Research

mTOR pathway gene expression in association with race and clinicopathological characteristics in Black and White breast cancer patients

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Abstract

Background Aberrant activation of the mammalian Target of Rapamycin (mTOR) pathway has been linked to obesity and endocrine therapy resistance, factors that may contribute to Black-White disparities in breast cancer outcomes. We evaluated associations of race and clinicopathological characteristics with mRNA expression of key mTOR pathway genes in breast tumors.

Methods Surgical tumor tissue blocks were collected from 367 newly diagnosed breast cancer patients (190 Black and 177 White). Gene expression of \( \text{AKT1} \), \( \text{EIF4BP1} \), \( \text{MTOR} \), \( \text{RPS6KB2} \), and \( \text{TSC1} \) were quantified by NanoString nCounter. Differential gene expression was assessed using linear regression on log2-transformed values. Gene expression and DNA methylation data from TCGA were used for validation and investigation of race-related differences.

Results Compared to White women, Black women had relative under-expression of \( \text{AKT1} \) (log2 fold-change = \(-0.31\), 95% CI \(-0.44, -0.18\)) and \( \text{RPS6KB2} \) (log2 fold-change = \(-0.11\), 95% CI \(-0.19, -0.03\)). Higher vs. lower tumor grade was associated with relative over-expression of \( \text{EIF4BP1} \) and \( \text{RPS6KB2} \), but with lower expression of \( \text{TSC1} \). Compared to luminal tumors, triple-negative tumors had relative under-expression of \( \text{TSC1} \) (log2 fold-change = \(-0.42\), 95% CI \(-0.22, -0.01\)). The results were similar in the TCGA breast cancer dataset. Post-hoc analyses identified differential CpG methylation within the \( \text{AKT1} \) and \( \text{RPS6KB2} \) locus between Black and White women.

Conclusions Over-expression of \( \text{RPS6KB2} \) and \( \text{EIF4BP1} \) and under-expression of \( \text{TSC1} \) might be indicators of more aggressive breast cancer phenotypes. Differential expression of \( \text{AKT1} \) and \( \text{RPS6KB2} \) by race warrants further investigation to elucidate their roles in racial disparities of treatment resistance and outcomes between Black and White women with breast cancer.

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1 Introduction

Breast cancer mortality is at least 40% higher in Black women compared to White women in the US [1]. The reason for this disparity gap is complex and likely involves both social and biological determinants. Breast tumors with more aggressive phenotypes, including higher grade, hormone receptor (HR)-negative, and triple-negative breast cancer (TNBC) subtype, are more common in Black women than in White women with breast cancer [2]. Obesity, an important prognostic factor of breast cancer [3, 4], likely contributes to the disparities in breast cancer mortality between Black and White women as obesity is more prevalent in Black women compared to White women [5]. In addition, Black women with HR-positive tumors have higher risks of recurrence and mortality relative to White women with the same breast cancer subtype [6, 7], which might be due to differences in resistance to endocrine therapy. Improving our understanding of biomarkers and molecular mechanisms involved in obesity-signaling pathways and endocrine therapy resistance and potentially different by race is important for planning mechanism-based therapeutic interventions to close the disparity gap in breast cancer mortality.

Genes in the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (mTOR) pathway are highly expressed in breast cancer and are related to prognosis [8–13]. Specifically, mTOR, which forms mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), functions as a serine/threonine protein kinase that regulates protein synthesis, proliferation, and autophagy [14, 15] (Fig. 1). mTORC1 regulates several proteins, including the p70 ribosomal S6 kinases (S6K1 and S6K2), which directly phosphorylate the activation domain of the estrogen receptor (ER), a mechanism for resistance to endocrine therapy in ER+ tumors [16–19]. mTORC1 also regulates protein synthesis through inhibiting 4E-binding protein-1 (4E-BP1) and eukaryotic initiation factor 4E (eIF-4E) [20–22]. In addition, mTORC1 is negatively regulated by tuberous sclerosis protein 1 and 2 (TSC1–TSC2) complex [23, 24]. Loss of TSC1 or TSC2 expression has been shown to activate mTORC1 and its downstream factors [23, 24].

The mTOR pathway can be promoted by positive energy imbalance, insulin resistance, and elevated insulin-like growth factors (IGFs) [25, 26]. Black women may be predisposed to higher mTOR activation as they have higher prevalence of obesity, insulin resistance, and diabetes compared to White women [13, 27]. Our recent data suggest that Black women have higher protein expression and activation levels of mTOR in their breast tumors than White women [13]. In addition, we found that higher levels of pathway activity—indicated by the expression of phosphorylated (p)-mTOR, p-AKT, and p-S6K1—were associated with lower tumor grade, smaller tumors, earlier stage at diagnosis, and luminal subtype. However, it remains unclear to what extent the expression levels of these genes differ by race and clinicopathological characteristics.

To address this gap in knowledge, we examined associations of race and breast tumor clinicopathological characteristics with the expression of key genes in the mTOR pathway, including \textit{AKT1}, \textit{MTOR}, \textit{RPS6KB2} (encodes S6K2), \textit{EIF4EBP1} (encodes eIF-4E), and \textit{TSC1}, in the mTOR pathway in Black and White women with newly diagnosed breast cancer. We hypothesized that the gene expression levels of \textit{AKT1}, \textit{MTOR}, and \textit{RPS6KB2} are higher in tumors from Black women compared to White women, but expression of \textit{EIF4EBP1} and \textit{TSC1}, two genes that may have lower expression when mTOR is overexpressed, are lower among Black women compared to White women. We also hypothesized that gene expression is higher in tumors with features indicative of more aggressive phenotype, including higher grade and ER- or TNBC subtypes, relative to less aggressive features. For validation purposes, we also examined gene expression and DNA methylation data from The Cancer Genome Atlas (TCGA).

2 Methods

2.1 Study population

Patients were selected from the Women’s Circle of Health Study (WCHS). Black patients were frequency matched to White patients by age at diagnosis and tumor subtype. To increase the representation of HER2 and triple-negative subtypes, which are less common than luminal subtype, we over-sampled HER2 and triple-negative subtypes for both Black and White groups. Because WCHS did not have enough cancer cases needed for the matching and oversampling design, we added patients from Pathology Network Shared Resources (PNSR) at the Roswell Park Comprehensive Cancer Center. As
a result, 190 Black patients and matched 177 White patients were selected (total N = 367). The design and data and biopsy specimen collection in WCHS have been described elsewhere [13, 28, 29]. Questionnaire data on known and suspected risk factors for breast cancer and anthropometric measurements were obtained during in-person interviews. Clinical and tumor characteristics and receptor status were obtained from pathology reports.

2.2 Tumor tissue processing and gene expression profiling

Formalin-fixed paraffin-embedded (FFPE) tumor tissue specimens were used for RNA extraction. Two 10 μm curls were cut from a FFPE block and RNA was extracted using the High Pure FFPE RNA Isolation Kit (Roche Molecular Systems, Inc., Pleasanton, CA, USA) and quantified using Qubit and Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). AKT1, EIF4EBP1, MTOR, RPS6KB2, TSC1 were added as custom content to the NanoString PanCancer Immune Panel and quantitated using NanoString nCounter® technology (NanoString Technologies, Seattle, WA, USA). Samples included 15 technical duplicates and one biological trio (from three different tumor blocks of the same case). All samples passed the QC measures following manufacturer's recommendations. Among the panel, the correlations of the gene counts were in the range of 0.98–0.99, with one exception of an R = 0.89, between each of the technical duplicative pairs. The correlations of the gene counts among the biological trio samples ranged from 0.98 to 0.99. In addition, DNA methylation data generated by Illumina 450 K BeadChip were available for 54 WCHS participants to examine the correlations between gene expression and DNA methylation [30].

2.3 Statistical analysis

Gene expression data were first normalized to positive controls included in the assays and then a panel of 40 housekeeping genes using geNorm algorithm, and the data were log2-transformed. Pairwise correlations of gene expression levels were assessed using Pearson correlation analysis. Multivariable linear regression analyses were performed to assess the log2 fold-change of gene expression across race and clinicopathological characteristics. Models included race (White, Black), age (≤ 40, 41–50, 51–65, > 65), tumor grade (low, intermediate, high), ER status (negative, positive), progesterone receptor (PR) status (negative, positive), HER2 status (negative, positive), and three-marker molecular subtype (Luminal: ER+/PR+/HER2+ or ER+/PR+/HER2-, HER2-overexpressed: ER-/PR-/HER2+, and triple-negative: ER-/PR-/HER2-). Because approximately 10% of the patients did not have data on breast cancer stage, we included the variable in separate models as a sensitivity analysis. Because Black women have a higher prevalence of obesity (BMI ≥ 30) than White women, and BMI is associated with mTOR activation [13], we conducted subgroup analysis additionally adjusting for BMI in 150 WCHS participants with available data. All statistical tests were 2-sided, and p-value < 0.05 was considered statistically significant. Analyses were performed using SAS version 9.3 (SAS Institute Inc.).
2.4 External validation of mTOR gene expression and DNA methylation analysis using data from The Cancer Genome Atlas (TCGA)

We downloaded GDC TCGA Breast cancer (BRCA) dataset from the University of California Santa Cruz (UCSC) Xena Portal (http://xena.ucsc.edu). We selected genomic data on AKT1, EIF4EBP1, MTOR, RPS6KB2, TSC1, as well as phenotypic data on race and age. We merged the gene expression data with the clinicopathologic data, including ER, PR, and HER2 status, PAM 50 intrinsic subtype annotation, and tumor stage classified by the American Joint Committee on Cancer (AJCC), from TCGA [8]. A total of 332 (30 Black and 302 White) breast cancer patients were included in the analysis. Since the number of Black women was low, the TCGA data were primarily used to validate our findings of gene expression associations with tumor characteristics in WCHS.

Given that DNA methylation may downregulate gene expression, a post-hoc analysis was conducted to explore differences between CpG site-specific methylation for AKT1 and RPS6KB2 by race using TCGA’s Illumina Human Methylation 450k array dataset from UCSC Xena portal. CpG island (CGI) positions were identified based on 1500 bp genomic length scale. Differences in DNA methylation (β), i.e., the ratio of methylation levels between methylated and unmethylated alleles, between 164 Black women and 670 White women were examined using one-way ANOVA; p-values were corrected for multiple comparisons using the False Discovery Rate (FDR) method at 0.05 level. We further determined the gene-level correlation of methylation level at CGI loci and gene expression using Pearson correlation. The DNA methylation analyses were performed using the SMART (Shiny Methylation Analysis Resource Tool) App.

3 Results

Table 1 shows the characteristics of the study participants overall and by race. Almost two-thirds (65.83%) had high-grade tumors; 50.4% were luminal tumors, 12.5% were HER2-overexpressed, and 37.1% were TNBC.

Pearson’s correlation coefficients among the mTOR pathway gene expressions overall and separately among Black women and White women are presented in Supplemental Table 1. Overall, there were weak positive correlations for AKT1 with MTOR and TSC1 (r = 0.17 and 0.19, respectively), but a moderate positive correlation with RPS6KB2 (r = 0.46). There was a moderate positive correlation for EIF4EBP1 with RPS6KB2 (r = 0.30) and a moderate negative correlation with TSC1 (r = −0.42). There were weak positive correlations for MTOR with RPS6KB2 and TSC1 (r = 0.18 and 0.12, respectively). These remained consistent in the race-specific analysis.

Table 2 shows means and standard deviations of log2-transformed gene expression levels by race and clinicopathological characteristics. Tumors from Black women had significantly lower AKT1 and RPS6KB2 expression compared to those from White women. By molecular subtype, the racial difference was observed in luminal and TNBC tumors for AKT1 (Fig. 2A) and luminal tumors for RPS6KB2 (Fig. 2D). Tumors with higher grade had higher expression of EIF4EBP1 and RPS6KB2 but lower expression of TSC1 relative to tumors with lower grade. ER+ tumors had higher expression levels of AKT1 and TSC1 but lower expression levels of EIF4EBP1 compared to ER- tumors. HER2+ tumors had higher expression of AKT1 and RPS6KB2 compared to HER2- tumors. Among the molecular subtypes, HER2-overexpressed tumors had the highest expression of AKT1, EIF4EBP1 and RPS6KB2.

In multivariable models (Table 3), Black women had relative under-expression of AKT1 (log2 fold-change = −0.31, 95% CI [−0.44, −0.18]) and RPS6KB2 (log2 fold-change = −0.11, 95% CI [−0.19, −0.03]) in their breast tumors compared to White women. High vs. low tumor grade had relative over-expression of EIF4EBP1 (log2 fold-change = 0.84, 95% CI [0.46, 1.22]) and RPS6KB2 (log2 fold-change = 0.33, 95% CI [0.15, 0.50]), but under-expression of TSC1 (log2 fold-change = −0.23, 95% CI [−0.38, −0.08]). HER2+ vs. HER2- status was associated with relative over-expression of AKT1 (log2 fold-change = 0.23, 95% CI [0.04, 0.42]) and RPS6KB2 (log2 fold-change = 0.13, 95% CI [0.01, 0.25]), but relative under-expression of TSC1 (log2 fold-change = −0.12, 95% CI [−0.22, −0.01]). In comparison to luminal tumors, TNBC subtype had lower expression of TSC1 (log2 fold-change = −0.42, 95% CI [−0.77, −0.07]). These associations remained similar after additionally adjusted for breast cancer stage (Supplemental Table 2), despite that a more advanced stage was associated with relative over-expression of AKT1 and RPS6KB2, compared to an earlier stage. Among patients with data on BMI, the subgroup analysis additionally adjusting for BMI showed consistent results (Supplemental Table 3).
3.1 External validation in the TCGA breast cancer dataset and the post-hoc analysis of DNA methylation data

Participant characteristics and the results of the analysis of TCGA data are shown in Supplemental Tables 4–6. Compared to luminal tumors, TSC1 gene expression was relatively under-expressed in HER2-enriched tumors and Basal-like tumors (Supplemental Table 6). These findings were consistent with our main analysis in WCHS. Additionally, EIF4EBP1 gene expression was relatively over-expressed in HER2-enriched, basal-like, and normal-like tumors, compared to Luminal tumors. WCHS data showed similar patterns, although the associations did not reach statistical significance.

Among methylation probes within AKT1, a gene that was relatively under-expressed in tumors from Black vs. White women, we observed two CpG island loci exhibiting significantly higher DNA methylation (β) values in Black than White women (cg07197515 and cg02884928) and one with a higher β value in White than Black women (cg15957959) (FDR corrected p < 0.05 for all three loci; Supplemental Fig. 1). The methylation levels of these loci were inversely associated with gene expression of AKT1 (r = −0.21, −0.22, and −0.29, respectively; Supplemental Fig. 2). A consistent
Table 2  Gene expression levels (log2-transformed values) of the mTOR pathway in breast cancer according to race, age, and clinicopathological characteristics (N = 367)

| Characteristic         | N     | AKT1 Mean ± SD | p-value | EIF4EBP1 Mean ± SD | p-value | MTOR Mean ± SD | p-value | RPS6KB2 Mean ± SD | p-value | TSC1 Mean ± SD | p-value |
|------------------------|-------|----------------|---------|--------------------|---------|----------------|---------|------------------|---------|----------------|---------|
| Race                   |       |                |         |                    |         |                |         |                  |         |                |         |
| White                  | 177   | 12.31 ± 0.63   | <0.0001 | 11.04 ± 0.86       | 0.7645  | 9.88 ± 0.30    | 0.6702  | 9.45 ± 0.40      | 0.0095  | 6.63 ± 0.38    | 0.6679  |
| Black                  | 190   | 12.00 ± 0.66   |         | 11.07 ± 0.97       |         | 9.87 ± 0.28    |         | 9.34 ± 0.40      |         | 9.62 ± 0.35    |         |
| Age                    |       |                |         |                    |         |                |         |                  |         |                |         |
| ≤ 40                   | 47    | 12.21 ± 0.58   | 0.7837  | 11.12 ± 0.80       | 0.9160  | 9.86 ± 0.30    | 0.4335  | 9.50 ± 0.42      | 0.2188  | 9.54 ± 0.35    | 0.2829  |
| 41—50                  | 103   | 12.18 ± 0.66   | <0.0001 | 11.06 ± 1.02       | <0.0001 | 9.90 ± 0.30    | <0.0001 | 9.39 ± 0.44      | <0.0001 | 9.66 ± 0.40    | 0.9911  |
| 51—65                  | 156   | 12.13 ± 0.72   | <0.0001 | 11.06 ± 0.93       | <0.0001 | 9.85 ± 0.29    | <0.0001 | 9.36 ± 0.39      | <0.0001 | 9.63 ± 0.34    | 0.9911  |
| > 65                   | 61    | 12.11 ± 0.58   | 0.4616  | 10.99 ± 0.81       | 0.9160  | 9.89 ± 0.26    | 0.4335  | 9.39 ± 0.37      | 0.2188  | 9.62 ± 0.36    | 0.2188  |
| Tumor grade            |       |                |         |                    |         |                |         |                  |         |                |         |
| Low                    | 26    | 12.03 ± 0.47   | <0.0001 | 10.18 ± 0.50       | <0.0001 | 9.92 ± 0.22    | <0.0001 | 9.10 ± 0.21      | <0.0001 | 9.92 ± 0.23    | <0.0001 |
| Intermediate           | 97    | 12.20 ± 0.61   | <0.0001 | 10.72 ± 0.80       | 0.8478  | 9.87 ± 0.29    | 0.3512  | 9.31 ± 0.42      | <0.0001 | 9.80 ± 0.32    | <0.0001 |
| High                   | 237   | 12.14 ± 0.71   | 0.0044  | 11.30 ± 0.88       | 0.0044  | 9.86 ± 0.30    | 0.0044  | 9.46 ± 0.40      | 0.0044  | 9.52 ± 0.35    | 0.0044  |
| ER status              |       |                |         |                    |         |                |         |                  |         |                |         |
| Positive               | 181   | 12.25 ± 0.63   | <0.0001 | 10.75 ± 0.86       | <0.0001 | 9.87 ± 0.28    | <0.0001 | 9.37 ± 0.44      | <0.0001 | 9.76 ± 0.34    | <0.0001 |
| Negative               | 186   | 12.05 ± 0.68   | <0.0001 | 11.35 ± 0.88       | 0.1461  | 9.87 ± 0.30    | 0.0795  | 9.41 ± 0.36      | <0.0001 | 9.49 ± 0.35    | <0.0001 |
| PR status              |       |                |         |                    |         |                |         |                  |         |                |         |
| Positive               | 154   | 12.21 ± 0.59   | <0.0001 | 10.71 ± 0.86       | <0.0001 | 9.88 ± 0.28    | 0.0795  | 9.35 ± 0.42      | <0.0001 | 9.76 ± 0.33    | <0.0001 |
| Negative               | 213   | 12.11 ± 0.71   | <0.0001 | 11.30 ± 0.88       | <0.0001 | 9.87 ± 0.30    | 0.0795  | 9.42 ± 0.39      | <0.0001 | 9.53 ± 0.36    | <0.0001 |
| HER2 status            |       |                |         |                    |         |                |         |                  |         |                |         |
| Negative               | 235   | 11.97 ± 0.58   | <0.0001 | 11.04 ± 0.92       | 0.7117  | 9.88 ± 0.29    | <0.0001 | 9.31 ± 0.38      | <0.0001 | 9.63 ± 0.38    | <0.0001 |
| Positive               | 132   | 12.47 ± 0.67   | <0.0001 | 11.08 ± 0.92       | 0.7117  | 9.86 ± 0.28    | 0.3447  | 9.53 ± 0.40      | <0.0001 | 9.63 ± 0.34    | <0.0001 |
| Molecular subtype      |       |                |         |                    |         |                |         |                  |         |                |         |
| Luminal                | 185   | 12.25 ± 0.62   | <0.0001 | 10.75 ± 0.85       | <0.0001 | 9.87 ± 0.28    | 0.0002  | 9.37 ± 0.44      | <0.0001 | 9.76 ± 0.33    | <0.0001 |
| HER2+                  | 46    | 12.58 ± 0.73   | <0.0001 | 11.40 ± 0.96       | <0.0001 | 9.93 ± 0.27    | <0.0001 | 9.62 ± 0.35      | <0.0001 | 9.52 ± 0.31    | <0.0001 |
| Triple-negative        | 136   | 11.88 ± 0.57   | <0.0001 | 11.35 ± 0.86       | <0.0001 | 9.86 ± 0.31    | 0.3447  | 9.35 ± 0.34      | 0.0002  | 9.47 ± 0.36    | 0.0002  |

Bolded values indicate statistically significant results with p < 0.05
Fig. 2 mTOR gene expression levels among Black women vs. White women, stratified by molecular subtypes. 

(A) Luminal, p-value = 0.0003; HER2+, p-value = 0.1696; Triple-negative, p-value = 0.0028, 

(B) Luminal, p-value = 0.8275; HER2+, p-value = 0.8599; Triple-negative, p-value = 0.9744, 

(C) Luminal, p-value = 0.7066; HER2+, p-value = 0.3314; Triple-negative, p-value = 0.8613, 

(D) Luminal, p-value = 0.0348; HER2+, p-value = 0.3720; Triple-negative, p-value = 0.1132, 

(E) Luminal, p-value = 0.7842; HER2+, p-value = 0.6807; Triple-negative, p-value = 0.3711
| Characteristic     | AKT1 Log2 fold-change (95% CI) | AKT1 p-value | EIF4EBP1 Log2 fold-change (95% CI) | EIF4EBP1 p-value | MTOR Log2 fold-change (95% CI) | MTOR p-value | RPS6KB2 Log2 fold-change (95% CI) | RPS6KB2 p-value | TSC1 Log2 fold-change (95% CI) | TSC1 p-value |
|-------------------|-------------------------------|--------------|----------------------------------|----------------|-----------------------------|--------------|---------------------------------|----------------|-----------------------------|--------------|
| Race              |                               |              |                                  |                |                             |              |                                 |                |                             |              |
| White             | Ref.                          |              | Ref.                             |                | Ref.                       |              | Ref.                            |                | Ref.                       |              |
| Black             | −0.31 (−0.44, −0.18)          | <0.0001      | −0.02 (−0.20, 0.16)              | 0.8320         | −0.02 (−0.08, 0.04)        | 0.5001       | −0.11 (−0.19, −0.03)            | 0.0105         | −0.02 (−0.09, 0.05)          | 0.5891       |
| Age               |                               |              |                                  |                |                             |              |                                 |                |                             |              |
| ≤ 40              | Ref.                          |              | Ref.                             |                | Ref.                       |              | Ref.                            |                | Ref.                       |              |
| 41–50             | 0.06 (−0.15, 0.27)            | 0.5850       | 0.03 (−0.26, 0.33)               | 0.8208         | 0.04 (−0.06, 0.14)         | 0.4647       | −0.05 (−0.18, 0.08)             | 0.4651         | 0.08 (−0.03, 0.20)           | 0.1589       |
| 51–65             | −0.01 (−0.21, 0.19)           | 0.9252       | 0.05 (−0.22, 0.33)               | 0.6959         | −0.02 (−0.11, 0.08)        | 0.7367       | −0.08 (−0.21, 0.04)             | 0.1869         | 0.05 (−0.06, 0.16)           | 0.3678       |
| > 65              | −0.14 (−0.37, 0.10)           | 0.2481       | −0.13 (−0.45, 0.19)              | 0.4360         | 0.03 (−0.08, 0.14)         | 0.5982       | −0.11 (−0.26, 0.03)             | 0.1293         | 0.08 (−0.05, 0.21)           | 0.2079       |
| Tumor grade       |                               |              |                                  |                |                             |              |                                 |                |                             |              |
| Low               | Ref.                          |              | Ref.                             |                | Ref.                       |              | Ref.                            |                | Ref.                       |              |
| Intermediate      | 0.18 (−0.09, 0.45)            | 0.2024       | 0.43 (0.06, 0.81)                | 0.0243         | −0.03 (−0.16, 0.10)        | 0.6809       | 0.19 (0.01, 0.36)               | 0.0332         | −0.04 (−0.18, 0.11)          | 0.6229       |
| High              | 0.16 (−0.11, 0.43)            | 0.2494       | 0.84 (0.46, 1.22)                | <0.0001        | −0.03 (−0.17, 0.10)        | 0.6104       | 0.33 (0.15, 0.50)               | 0.0002         | −0.23 (−0.38, −0.08)         | 0.0024       |
| ER status         |                               |              |                                  |                |                             |              |                                 |                |                             |              |
| Positive          | Ref.                          |              | Ref.                             |                | Ref.                       |              | Ref.                            |                | Ref.                       |              |
| Negative          | 0.002 (−0.61, 0.61)           | 0.9950       | −0.13 (−0.98, 0.71)              | 0.7556         | −0.05 (−0.35, 0.25)        | 0.7442       | −0.06 (−0.45, 0.32)             | 0.7435         | 0.10 (−0.23, 0.43)           | 0.5607       |
| PR status         |                               |              |                                  |                |                             |              |                                 |                |                             |              |
| Positive          | Ref.                          |              | Ref.                             |                | Ref.                       |              | Ref.                            |                | Ref.                       |              |
| Negative          | 0.20 (−0.04, 0.44)            | 0.0970       | 0.09 (−0.24, 0.42)               | 0.5917         | −0.04 (−0.16, 0.07)        | 0.4701       | 0.08 (−0.07, 0.23)              | 0.3074         | 0.07 (−0.06, 0.20)           | 0.2746       |
| HER2 status       |                               |              |                                  |                |                             |              |                                 |                |                             |              |
| Negative          | Ref.                          |              | Ref.                             |                | Ref.                       |              | Ref.                            |                | Ref.                       |              |
| Positive          | 0.23 (0.04, 0.42)             | 0.0172       | 0.13 (−0.13, 0.40)               | 0.3229         | −0.07 (−0.17, 0.02)        | 0.1229       | 0.13 (0.01, 0.25)               | 0.0338         | −0.12 (−0.22, −0.01)         | 0.0289       |
| Molecular subtype |                               |              |                                  |                |                             |              |                                 |                |                             |              |
| Luminal           | Ref.                          |              | Ref.                             |                | Ref.                       |              | Ref.                            |                | Ref.                       |              |
| HER2+             | 0.07 (−0.61, 0.76)            | 0.8390       | 0.40 (−0.55, 1.35)               | 0.4080         | 0.18 (−0.15, 0.52)         | 0.2789       | 0.09 (−0.34, 0.53)              | 0.6689         | −0.26 (−0.64, 0.11)          | 0.1625       |
| Triple-negative   | −0.43 (−1.08, 0.21)           | 0.1879       | 0.49 (−0.41, 1.38)               | 0.2847         | 0.05 (−0.27, 0.36)         | 0.7712       | −0.05 (−0.45, 0.36)             | 0.8274         | −0.42 (−0.77, −0.07)         | 0.0197       |

CI: confidence interval; Bolded values indicate statistically significant results with p < 0.05

*Linear regression models included race, age, tumor grade, ER status, PR status, HER2 status, and molecular subtype.
finding on the correlation was observed in WCHS participants between methylation of cg02884928 and AKT1 expression \((r = −0.24, p = 0.08; \text{data not shown})\). One CpG island locus in RPS6KB2 showed higher DNA methylation in Black women compared to White women \((\text{cg18905855, FDR corrected } p\text{-value } = 7.65\times10^{-8}; \text{Supplemental Fig. 3})\). There was a weak positive correlation between methylation of cg18905855 and gene expression of RPS6KB2 \((r = 0.10; \text{Supplemental Fig. 4})\).

4 Discussion

In this study of breast tumors from Black and White women, race, grade, HER2 status, and molecular subtype were associated with expression levels of genes we examined in the mTOR pathway in breast tumors. AKT1 and RPS6KB2 were relatively under-expressed in tumors from Black women compared to White women, a finding that was in the opposite direction of our hypothesis.

AKT is one of the most activated proteins in breast cancer [31–33]. We observed differential gene expression of AKT1 by HER2 status and stage among the study individuals and by intrinsic subtype and stage among the TCGA individuals. High vs. normal BMI is associated with higher levels of gene expression for AKT-mTOR pathway genes in breast cancer [26, 34–36], but in our analysis, the association of race and AKT1 expression did not change after adjusting for BMI. We are unable to explore other factors (e.g., frequencies of mutations and circulating IGF levels), which may differ by race and potentially influence AKT1 gene expression. Our post-hoc analysis showed some indications that differential DNA methylation might play a role in the relative under-expression of AKT1 among Black women compared to White women. We demonstrated that two CpG probes in AKT1 exhibited significantly higher methylation in Black patients compared to White patients and the methylation levels were inversely associated with gene expression levels.

Amplification and expression of RPS6KB2 in breast cancer tissues are correlated with decreased tamoxifen responsiveness and poor prognosis in ER+/PR+ tumors [11]. The resistance to endocrine therapy is likely an important contributor to the racial disparity in breast cancer mortality because it is clear that Black women with ER+/PR+ tumors have higher mortality [6] as well as breast cancer recurrence [7], compared to White women with ER+/PR+ tumors, and the difference is not observed among Black and White women with ER−/PR− tumors. However, we observed a relative under-expression of RPS6KB2 in breast cancer overall and in luminal tumors from Black women compared to White women, a finding that was opposite to our hypothesis. The explanation is not clear. With regard to clinicopathological factors, we found that RPS6KB2 was relatively over-expressed in higher grade and HER2+ tumors in our samples. In TCGA, RPS6KB2 was relatively over-expressed in HER2-enriched and basal-like tumors, relative to luminal tumors, although these associations were not statistically significant. We found one CpG probe with differential DNA methylation in RPS6KB2, although the methylation levels were not inversely associated with the gene expression levels. These associations may require further examination by including information on endocrine therapy as well as more upstream and downstream factors of the mTOR pathway and DNA methylation.

Along with RPS6KB2, high expression of EIF4EBP1 might be indicative of more aggressive breast tumor phenotypes as overexpression of EIF4EBP1 is associated with poor breast cancer prognosis [37]. We observed an association between higher tumor grade and higher gene expression of EIF4EBP1. This finding is consistent with the literature reporting that p-4E-BP1 is expressed in poorly differentiated tumors and associated with high pathologic grade and poor breast cancer prognosis [38]. In TCGA, we observed that EIF4EBP1 was relatively over-expressed in HER2-enriched and basal-like tumors. We observed the same patterns in our sample, although the associations did not reach statistical significance.

We observed associations of higher grade, HER2+, and triple-negative breast tumors with lower gene expression of TSC1, a result consistent with our analysis in TCGA for HER2-enriched and basal-like tumors. Invasive breast tumors have lower protein expression of TSC1 and TSC2 compared to the normal mammary epithelium [39]. TSC1 is a tumor suppressor gene that encodes hamartin, which interacts with and stabilizes the GTPase activating protein, tuberin, encoded by TSC2 gene. The hamartin-tuberin complex is a negative regulator of mTORC1 signaling [24]. Dysregulated TSC1/TSC2 complex results in the overactivation of mTOR signaling leading to cell proliferation [39]. Thus, lower expression of TSC1, along with higher expression of RPS6KB2 and EIF4EBP1, can be indicative of more aggressive breast cancer phenotypes.

Observations from our data suggest that the patterns of tumor characteristics in relation to gene expression may not be consistent with the patterns of the phosphorylation of proteins through which the mTOR pathway responds to the extracellular environment, e.g., energy and amino acid influx. In our previous study using tissue microarray data from the WCHS participants, phosphoprotein expression of MTOR, AKT1, and S6K1 were consistently lower in patients with a higher grade tumor, larger tumor, more advanced disease, or TNBC [13]. However, in the current study, MTOR and AKT1...
Gene expression was not associated with these tumor characteristics. For \textit{RPS6KB2}, gene expression seemed to indicate more aggressive tumor characteristics. A limitation of this comparison was that \textit{RPS6KB2} encodes protein S6K2, not S6K1 for which we had data on the phosphoprotein expression. Although S6K1 and S6K2 may have a similar function in the mTOR pathway, a more direct comparison between expression levels of \textit{RPS6KB2} and p-S6K2 is needed. Also, we were unable to include tumor size in the analyses because the data were unavailable among the patients selected from PNSR although patients from WCHS had the variables. The exact reason for the discrepancies between gene and phosphoprotein expression in relation to tumor characteristics is unclear. A hypothesis is that comparing to gene expression, protein phosphorylation may be more likely to be suppressed or lose its function in breast tumors with more aggressive features.

Limitations of this study should be noted. Our study only utilized data on a small panel of mTOR pathway genes. The results would inform a more comprehensive gene set analysis that would yield more robust results. Also, data on tumor grade was unavailable in the TCGA data. In the post hoc analysis, while methylation loci that impact gene expression tend to be located in gene promoter regions, we were unable to confirm whether the significant CpG loci found in our exploratory analysis of DNA methylation were in the promoter regions of the genes studied here. The Illumina Infinium Human Methylation 450k array probes were ambiguously mapped to the human genome [40], and the assay may be biased towards CGI and promoters [41]. Also, the loci of \textit{AKT1} and \textit{RPS6KB2} that were differentially methylated between tumors from Black women and White women were scattered throughout the genes. DNA methylation results should be interpreted with caution, and an in-depth DNA methylation analysis is warranted.

In conclusion, \textit{AKT1} and \textit{RPS6KB2}, two important prognostic predictors in the mTOR pathway, are expressed differently in breast tumors in Black and White women. Whether DNA methylation plays a role in Black-White gene expression differences specific to the mTOR pathway warrants further research. Race and clinicopathological variables of breast cancer should be considered in studying the prognosis and endocrine therapy resistance related to mTOR pathway genes. Our findings may inform more comprehensive investigations of gene expression in the mTOR pathway in relation to prognosis and treatment resistance overall and by race.
References

1. Centers for Disease Control and Prevention (CDC). Breast cancer rates among Black women and White women. Centers for Disease Control and Prevention: Atlanta; 2018.

2. Desantis CE, Sauer AG, Jemal A, Siegel RL, Miller KD. Breast cancer statistics, 2019. CA Cancer J Clin. 2019;69:438–51.

3. Huang J, Dibble CC, Matsuzaki M, Manning BD. The TSC1–TSC2 complex is required for proper activation of mTOR complex 2. Mol Cell 2001;15(21):2852–64.

4. Huang J, Dibble CC, Matsuzaki M, Manning BD. The TSC1–TSC2 complex: a molecular switchboard controlling cell growth. Biochem J. 2009;412(2):179–90.

5. Creighton CJ, Casa AJ, Lazarad ZW, Huang S, Tsirakis M, Hilsenbeck SG, et al. Insulin-like growth factor-I activates gene transcription programs strongly associated with poor breast cancer prognosis. J Clin Oncol. 2008;26(25):4078–85.

6. Fuentes-mattei E, Velazquez-torres G, Phan L, Zhang Y, Chong J, et al. Effects of obesity on transcriptomic changes and cancer hallmarks in estrogen receptor—positive breast cancer. J Natl Cancer Inst. 2014;106(7):dju158.

7. Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev. 2004. https://doi.org/10.1101/gad.1212704.

8. The Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature. 2012;490:1–10.

9. Araki K, Miyoshi Y. Mechanism of resistance to endocrine therapy in breast cancer: the important role of PI3K/Akt/mTOR in estrogen receptor—positive, HER2-negative breast cancer. Breast Cancer. 2018;25(4):392–401.

10. Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K, et al. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. Cancer Cell. 2007;12(4):395–402.

11. Pérez-Tenorio G, Stål O, Southeast Sweden Breast Cancer. Activation of AKT/PKB in breast cancer predicts a worse outcome among endocrine treated patients. Br J Cancer. 2002;86(4):540–5.

12. Lipton JO, Sahin M. The neurology of mTOR. Neuron. 2014;84(2):275–91.

13. Cheng TD, Omilarian AR, Yao S, Sanchez PV, Polk L, Zhang W, et al. Body fatness and mTOR pathway activation of breast cancer in the Women’s Circle of Health Study. npj Breast Cancer. 2020;6(45):1–9.

14. Creighton CJ, Fu X, Hennessy BT, Casa AJ, Zhang Y, Gonzalez-AM, et al. Proteomic and transcriptomic profiling reveals a link between the PI3K pathway and lower estrogen-receptor (ER) levels and activity in ER + breast cancer. Cancer Res. 2010;70(3):R40.

15. Karlsson E, Pérez-Tenorio G, Amin R, Bostjen J, Skog L, Forndor, et al. The mTOR effectors 4EBP1 and S6K2 are frequently coexpressed, and associated with a poor prognosis and endocrine resistance in breast cancer: A retrospective study including patients from the randomized Stockholm tamoxifen trials. Breast Cancer Res. 2013;15(5):1–14.

16. Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev. 2004. https://doi.org/10.1101/gad.1212704.

17. Berns K, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K, et al. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. Cancer Cell. 2007;12(4):395–402.

18. Pérez-Tenorio G, Stål O, Southeast Sweden Breast Cancer. Activation of AKT/PKB in breast cancer predicts a worse outcome among endocrine treated patients. Br J Cancer. 2002;86(4):540–5.

19. Kim EKH, Soo J, Suk M, Kim KK, Inn J. Phosphorylated S6K1 is a possible marker for endocrine therapy resistance in hormone receptor—positive breast cancer. Breast Cancer Res. 2011;126(1):93–9.

20. Wang X, Heung K, Murakami M, Yamanaka S, Proud CG. Distinct signaling events downstream of mTOR cooperate to mediate the effects of amino acids and insulin on initiation factor 4E-binding proteins. Mol Cell Biol. 2005;25(7):2558–72.

21. Gingras A, Gygi SP, Raught B, Polakiewicz RD, Abraham RT, Hoencka MF, et al. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. Genes Dev. 1999;13(11):1422–37.

22. Gingras A, Raught B, Gygi SP, Niedzwiecka A, Miron M, Burley SK, et al. Hierarchical phosphorylation of the translation inhibitor 4E-BP1. Genes Dev. 2001;15(21):2852–64.

23. Huang J, Dibble CC, Matsuzaki M, Manning BD. The TSC1–TSC2 complex is required for proper activation of mTOR complex 2. Mol Cell Biol. 2008;28(12):4104–15.

24. Huang J, Manning BD. The TSC1–TSC2 complex: a molecular switchboard controlling cell growth. Biochem J. 2009;412(2):179–90.

25. Creighton CJ, Casa AJ, Lazarad ZW, Huang S, Tsirakis M, Hilsenbeck SG, et al. Insulin-like growth factor-I activates gene transcription programs strongly associated with poor breast cancer prognosis. J Clin Oncol. 2008;26(25):4078–85.

26. Fuentes-mattei E, Velazquez-torres G, Phan L, Zhang Y, Chong J, et al. Effects of obesity on transcriptomic changes and cancer hallmarks in estrogen receptor—positive breast cancer. J Natl Cancer Inst. 2014;106(7):dju158.

27. Hasson RE. In: Bowen DJ, Denis GV, Berger NA, editors. Impact of energy balance on cancer disparities. Switzerland: Springer International Publishing; 2014. p. 165–99.

28. Ambrosio CB, Clupak GL, Bandera EV, Jandorf L, Bovbjerg DH, Zipori G, et al. Conducting molecular epidemiological research in the age of HIPAA: a multi-institutional case-control study of breast cancer in African-American and European-American women. J Oncol. 2009;2009:871250.

29. Hao S, Cheng TYD, Elkhmanay A, Yan L, Omilarian A, Abrams S, et al. Breast tumor microenvironment in Black women: a distinct signature of CD8 + T-Cell exhaustion. J Natl Cancer Inst. 2021;113(8):1036–43.

30. Espinal AC, Buas MF, Wang D, Cheng DTY, Sucheston-Campbell L, Hu Q, et al. FOXA1 hypermethylation: link between parity and ER—negative breast cancer in African American women? Breast Cancer Res Treat. 2017;166(2):559–68.

31. Bose S, Chandran S, Mrocha JM, Bose N. The Akt pathway in human breast cancer: a tissue-array-based analysis. Mod Pathol. 2006;19:238–45.

32. Wu Y, Mohamed H, Chiller R, Ali I, Clayton S, Slamon D, et al. Clinical significance of Akt and HER2/neu overexpression in African-American and Latina women with breast cancer. Breast Cancer Res. 2008;10(1):1–19.
33. Tokunaga E, Kimura Y, Oki E, Ueda N, Futatsugi M, Mashino K, et al. Akt is frequently activated in HER2/neu-positive breast cancers and associated with poor prognosis among hormone-treated patients. Int J Cancer. 2006;118(2):284–9.

34. Creighton CJ. A gene transcription signature of the Akt / mTOR pathway in clinical breast tumors. Oncogene. 2007;26:4648–55.

35. Toro AL, Costantino NS, Shriver CD, Ellsworth DL, Ellsworth RE. Effect of obesity on molecular characteristics of invasive breast tumors: gene expression analysis in a large cohort of female patients. BMC Obes. 2016. https://doi.org/10.1186/s40608-016-0103-7.

36. Heng YJ, Wang J, Ahearn TU, Brown SB, Zhang X. Molecular mechanisms linking high body mass index to breast cancer etiology in post-menopausal breast tumor and tumor-adjacent tissues. Breast Cancer Res Treat. 2019;173(3):667–77.

37. Rutkovsky AC, Yeh ES, Guest ST, Findlay VJ, Muise-helmericks RC, Armeson K, et al. Eukaryotic initiation factor 4E-binding protein as an oncogene in breast cancer. BMC Cancer. 2019;19(491):1–15.

38. Rojo F, Najera L, Lirola J, Jimenez J, Guzma M, Sabadell MD, et al. 4E-binding protein 1, a cell signaling hallmark in breast cancer that correlates with pathologic grade and prognosis. Clin Cancer Res. 2007;13(1):81–90.

39. Mehta MS, Vazquez A, Kulkarni DA, Kerrigan JE, Atwal G, Metsugi S, et al. Polymorphic variants in TSC1 and TSC2 and their association with breast cancer phenotypes. Breast Cancer Res Treat. 2013;125(3):1–15.

40. Zhang W. On the analysis of the Illumina 450K array data: probes ambiguously mapped to the human genome. Front Genet. 2012;3:6367.

41. Bibikova M, Barnes B, Tsan C, Ho V, Klotzle B, Le JM, et al. High density DNA methylation array with single CpG site resolution. Genomics. 2011;98(4):288–95.

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