	SIX homeobox 2	ECD
CELA2A	6.1 ± 1.2	chymotrypsin like elastase 2A	P, ECD
SERPINA5	5.4 ± 1.6	serpin family A member 5	P
CAPN13	5.1 ± 1.2	calpain 13	P
CGA	4.9 ± 1.3	glycoprotein hormones, alpha polypeptide	P

PRSS3	4.6 ± 1.4	serine protease 3	HIR, P
IGKV3-20	4.6 ± 1.4	immunoglobulin kappa variable 3-20	HIR
CUZD1	4.6 ± 1.4	CUB and zona pellucida like domains 1	P
PAX5	4.8 ± 1.1	paired box 5	HIR
IHH	4.6 ± 1.2	Indian hedgehog signaling molecule	P, ECD
PERCC1	4.2 ± 1	proline and glutamate rich with coiled coil 1	ECD
IGHG1	4 ± 1	immunoglobulin heavy constant gamma 1 (G1m marker)	HIR
IGLC2	3.9 ± 0.9	immunoglobulin lambda constant 2	HIR
CTRC	3.8 ± 0.9	chymotrypsin C	P
CPB1	3.7 ± 0.9	carboxypeptidase B1	P
SLPI	3.5 ± 1	secretory leukocyte peptidase inhibitor	HIR, P
EGF	3.5 ± 1	epidermal growth factor	P
ADGB	3.5 ± 0.9	androglobin	P
VTN	3.2 ± 0.9	vitronectin	HIR, P
IGLL5	3.1 ± 0.9	immunoglobulin lambda like polypeptide 5	HIR
EGLN3	3 ± 0.8	egl-9 family hypoxia inducible factor 3	P
ADRA2A	3 ± 0.7	adrenoceptor alpha 2A	P
NKD2	2.9 ± 0.8	NKD inhibitor of WNT signaling pathway 2	P
S100B	-2 ± 0.5	S100 calcium binding protein B	NCD
VEGFC	-2 ± 0.5	vascular endothelial growth factor C	NCD
DLX6	-2.1 ± 0.6	distal-less homeobox 6	NCD
FGF9	-2.2 ± 0.7	fibroblast growth factor 9	NCD
PGAP1	-2 ± 0.5	post-GPI attachment to proteins inositol deacylase 1	NCD
ASTN1	-2.3 ± 0.7	astrotactin 1	NCD
PPP1R16B	-2.3 ± 0.7	protein phosphatase 1 regulatory subunit 16B	NCD
CHD5	-2.4 ± 0.7	chromodomain helicase DNA binding protein 5	NCD
DCC	-2.4 ± 0.6	DCC netrin 1 receptor	NCD
HOXD3	-2.6 ± 0.8	homeobox D3	NCD
UNC5C	-2.7 ± 0.8	unc-5 netrin receptor C	NCD
CNGB1	-2.7 ± 0.7	cyclic nucleotide gated channel subunit beta 1	NCD
PRTG	-2.8 ± 0.8	protogenin	NCD
TRPC5	-2.9 ± 0.8	transient receptor potential cation channel subfamily C member 5	NCD
CNTNAP2	-3.3 ± 0.7	contactin associated protein 2	NCD
RNF112	-3.7 ± 1	ring finger protein 112	NCD
FEZF2	-3.6 ± 0.8	FEZ family zinc finger 2	NCD
NCAM2	-3.8 ± 0.9	neural cell adhesion molecule 2	NCD
GRIP1	-3.8 ± 0.8	glutamate receptor interacting protein 1	NCD
FAIM2	-4.2 ± 1.1	Fas apoptotic inhibitory molecule 2	NCD
GABRB1	-4.9 ± 1.5	gamma-aminobutyric acid type A receptor subunit beta1	NCD
TDRD5	-4.6 ± 1.2	tudor domain containing 5	NCD
C14orf39	-5.1 ± 1.5	chromosome 14 open reading frame 39	NCD
PENK	-5.6 ± 1.6	proenkephalin	NCD
PITX1	-5.4 ± 1.3	paired like homeodomain 1	NCD
ZNF804A	-5.7 ± 1.5	zinc finger protein 804A	NCD
GRIK1	-5.7 ± 1.5	glutamate ionotropic receptor kainate type subunit 1	NCD
HCN1	-5.8 ± 1.3	hyperpolarization activated cyclic nucleotide gated K channel 1	NCD
TAFA3	-11.4 ± 2.4	TAFA chemokine like family member 3	NCD
NEUROD4	-14.3 ± 4.1	neuronal differentiation 4	NCD
TLX2	-13.4 ± 2.9	T cell leukemia homeobox 2	NCD

DLX2	-25.8 ± 4.2	distal-less homeobox 2	NCD
CHRM1	-28.2 ± 3.7	cholinergic receptor muscarinic 1	NCD

*Direction refers to the expression level of the gene in Black patients compared to White
**NCD = Neurogenesis & Cell Development, ECD = Epithelial Cell Differentiation, HIR = Humoral Immune Response, P = Proteolysis

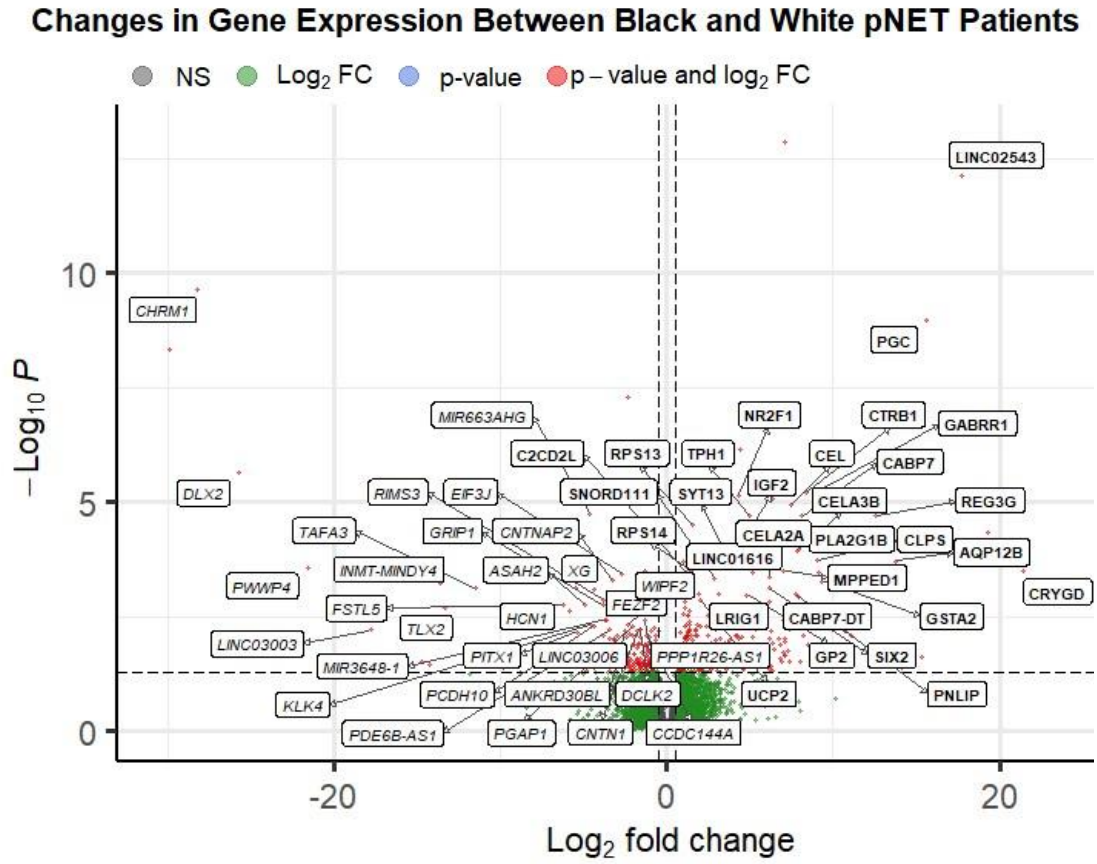


Figure 3. Differentially Expressed Genes Between Black and White PNET Patients. 414 genes met cutoffs for differential gene expression (qv < 0.05 & absolute L2FC > 1). Genes with the top 30 L2FC values in each direction are labeled. Genes with italicized labels have lower expression in Black PNET patients, while those with bold labels have higher expression.

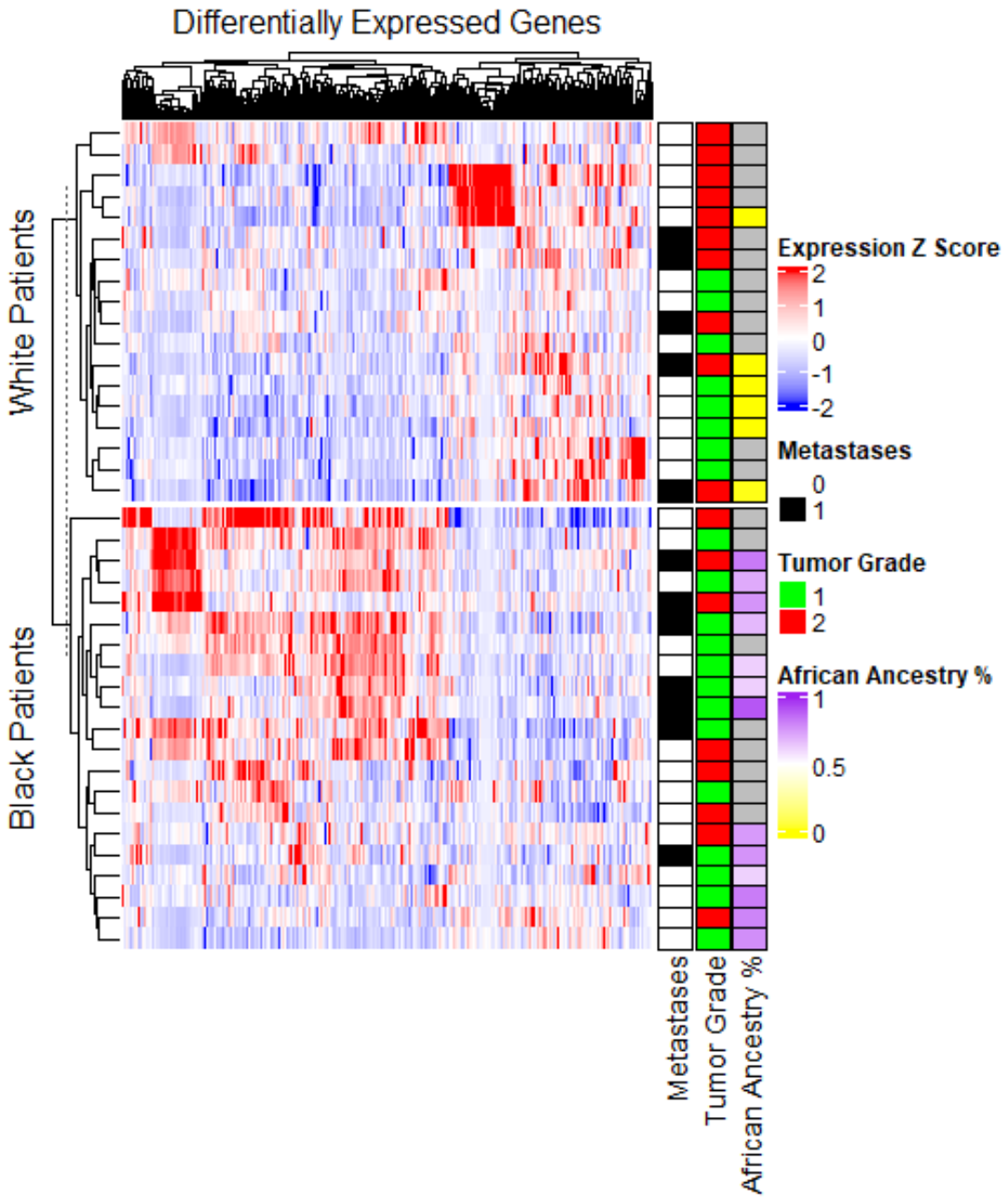


Figure 4. Heatmap of Differentially Expressed Genes Between Black and White PNET Patients. Z scores of gene expression are depicted and patients clustered into groups based on similar gene expression profiles, illustrating the high level of variation between racial groups. The presence of malignancy, tumor grade, and levels of African ancestry estimates (colored grey, where unavailable) are depicted alongside gene expression (right).

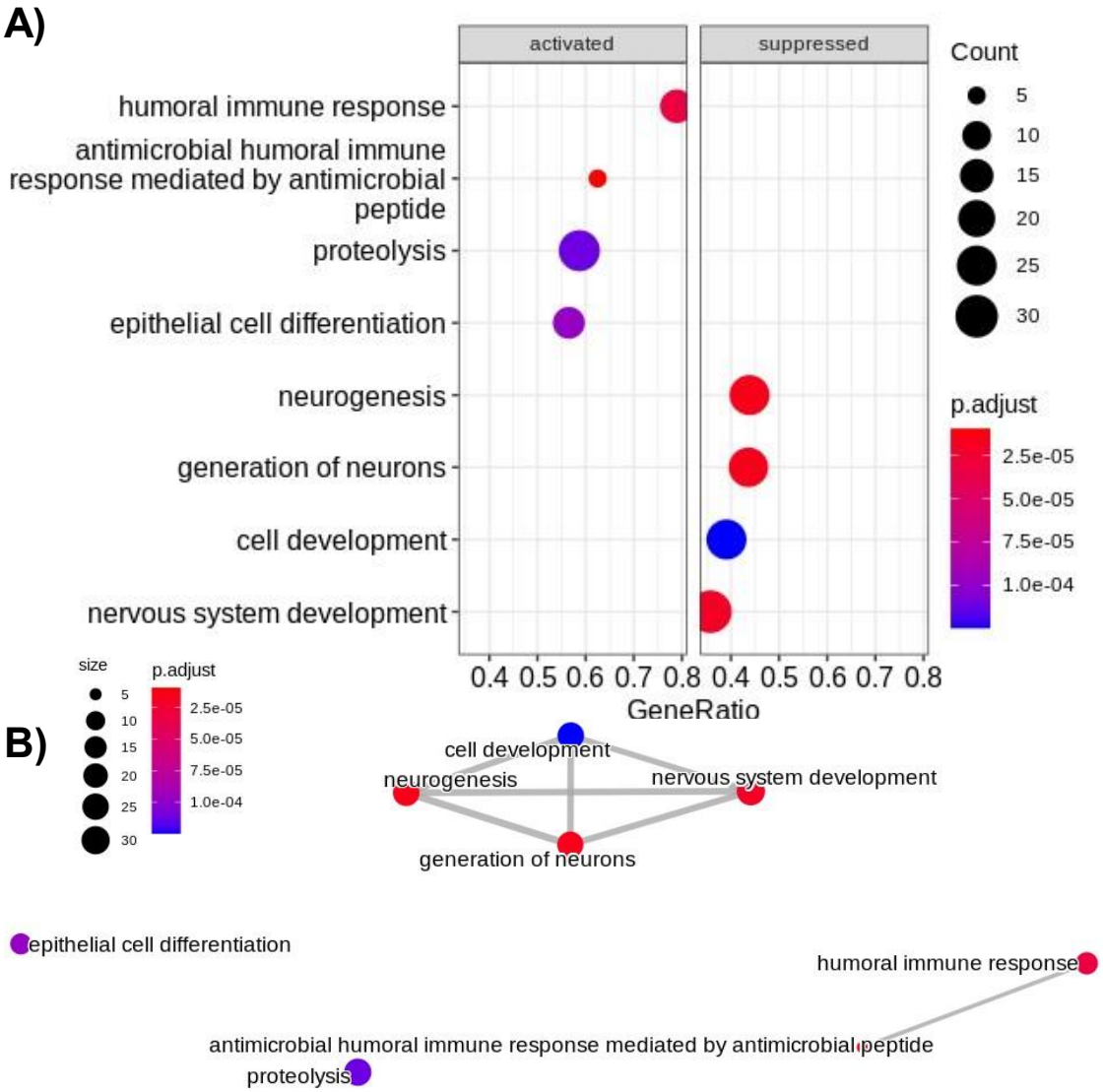


Figure 5) Gene Set Enrichment Analysis of Differentially Expressed Genes. **A)** Dotplot depicting significantly enriched gene sets that are functionally activated or suppressed in the Black patient cohort compared to the White patient cohort. **B)** Network plot of enriched gene sets. Functionally, gene sets segregated into 4 general network categories based on shared genes and their expression levels: neurogenesis & cell development, epithelial cell differentiation, the humoral immune response, and proteolysis.

Genetic Ancestry. Ancestry estimates were successfully generated for 6 White and 13 Black patients. Ancestry data were incomplete for the remaining 20 patients at the time of submission. The mean African ancestry estimates were 74.5% ± 9.1% and 0.17% ± 0.4% for the Black and White patient cohorts, respectively. Upon differential gene expression analysis based on estimates of African ancestry, 108 genes were found to meet the above-described cutoffs for differential gene expression (**Fig. 6**; **Supplementary Data 3**). Notably, 23 of these genes were among those found to be differentially expressed based upon SRR in the analysis of the whole cohort. Among these genes, eight were within the leading edge of the GSEA, including *CPA2*, *CTRB1*, *CTRC*, *EGF*, *IFLC2*, *PERCC1, REG3A*, and *REG3G*. Upon univariate correlation between rlog-normalized expression values and African ancestry estimates, 2100 genes met criterion for significance ($qv < 0.05$ & absolute r value > 0.3). Multivariable linear regression was then used to further evaluate the relationship between gene expression and African ancestry. Tumor grade, patient sex, sample age, and sequencing batch were included in the models, as likelihood ratio tests indicated a significant relationship between these variables and gene expression. 2360 genes were found to be significantly associated with African ancestry; of which 109 genes were among DEGs determined by SRR, and 35 were within the leading edge of the GSEA by SRR. 19 genes were significant across these three analyses (**Table 3**). Notably, 7/19 genes were within the leading edge of GSEA. *REG3A*, *REG3G*, *EGF*, *CPA2*, *IGLC2*, *CTRC*, and *CTRB1*.

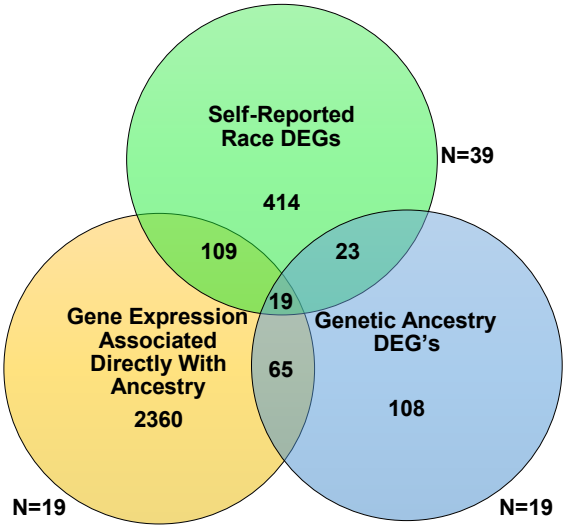


Figure 6. Genes Associated with Genetic Ancestry, and those Differentially Expressed by models based on SRR and Genetic Ancestry.

Table 3) Differentially Expressed Genes Associated with Genetic Ancestry				
HGNC Symbol	**Description**	**L2FC***	**L2FC SE***	**Q value**
REG3G	regenerating family member 3 gamma	12.52	2.21	< 0.0001
REG3A	regenerating family member 3 alpha	10.69	2.56	0.0064
PNLIPRP2	pancreatic lipase related protein 2	9.17	1.82	0.0003
	chymotrypsinogen B (CTRB) pseudogene	8.49	2.16	0.0128
CTRB1	chymotrypsinogen B1	8.4	1.41	< 0.0001
CEL	carboxyl ester lipase	7.49	1.29	< 0.0001
CPA2	carboxypeptidase A2	7.13	1.93	0.0219
AQP8	aquaporin 8	6.8	1.73	0.0128
SLC39A5	solute carrier family 39 member 5	5.37	1.27	0.006
GP2	glycoprotein 2	4.82	1.02	0.0011
IGLC2	immunoglobulin lambda constant 2	3.94	0.92	0.0048
CTRC	chymotrypsin C	3.82	0.87	0.0037
EGF	epidermal growth factor	3.49	0.97	0.0286
ENC1	ectodermal-neural cortex 1	2	0.52	0.0162
LRP5	LDL receptor related protein 5	1.71	0.47	0.0229
RPL35	ribosomal protein L35	1.26	0.31	0.0091
SRPRA	SRP receptor subunit alpha	0.86	0.25	0.0384
	novel piRNA host transcript	-2.31	0.62	0.0205
XG	Xg glycoprotein (Xg blood group)	-4.41	0.92	0.0008

* L2FC = $\log_2$ Fold Change, SE= standard error

Differentially Expressed Genes Among Racial Groups Influence Clinical Outcomes. Given our previous observations that there are differences in the rate of

metastatic disease between Black and White patients, the relationship between gene expression and various clinical outcomes was evaluated. Due to the relatively low number of events in our datasets, a composite metric termed here as "malignancy" (comprised of LNM at resection, metastasis at resection, and progression) was used as the outcome of interest. Logistic regression models were generated for genes previously found to be differentially expressed between racial groups. Six DEGs met cutoffs (Wald test p-value < 0.05 and 95% Confidence Intervals for Odds Ratios both > 1 or < 1) for significance, including *MEGF8*, *TDRD5*, *FSTL5*, *RN7SKP239*, *GSTM2*, and *FMN1*.. Similarly, Cox Proportional-Hazards (Cox-PH) models were used to evaluate the influence of gene expression on progression-free survival. Eight DEGs met criterion for significance, including *BPIFB2*, *VTN*, *CASZ1*, *REG3G*, *N4BP3*, *VPS51*, *REG1CP*, and *MTCO1P2*. In total, 14 differentially expressed genes had a robust relationship with better and worse outcomes, respectively (**Fig. 7**). Subsequently, expression levels of significant DEGs were evaluated to determine optimized cutoffs for Kaplan-Meier analysis, and patients binned into groups based on high or low expression. Expression cutoffs were significantly associated with PFS for VTN and MTCO1P2, N4BP3, VPS51, and CASZ1 (**Fig. 8**). High expression of MTCO1P2 and VTN were both independently predictive of decreased PFS, while N4BP3, VPS51, and CASZ1 expression predicted increased PFS. While expression cutoffs for REG1CP and BPIFB2 approached significance (p= 0.057 and 0.055), those for REG3G did not (p=0.26).

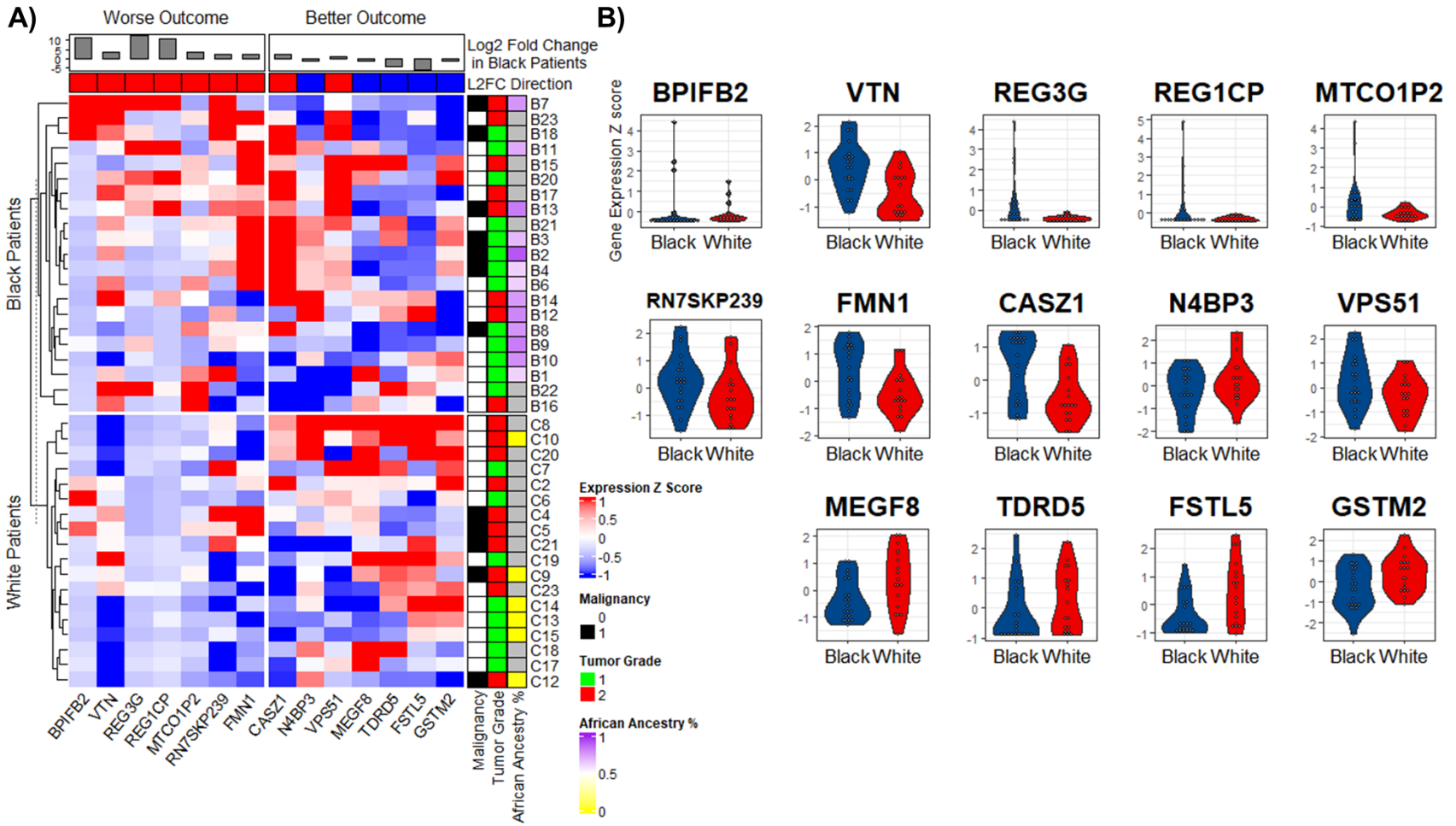


Figure 7) Differentially Expressed Genes Associated with Clinical Outcomes. **A)** Heatmap of differentially expressed genes associated with risk of malignancy and progression-free survival. Per-gene $\log_2$ fold-change values are depicted above the heatmap. **B)** Violin plots of gene expression Z scores by racial group, depicting the distribution of gene expression values with respect to the cohort mean. Two novel transcripts and a U2 spliceosomal RNA are not pictured.

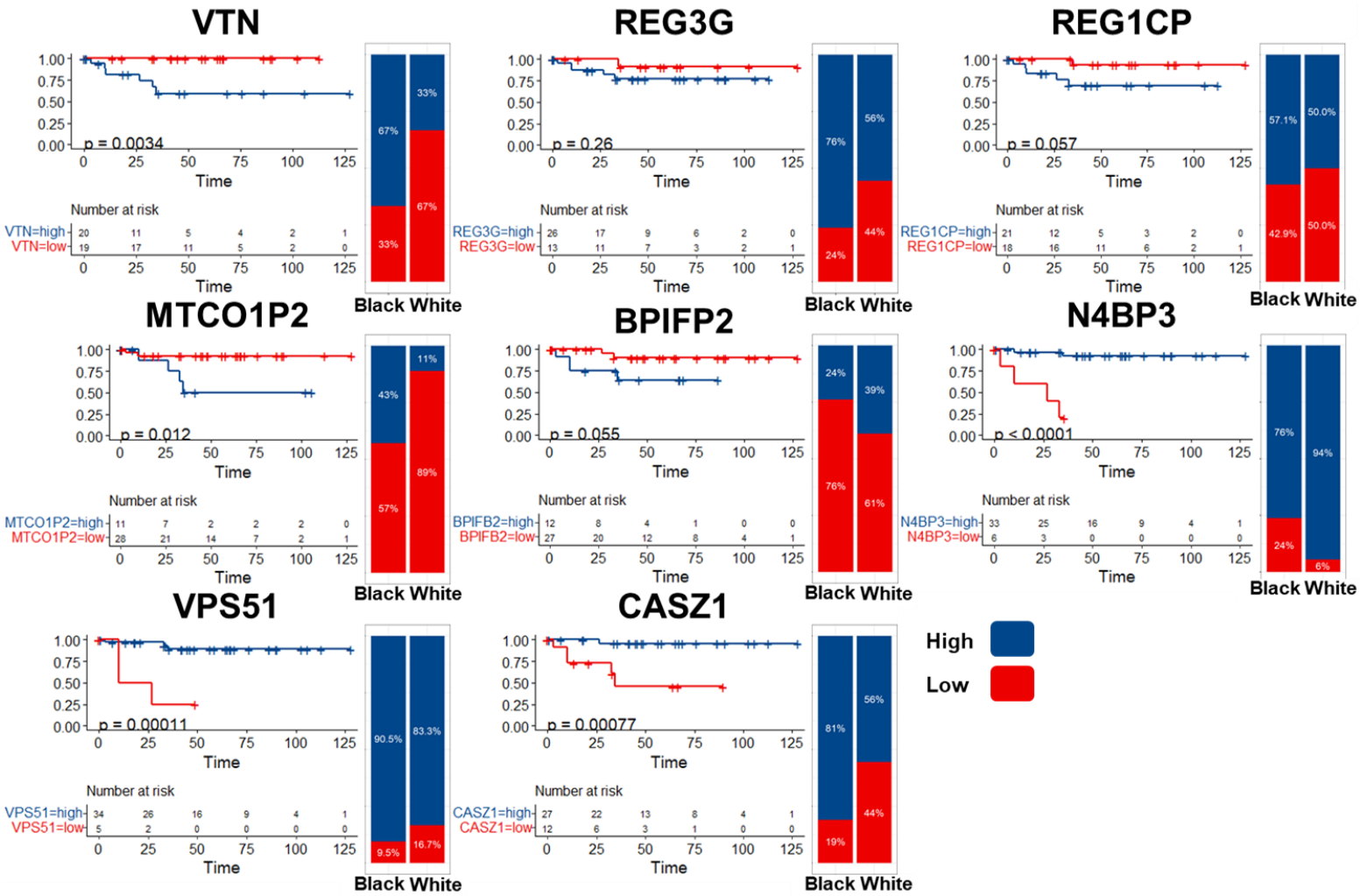


Figure 8) Kaplan-Meier plots of PFS for DEGs significant in Cox-PH models. Cutoffs were optimized for their relationship with PFS using CutoffFinder.

Differences in Protein Biomarker Expression Among Racial Groups

While the implementation of next-generation sequencing panels and polymerase chain reaction arrays for cancer care is increasing in tertiary care centers and steadily becoming more cost-efficient, widespread adoption of these assays in routine clinical practice faces numerous barriers[36,37] The clinical utility of NGS panels is not well known for many cancers, particularly in minority populations for which key allele frequencies and the pathogenicity of many variants are unknown due to their underrepresentation in genomic studies[38,39]. These groups also may not readily have access to tertiary care facilities, adequate insurance, or sufficient income to undergo this testing[40-43]. Contrarily, IHC evaluation is ubiquitous in clinical practice and can be interpreted by any board-certified pathologist. As such, numerous IHC biomarkers have been characterized to be predictive of clinical outcomes in pNETs[44,45]. Expression of SSTR2/5 is associated with better prognosis, while loss of p53/Rb and alpha-internexin are associated with worse prognosis [46-48]. Evidence is conflicting as to the impact of DAXX/ATRX mutation on pNETs, possibly owing to racial differences in their impact and influence on pNET progression as we hypothesize . Recent studies have also found that DAXX/ATRX alterations are correlated with increased infiltration of tumor associated macrophages (TAMs), which is itself associated with worse outcomes[49]. Other TIM markers have also been shown to have prognostic value in pNETs, including PD-L1 and tumor-infiltrating lymphocytes[50-52]. However, many of these biomarkers have never been evaluated among diverse racial groups in PNETs. To do so, a cohort of pre-existing TMAs, containing resected PNET cores in duplicate from 28 White and 8 Black patients were stained with a multiplexed panel of 21 antibodies to PNET identifying markers, immune cell markers,

and proteins identified in previous literature to influence PNET prognosis. pNET-identifying markers (chromogranin A, synaptophysin), prognostic pNETs biomarkers (PAM[53], SSTR2/5[47], the progesterone receptor,[54], alpha-internexin[48]), prognostic and pro/anti-tumor markers of the TIM (CD4, CD8, PD-1/PDL-1 & CD68)[55].

Differential Immune Cell Infiltration. Random-forest classifiers were then trained and implemented to identify PNET cells and immune cells, and density maps generated to identify cells infiltrating into tumor nests (**Fig. 9**). There was no significant difference in the number of total cells or tumor cells per core between the Black and White patient cohorts (56,110 and 44,200, p=0.33; 34,183, 27,504, p=0.47), although there was significant deviation within each group (SD =28,295 and 23,597). The mean tumor makeup of cores was 57.6% and 56.7% for the Black and White patient cohorts, respectively (**Fig. S1**). In order to evaluate the relationship between patient race and infiltrating T cells, infiltrating macrophages, and tumor cells thresholding positivity for PD-L1+, multivariable gaussian regression models were generated to predict these respective values. Proportions of these cells to the total number of tumor cells in the tumor beds (infiltrating immune cells) and entire cores (PD-L1+) are depicted in **Fig. 10A.** The number of total tumor cells within tumor beds was included as a covariate. Tumor grade was not a significant predictor in any model and resulted in worse fitment; therefore, grade was excluded from modeling. Overall models were significant for both T cell (adj. r^2=0.34, p = 0.0002) and macrophage (adj. r^2=0.35, p = 0.0003) infiltration, but not for PD-L1+ Tumor cells (adj. r^2=0.0006, p = 0.37). Interestingly, upon inclusion of total T cells and macrophages into the PD-L1 model, fitment was improved, but the model remained not significant (adj. r^2=0.11, p = 0.11). Expectedly, the total number of tumor cells was predictive of both T cell (B = 0.004, p <

0.0001) and macrophage (B =0.006, p < 0.0001) infiltration; however total tumor cells did not predict the number of PD-L1+ tumor cells (B=0.171, p = 0.37). Black Race was a significant negative predictor of CD8/CD4+ T cell infiltration (B = -1053, p = 0.007), but not macrophage infiltration (B = -103, p=0.6) or PD-L1+.

Differential Expression of Prognostic Protein Biomarkers. While the implementation of next-generation sequencing panels and polymerase chain reaction arrays for cancer care is increasing in tertiary care centers and steadily becoming more cost-efficient, widespread adoption of these assays in routine clinical practice faces numerous barriers [36,37] The clinical utility of NGS panels is not well known for many cancers, particularly in minority populations for which key allele frequencies and the pathogenicity of many variants are unknown due to their underrepresentation in genomic studies[38,39]. These groups also may not readily have access to tertiary care facilities, adequate insurance, or sufficient income to undergo this testing [40-43]. Contrarily, IHC evaluation is ubiquitous in clinical practice and can be interpreted by any board-certified pathologist. As such, numerous IHC biomarkers have been characterized to be predictive of clinical outcomes in pNETs[44,45]. Expression of SSTR2/5 is associated with better prognosis, while loss of p53/Rb and alpha-internexin are associated with worse prognosis [46-48]. Evidence is conflicting as to the impact of DAXX/ATRX mutation on pNETs, possibly owing to racial differences in their impact and influence on pNET progression as we hypothesize . Recent studies have also found that DAXX/ATRX alterations are correlated with increased infiltration of tumor associated macrophages (TAMs), which is

itself associated with worse outcomes [49]. Other tumor-immune microenvironment (TIM) markers have also been shown to have prognostic value in pNETs, including PD-L1 and tumor-infiltrating lymphocytes[50-52]. However, many of these biomarkers have never been evaluated among diverse racial groups in PNETs. Therefore, using a cohort of pre-existing TMAs, pNET-identifying markers (chromogranin A, synaptophysin), prognostic pNETs biomarkers (PAM[53], SSTR2/5[47], the progesterone receptor,[54], alpha-internexin[48]), prognostic and pro/anti-tumor markers of the TIM (CD4, CD8, PD-1/PDL-1 & CD68)[55]. Significant expression differences in tumor cells were present among all biomarkers evaluated, including podoplanin, peptidyl alpha-amidating monooxygenase (PAM), somatostatin receptors 2 & 5 (SSTR2 & SSTR5), alpha-internexin, PD-L1, and the progesterone receptor (PR). Notably, PAM, SSTR5, PR, PD-L1, and podoplanin were all lower in the Black patient group, while alpha-internexin and SSTR2 were higher in the Black patient group (**Table 4**, **Fig. 10B**). Alpha-internexin is an intermediate-filament protein expressed primarily in neurons that acts as a component of the axon's scaffold during early axonal development. Its expression is mostly absent in normal islets, but peaks in the early stages of PNET development and has been associated with worse overall survival in PNETs[48,56]. Notably, the difference in alpha-internexin expression was the least significant of all the biomarkers evaluated and likely represents no biologically relevant difference. In concordance, there was no significant difference in alpha-internexin expression in the sequencing cohort. However, this was not the case for the remaining biomarkers evaluated. Peptidylglycine α-amidating monooxygenase (PAM) is an enzyme expressed healthy neuroendocrine cells, functioning in the maturation of numerous secreted peptide hormones and chromogranin.[53] Its loss thus serves as a proxy

for early tumor de-differentiation, and has been associated with worse survival outcomes in NETs of multiple primary sites irrespective of tumor grade and stage. Notably, PAM expression was lower in the Black patient cohort than the White patient group. However, its expression was conversely significantly higher among Black patients in our sequencing cohort (L2FC = 1.37 ± 0.32, qv = 0.006). SSTR2 and SSTR5 are G-protein-coupled receptors (GPCRs) that have complex functions in regulating hormone secretion, metabolism, immune cell function, and gastric acid secretion in normal tissues, and are known to inhibit hormone secretion, proliferation, angiogenesis, and cell migration in PNETs[47,57]. They also serve as targets for tumor imaging and the delivery of radiotherapeutics. As such, they have been associated with broadly improved survival outcomes. SSTR2 was higher in the Black patient cohort than the White patient cohort, while SSTR5 was lower in Black patients. Neither of these genes differed significantly in the sequencing cohort, however. The progesterone receptor (PR) has likewise previously been associated with improved disease-free survival in PNETs. Its expression was lower in the Black patient cohort compared to the White patient cohort, but did not differ significantly in sequencing analyses. PD-L1 is a well-established immunosuppressive biomarker that has been shown to indicate higher tumor grade and worse survival in PNETs. Coinciding with our analysis of PD-L1+ tumor cells, PD-L1 expression levels were lower in Black patients than in White patients. This was the opposite in our sequencing analysis, but did not achieve statistical significance.

Table 4. Expression of Protein Biomarkers in PNET TMAs

Protein	White patients (n=28)	Black patients (n=8)	RNA-Seq Cohort L2FC ± SE	RNA-Seq Cohort QV
PAM**	9.41 ± 7.4	8.82 ± 9.7	1.376 ± 0.33	0.006
Podoplanin**	1.16 ± 1.3	0.45 ± 3.2	-0.99 ± 0.67	0.51
SSTR2**	39 ± 87.8	79.7 ± 45.5	-0.442 ± 0.43	0.68
SSTR5**	7.1 ± 4.9	5.04 ± 9.3	0.138 ± 0.92	0.96
Alpha-internexin*	0.51 ± 1.3	0.52 ± 1.2	0.551 ± 0.42	0.57
PR**	2.96 ± 1.4	1.91 ± 4	-0.394 ± 0.71	0.85
PD-L1**	2.23 ± 0.9	0.7 ± 2.9	-0.612 ± 0.54	0.6

Expression values are depicted as mean ± standard deviation
*p = 0.02, ** p < 0.00001

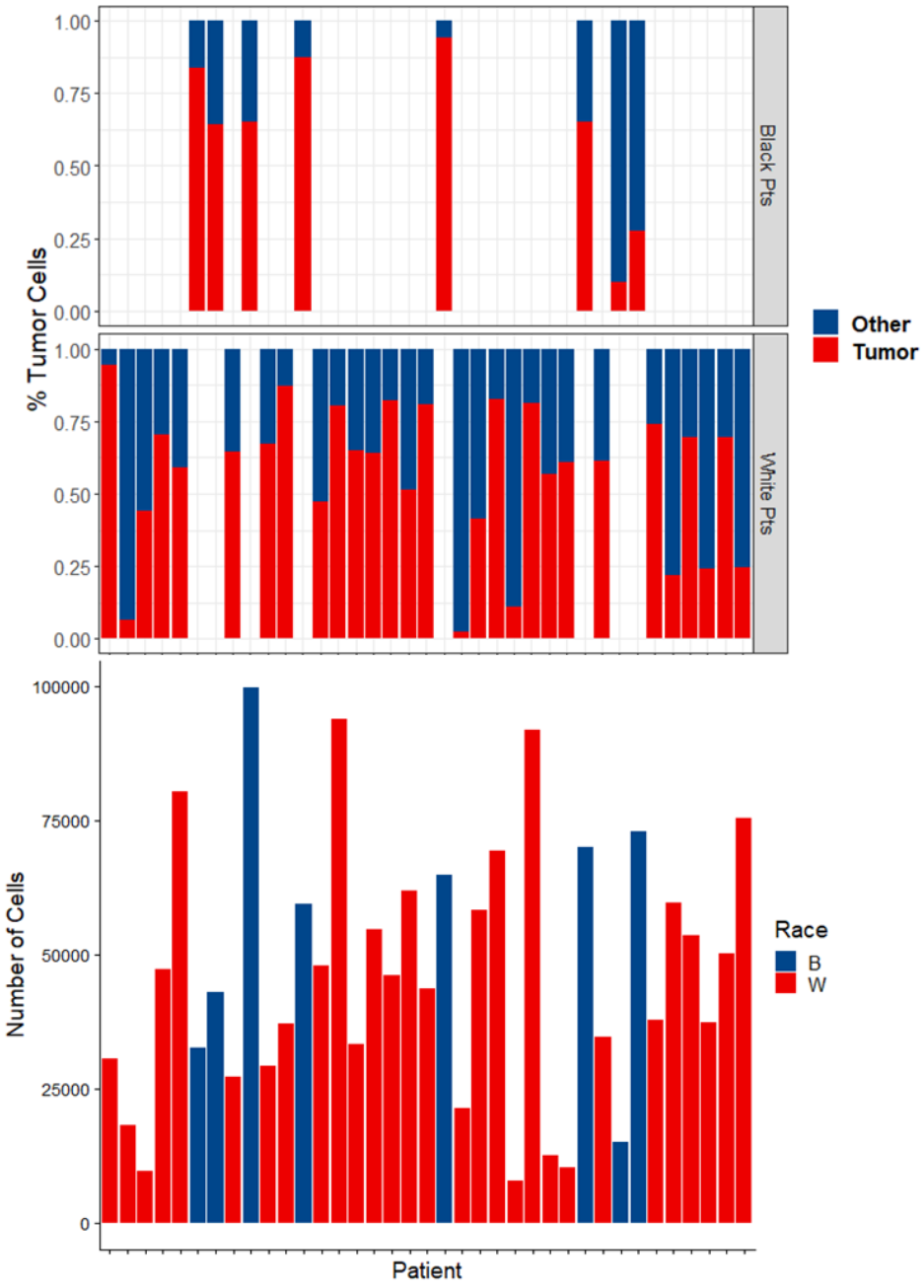


Figure S1) Makeup of TMA Cores by Racial Group. The proportion of tumor cells in each patient's tumor cores (top), and the total number of all cells detected in their respective cores (bottom).

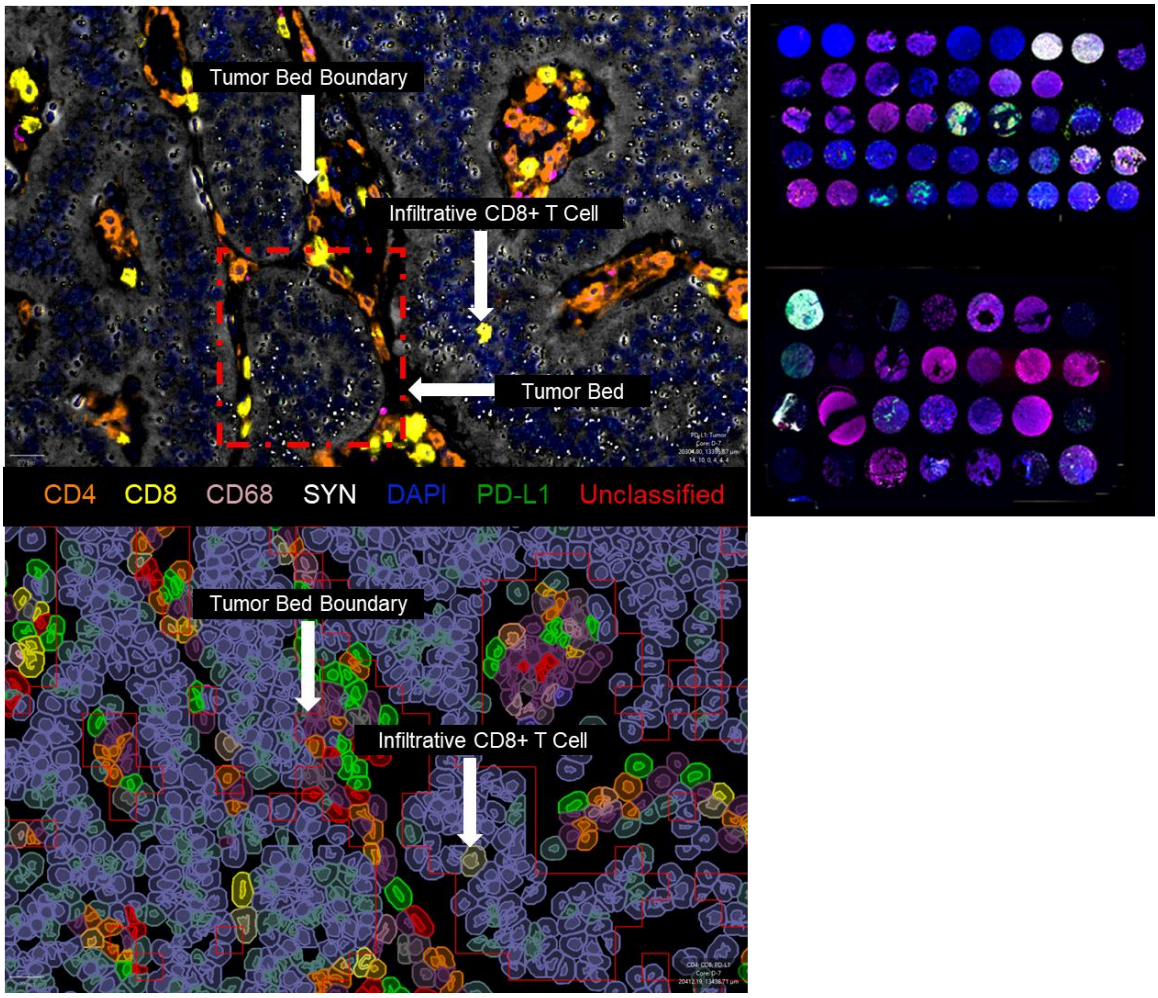


Figure 9) Multiplexed Immunofluorescence of PNET Tissue Microarrays. Microarrays containing resected G1 and G2 PNETs from 8 Black and 28 White patients were stained for PNET and immune cell identifying markers. Random forest classifiers were then trained to classify cells into populations of interest and density maps used to generate tumor bed boundaries, thereby identifying infiltrative immune cells.

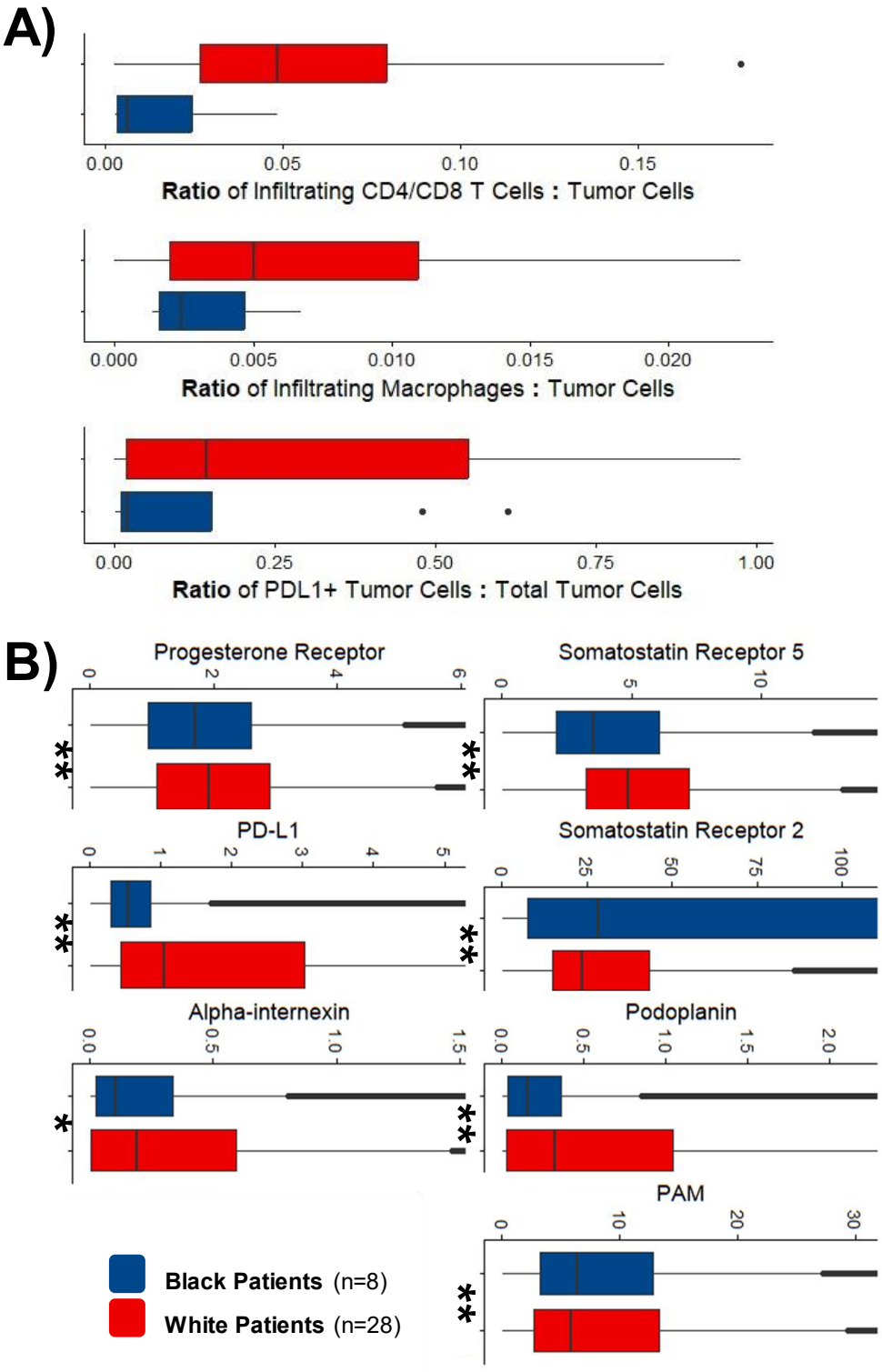


Figure 10) A. Ratio of Infiltrating Immune Cells and PD-L1 Positive Tumor Cells to Total Tumor Cells. B. Expression of protein biomarkers previously associated with outcomes in PNETs. *p = 0.02, ** p < 0.00001.

Discussion

In the present study, numerous genes were found to be differentially expressed between racial groups. These genes were enriched for those relating to the humoral immune response, proteolysis, neurogenesis and cell development, and epithelial cell differentiation, with 79 genes contributing most to their enrichment. Interestingly, most (n=33) core enrichment genes were related to neurogenesis and cell development, which is a pathway known to play a role in the biology of PNET progression. [58] This was followed by those in the proteolysis gene set (n=27), humoral immune response (n=19) and finally the epithelial cell differentiation gene set (n=13). Those enriched in the humoral immune response set, excepting 4 immunoglobulin genes (*IGKV3-20*, *IGHG1*, *IGLC2*, and *IGLL5*), overlapped with proteolysis (*PRSS2*, *PRSS3* , *SLPI*, *VTN*, *PGC*), or also have myriad other distinct functions that heavily implicate them in cancer biology (*PAX5*, *VTN*, *REG3A*, *REG3G*, *REG1B*). The multitude of DEGs for proteases and proteolytic enzymes upregulated in the Black patient cohort is particularly interesting given the lack of pancreatitis in the Black patient cohort. Many of these genes have previously been associated with pro-tumorigenic roles in pancreatic adenocarcinoma- which has a better-characterized relationship with pancreatitis- but have not traditionally been associated with PNETs [59].

Notably, a number of DEGs were found to independently predict clinical outcomes, specifically those tied to progression and metastatic disease. This relationship was present without respect to racial groups, indicating that the differential expression of genes may indeed be influential in driving racial disparities between Black and White patients with PNETs. *REG3G*, *BPIFB2*, *REG1CP*, *VTN*, *FMN1*, *RN7SKP239*, and *MTCO1P2* were all

associated with worse outcomes. The *REG3G* gene (previously pancreatitis-associated protein 1B [*PAP1B*]) encodes a member of the regenerating islet-derived protein family, which are secreted c-type lectins that promote islet growth in response to inflammation or injury. *REG3G* has been shown to promote pancreatic carcinogenesis and suppress the immune response by inducing regulatory T cell differentiation and toleration among dendritic cells.[60-62]. Alongside *REG3G*, the lncRNA *REG1CP* was also predictive of worse outcomes. Interestingly, *REG1CP* has been shown to promote tumorigenesis via multiple mechanisms, but namely through its action to facilitate the unwinding of DNA at the *REG3A* promoter[63]. *REG3A*, which shares 85% protein homology with *REG3G*, has been robustly shown to increase tumor cell migration and invasion, and predict worse outcomes in multiple cancers.[63-67]. Furthermore, it is similarly broadly overexpressed to *REG3G* and *REG1CP* in the Black patient cohort compared to the White patient cohort (Z score < 0 = 47% vs 11%). Formin 1 (FMN1) is an actin-nucleating protein that serves to facilitate interactions between the cytoskeleton and signal transduction, playing key roles in focal adhesion of primary epithelia as well as in dendrite and synaptogenesis in neurons.[68] It has been robustly shown to regulate motility and invasion in glioblastoma cells and, while allowing for the formation of strong focal adhesions in cells via its effects on the microtubule lattice, dynamically regulates those adhesions to generate a "hurdling" pattern of cellular motility and invasion. Bactericidal/permeability-increasing protein, fold-containing family B, member 2 (*BPIFB2*) encodes a lipid transfer/lipopolysaccharide-binding protein that has been sparsely investigated, but previously studied for its relationship with immunotherapeutic outcomes in gastric cancer, high expression in immunologically "cold" lung adenocarcinoma, and ability to

reduce CD8+ T-cell chemotaxis[69,70]. *RNASK9239* and *MTCO1P2* are both pseudogenes that are poorly understood. Conversely, *N4BP3,* and *CASZ1* expression were both positively associated with PFS. *N4BP3* encodes a poorly-characterized protein that interacts with the NEDD4 E3 ubiquitin ligase and has been shown to play a role in anterior neural development, neural crest migration, and axon arborization[71,72]. It has previously been associated with angiogenesis in preclinical studies.[73] Castor zinc finger 1 (*CASZ1)* is a transcription factor that plays a role in the development of neural crest derived cells. It has been previously shown to act as a tumor suppressor in neuroblastoma and is associated with improved outcomes in in hepatocellular carcinoma. [74,75]

While the present study depicts that differentially expressed genes among racial groups have a relationship with clinical outcomes, the spectrum of gene expression does not mutually exclude certain racial groups. This is depicted well in the case of *VTN*, where high expression is present in most Black patients, but select few White patients (**Fig. 7**). Indeed, there are multiple possible explanations for this phenomenon. It is quite possible that there are molecular subtypes or conditions of PNET that simply occur with a higher frequency in certain racial groups due to a confluence of factors, altering gene expression. This phenomenon is well-characterized in prostate and breast cancer and may also be likened to- or even the result of- differences in the occurrence of pathogenic variants among racial groups.[76-80] Another possible explanation is that similar environmental exposures are influencing gene expression, and these environmental factors are themselves associated with race, thus being captured within this study by proxy. It should be noted however, that the authors are unaware of any research into the potential prognostic implications (in PNETs) of the differentially expressed genes

associated with survival outcomes in the present study. Currently, two well-known molecular signatures, the NETest and PPQ, have been used to predict progression and response to various therapies as both blood and tumor-based gene expression panels.[81-84] Among the genes identified in this study as having a relationship with malignancy and progression, only 4 genes (*TECPR2, ZZZ3, KRAS, PHF21A*) are present in the collective 59 genes used in these tests. Assuming that the DEGS identified in this study are indeed differentially expressed in PNETs among racial groups, the absence of their discussion in the current literature stems from the paucity of diversity in the study of these tumors. Furthermore, it becomes evident that while Black patients in particular may benefit less or even be harmed from personalized medicine approaches in PNETs as a result of their relative exclusion, these genes remain understudied with respect to their potential implications for all patients with PNETs. Improved representation of patients from diverse backgrounds is imperative for understanding variation in the oncogenesis and progression of these heterogeneous tumors so that this information can be leveraged to improve patient outcomes.

The evaluation of previously established prognostic biomarkers between Black and White patients found significant differences in the expression of all proteins. Overall, these data indicate differences in the expression levels of these biomarkers among racial groups. However, only PAM expression agreed by racial group with gene expression data from the sequencing cohort, while PD-L1 was significantly opposite its protein level, having higher expression in the Black patient sequencing cohort. Of note, these data are from different patient samples entirely, and concordance between gene and protein expression is dependent on multiple factors, including transcriptional and protein

regulation. Furthermore, the relatively low number of Black patients and the high number of metastatic lesions represented in this dataset severely limit the ability to make conclusions based on these data. Further studies will be necessary to definitively evaluate these protein biomarkers and may benefit from having orthogonal validation at the sequencing level.

Limitations and *Future Directions*

Use of FFPE tissue. There are numerous limitations to the present study. Due to the relative rarity of PNETs, frequent lack of a conclusive presurgical diagnosis, and more streamlined microdissection of tumor cells, FFPE tissue was used rather than fresh-frozen tissue for the isolation of RNA for sequencing. While RNA from FFPE tissues is often degraded and modified by the fixation process, recent studies have shown a high concordance between the sequencing results of fresh-frozen and FFPE tissues using modern methods for deparaffinization, relief of formalin-induced crosslinking, ribosome-depletion, and the generation of 3' sequencing libraries to optimize the reading and alignment of fragmented RNA from FFPE specimens [85-87]. Furthermore, the exclusion of older samples, those with extensive RNA degradation, and those with poor quality sequencing libraries served to mitigate the influence of potential nucleotide degradation. Considering that certain RNA species have a higher propensity to undergo degradation over time, the age of the sample was included as a covariate in differential expression and regression analysis of sequencing data.

Sample size. Another critical limitation of this study is the relatively low sample size for the studies herein. In addition to limiting our ability to identify truly differentially expressed genes, the low number of samples corresponds to the low number of

progression events that occurred. Naturally, this further limited our ability to assess the relationship between gene expression and clinical outcomes. However, while the sample sizes were relatively low, the patients within this cohort represented a thoroughly curated set of patients that were selected to mitigate the influence of potential confounding.

Environmental Influences, Clinical Influences, and the Limitations of RNA. Relatedly, this study did not evaluate associations with the numerous additional clinical and environmental factors that may influence gene expression. Given our previous findings that epigenetic regulatory genes are differentially mutated between Black and White patients, the likelihood of mutational dynamics and tumor epigenetics influencing differential gene expression is high. Indeed, integrated analyses of these data on this study population are forthcoming and will provide further structure to these data that will facilitate the identification of differential tumor biology between these groups. Furthermore, there may be variation in influential comorbidities that have not yet been captured within these data due to both the small sample size, as well as potential variation in the fidelity of EMR data. While extensive chart review and interviewing were conducted, many medical conditions go unreported or undiagnosed. Notably, the risk of chronic pancreatitis is two to three-fold higher for Black patients than for White patients.[88] In particular, given the differential expression of many genes related to pancreatitis in this cohort, it is possible that chronic, subclinical pancreatitis was present at resection that was not indicated via laboratory studies, the patients' clinical courses, or during their operation. Future studies to confirm these findings will necessitate intensive evaluation of adjacent tissue for signs of pancreatitis as well as an analysis of high-dimensional clinical data integrated with multi-omics analyses to advance our knowledge in this domain. In

addition, future studies will require functional analyses to evaluate DEGs associated with clinical outcomes for causative relationships. While some of the genes identified herein were previously characterized as such in other cancers, recently discovered transcripts and pseudogenes without functionally characterized noncoding RNAs may well be associated with outcomes because they are the byproduct of a highly dysregulated transcriptional environment that itself bears a causative relationship with progression via another mechanism.

This study represents the first of its kind for NETs as a whole, nearly doubling the number of identifiably Black patients in the genomic literature of pancreatic NETs. While further studies will undoubtedly be necessary to better characterize the influence that differentially expressed genes and proteins and have on clinical outcomes, improvements in the availability and utility of personalized medicine portend that molecular tumor characteristics will be more influential in guiding cancer care in the future. Parity of racial representation in studies to identify clinically impactful molecular characteristics in PNETs will ensure that those characteristics enriched in certain racial groups are considered in future studies and clinical panels used to inform clinical decision-making.

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CONCLUSION

In summary, there is little representation of racial groups in the current genomic, transcriptomic, and epigenomic literature in GEP-NENs, PNETs included. As PNETs are the most studied tumor in GEP-NEN genomics, this is particularly concerning. Multiple genes appear to be differentially mutated between Black and White patients with PNETs. Among these genes is MEN1, which has traditionally been considered to be the most frequently mutated gene in PNETs. This raises important questions as to what the more dominant risk genes may be for different populations. The studies herein further support the hypothesis that there is variation in numerous aspects of the transcriptome, tumor microenvironment, and protein expression between Black and White patients with PNETs and indicates that these differences may be influencing racial disparities in clinical outcomes. Of particular note, multiple DEGs were specifically association with African ancestry and are associated with tumor progression and metastatic disease. These genes should be further explored in preclinical studies to determine if they bear a causal relationship with these outcomes. Further studies will be necessary to validate these findings and more thoroughly characterize interracial differences in PNET genomics. High-dimensional data will be imperative to identify specific environmental, lifestyle, and clinical factors that may confound these studies. This body of work will serve as the basis for identifying and understanding specific nodes of variation that can be evaluated in more diverse patient populations to hopefully guide more equitable personalized medicine and effective treatment for patients with PNETs in the future.

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APPENDIX A

INSTITUTIONAL REVIEW BOARD APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Office of the Institutional Review Board for Human Use

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APPROVAL LETTER

TO: Rose, John Bart; Herring, Brendon

FROM: University of Alabama at Birmingham Institutional Review Board
Federalwide Assurance # FWA00005960
IORG Registration # IRB00000196 (IRB 01)
IORG Registration # IRB00000726 (IRB 02)
IORG Registration # IRB00012550 (IRB 03)

DATE: 18-May-2021

RE: IRB-300006067
IRB-300006067-005
Differential Gene Expression and Methylation in Gastroenteropancreatic Neuroendocrine Tumors Among Ethnic Populations

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The IRB reviewed and approved the Revision/Amendment submitted on 17-May-2021 for the above referenced project. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services.

Type of Review: Exempt
Exempt Categories: 4
Determination: Exempt
Approval Date: 18-May-2021

Documents Included in Review:

- IRB EPORTFOLIO

To access stamped consent/assent forms (full and expedited protocols only) and/or other approved documents:
1. Open your protocol in IRAP.
2. On the Submissions page, open the submission corresponding to this approval letter. NOTE: The Determination for the submission will be "Approved."
3. In the list of documents, select and download the desired approved documents. The stamped consent/assent form(s) will be listed with a category of Consent/Assent Document (CF, AF, Info Sheet, Phone Script, etc.)