Genetic variants in the mTOR pathway and breast cancer risk in African American women

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Abstract

The phosphatidylinositol 3-kinase–AKT–mammalian target of rapamycin (mTOR) pathway has been implicated in breast carcinogenesis. However, there has been no large-scale investigation of genetic variants in the mTOR pathway and breast cancer risk. We examined 28847 single-nucleotide polymorphisms (SNPs) in 61 mTOR pathway genes in the African American Breast Cancer Epidemiology and Risk consortium of 3663 cases [1983 estrogen receptor-positive (ER+) and 1098 ER-negative (ER−)] and 4687 controls. Gene-level analyses were conducted using the adaptive rank truncated product (ARTP) test for 10773 SNPs that were not highly correlated ($r^2 < 0.8$), and SNP-level analyses were conducted with logistic regression. Among genes that were prioritized (nominal $P < 0.05$, ARTP tests), associations were observed for intronic SNPs $TSC2$ rs181088346 [odds ratio (OR) of each copy of variant allele = 0.77, 95% confidence interval (CI) = 0.65–0.88 for all breast cancer] and $BRAF$ rs114729114 (OR = 1.53, 95% CI = 1.24–1.91 for all breast cancer and OR = 2.03, 95% CI = 1.50–2.76 for ER− tumors). For ER− tumors, intronic SNPs $PGF$ rs11542848 (OR = 1.38, 95% CI = 1.15–1.66) and rs61759375 (OR = 1.34, 95% CI = 1.14–1.57) and $MAPK3$ rs78564187 (OR = 1.26, 95% CI = 1.11–1.43) were associated with increased risk. The variant allele of $RPS6KB2$ rs35363135, a synonymous coding SNP, was more likely to be observed in ER− than ER+ tumors (OR = 1.18, 95% CI = 1.05–1.31, gene-wide Bonferroni-corrected $P = 0.06$). In conclusion, specific mTOR pathway genes are potentially important to breast cancer risk and to the ER negativity in African American women.

Introduction

African American (AA) women have the highest prevalence of obesity (58.6% with body mass index >30 kg/m2) (1) among racial/ethnic groups in the USA. AA women are also more likely to have central obesity than white women (2), which has been associated with hyperinsulinemia and insulin resistance, both of which are implicated in breast cancer (3). Among AA women,
the association of obesity with breast cancer risk may differ by tumor subtypes defined by receptor status, including estrogen receptor (ER) (4,5). Although research has suggested several biological pathways relevant to obesity (e.g. inflammation and hormonal factors), the mechanisms by which body size influences breast cancer risk are largely unclear (6). Because a key causal factor for obesity is positive energy imbalance, that is, energy intake being greater than expenditure, pathways related to energy signaling may be important to the underlying mechanism behind the influence of obesity on breast cancer risk.

The phosphatidylinositol 3-kinase–AKT–mammalian target of rapamycin (mTOR) pathway can sense both cellular growth conditions and energy signaling (Figure 1). In cells with excess energy, adenosine monophosphate signals the mTOR complex 1 (mTORC1), activating a variety of downstream responses including cell proliferation, angiogenesis and blockage of cell autophagy (7). In addition, mTORC2 receives signals from growth factors (e.g. glucose and insulin) and further stimulates AKT and mTORC1 (8). AKT1 and MTOR mutations are observed in breast cancer tumor tissue (9). Thus, the mTOR pathway may be important in breast carcinogenesis, and investigating genetic polymorphisms in this pathway may shed light on associations between obesity and breast cancer risk. To date, there are few studies examining the association of genetic variation in the mTOR pathway and breast cancer risk (10) and subtypes (10,11), and only a small number of single-nucleotide polymorphisms (SNPs) have been examined. Also, to our knowledge, no published study has assessed this association among AA women. Here, we investigated the association of genetic variants in the mTOR pathway with breast cancer risk in a large sample of AA women. We examined the association of variants in genes in the mTOR pathway with overall breast cancer risk, as well as with ER-positive (ER+) and ER-negative (ER−) breast cancer risk separately because of potential differences in etiology related to obesity (4,5). We also investigated the association of variants with ER negativity in case-only analysis.

**Methods**

**Study population**

We included women with incident invasive breast cancer or ductal carcinoma in situ and controls with available DNA for genotyping in the African American Breast Cancer Epidemiology and Risk (AMBERR) consortium (12,13). The AMBERR consortium pools data from four studies with large numbers of AA women: the Carolina Breast Cancer Study (CBCS), the Women’s Circle of Health Study (WCHS), the Black Women’s Health Study (BWHS) and the Multiethnic Cohort (MEC) Study. Briefly, the CBCS is a population-based case-control study of women aged 20–74 years in North Carolina conducted 1993–1996 (phase I) and 1996–2001 (phase II) (14). Breast cancer cases were identified through the North Carolina Central Cancer Registry; controls were identified from Division of Motor Vehicle lists (age <65) or from Health Care Financing Administration lists (age ≥65). Controls were frequency matched to cases on age in 5-year age groups. The current study also included participants from CBCS phase III, a case-only prospective study started in 2008. Home visits were conducted to collect information on breast cancer risk factors and to obtain biospecimens. The WCHS is a case-control study of women aged 20–74 years that began in New York in 2003, subsequently expanded to New Jersey (15), and currently enrolling only AA participants in New Jersey. Breast cancer cases were identified through major hospitals in New York City and through the New Jersey State Cancer Registry. Controls were identified through random digit dialing and through community-based recruitment (16). Controls were frequency matched to cases on 5-year age group. Data on epidemiologic risk factors and samples for DNA analysis were obtained during home interviews (4). The BWHS is a prospective cohort study of 59000 AA women 21–69 years of age recruited from 17 states in 1995 (17). Breast cancer diagnoses were self-reported on the biennial follow-up questionnaires or identified through state cancer registries and the National Death Index. Approximately 27000 BWHS participants provided saliva samples for DNA analysis. The MEC is a prospective study started in 1993–1996 that includes 16594 AA women 45–75 years of age (18). Data were collected through questionnaires mailed at 5-year intervals; blood samples were obtained for DNA analysis. Cases were identified by linkage to the Hawaii Tumor Registry, the Cancer Surveillance Program for Los Angeles County and the California State Cancer Registry. For BWHS and MEC cohorts, controls were selected among study participants who had not been diagnosed with breast cancer. For all studies, ER status was based on immunohistochemistry results from hospital pathology records and/or cancer registry data. All participants were self-identified AA women. Each study obtained informed consent from all participants and was approved by the relevant Institutional Review Boards.

**Genotyping**

DNA was isolated from blood in CBCS and MEC, from saliva obtained using Oragene kits in WCHS and from saliva obtained using a mouthwash-swish method in BWHS (19). A total of 61 candidate genes of the mTOR pathway were selected based on pathway information provided by BioCarta (20) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Supplementary Table 1, available at Carcinogenesis Online). The gene set included key proteins of the mitogen-activated protein kinases (MAPK) pathway, which can signal the mTOR pathway (10). Tag SNPs were selected based on linkage disequilibrium (r² ≥ 0.8) with minor allele frequency ≥10%, according to the haplotype structure of the Yoruban population in 1000 Genomes. Genotyping using the illumina Human Exome Beadchip v1.1 with custom content was performed on samples from CBCS, WCHS and BWHS by the Center for Inherited Disease Research (CIDR). Genotypes were attempted for 6936 study subjects from the BWHS, CBCS and WCHS, and completed with call rate >98% for 6828 (1103 cases and 3698 controls). Prior to imputation, we omitted SNPs that were monomorphic, were positional duplicates, were on the Y chromosome, had a P value for Hardy–Weinberg equilibrium <1×10⁻⁴, had call rate <0.98, had >1 Mendelian errors in HapMap trios or had >2 discordant calls in duplicate samples. Imputation was performed at the University of Washington, Seattle, WA, using IMPUTE2 software and the 1000 Genomes Phase I reference panel (release date: 21 May 2011; December 2013 released haplotypes) (21). SNPs from the standard and custom content of the exome chip were used to impute the 1000 Genomes variants present with ≥2 minor alleles on the AFR and EUR panels. As the imputation backbone for this study was not as dense as typical genome-wide association study chips, imputation quality metrics in regions with sparse coverage was lower than for genome-wide association study chips. However, 57% of all 1000 Genomes imputed variants within 60kb of at least one genotyped SNP on our panel had r² > 0.5. Using the masking analysis in IMPUTE2 to compare imputed to true genotypes, that is, imputation r², variants with MAF ≥ 0.05 had median r² = 0.93, and variants with MAF <0.05 had median r² = 0.53. For the MEC study, genetic data from 533

**Abbreviations**

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<tr>
<td>AA</td>
<td>African American</td>
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<td>AMBER</td>
<td>African American Breast Cancer Epidemiology and Risk</td>
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<td>AKTP</td>
<td>adaptive rank truncated product</td>
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<td>BWHS</td>
<td>Black Women’s Health Study</td>
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<td>CBCS</td>
<td>Carolina Breast Cancer Study</td>
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<td>CI</td>
<td>confidence interval</td>
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<td>ER</td>
<td>estrogen receptor</td>
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<td>MAPK</td>
<td>mitogen-activated protein kinases</td>
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<td>MEC</td>
<td>Multiethnic Cohort</td>
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<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<td>mTORC</td>
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<td>OR</td>
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<td>SNPs</td>
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<td>WCHS</td>
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cases and 989 controls were already available, including Illumina Human 1M-Duo chip data and SNPs imputed from 1000 Genomes. The imputed genotypes from the BWHS, CBCS and WCHS were combined with the imputed genotypes from MEC into a final data set. Variants were included in the combined data set if the allele frequencies in the two subsets differed by ≤0.15 or if the imputation $r^2$ was ≥0.5 in either study. The criterion of imputation $r^2$ was ≥0.2–0.3 in either study. Two approaches were used to examine associations of SNPs and breast cancer risk: gene-based and SNP-based analyses. The gene-based analysis was performed using the adaptive rank truncated product (ARTP) test implemented in the R package PIGE. The ARTP combined the optimal number of most significant $P$-values from among the top 10 SNPs for each gene. We selected a set of 10773 SNPs that were not highly correlated for implementing the ARTP method to avoid capturing only a few association signals for some genes due to correlations between their top SNPs. One SNP of every pair of SNPs with correlation $r^2$ ≥ 0.8 were excluded from the gene-based tests using the filter.R2 option in the R package AdaJoint.

SNP-level association analyses were performed for SNPs in genes with nominal $P < 0.05$ in the ARTP tests. We used logistic regression with case status as outcome, and an additive model for genotype, adjusting for age (10-year groups), study, geographic location, DNA source and principal components of the genotypes. $P$-values were corrected by the Bonferroni method for the number of SNPs tested within each gene ($P_{corr}$). Imputed SNPs with minor allele frequency (MAF) <0.02 were excluded due to low imputation quality. In addition, to avoid missing any potentially meaningful associations, we report top SNP-level associations for genes with nominal $P < 0.05$. P-values for heterogeneity between the risk of ER− and ER+ subtypes were calculated using a case–case only logistic regression model.

Statistical analyses were performed using PLINK (version 1.07) and R software. Functional follow-up was performed in the ENCODE (Encyclopedia of DNA Elements), including HaploReg v3 and RegulomeDB databases.

Results

Table 1 shows the ER status and age distributions of the study participants with genotype data (3663 cases and 4687 controls) in each study. Overall, 35.6% cases had ER− tumors, with CBCS having the highest percentage of young cases.

No gene-level associations were significant after Bonferroni corrections for the number of genes tested ($n = 61$). Table 2 shows

Figure 1. Overview of the mTOR pathway. 4E-BP1, 4E-binding protein-1; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; ATP, adenosine triphosphate; eIF-4E, eukaryotic initiation factor-4E; ER, estrogen receptor; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; MLST8, mTOR-associated protein, LST8 homolog; mSIN1, mammalian stress-activated protein kinase interacting protein 1; PGF, placental growth factor; PRAS40, proline-rich Akt substrate 40 kDa; Proctor, protein observed with Rictor; PTEN, phosphatase and tensin homologue; Raf, Raf-1 proto-oncogene, serine/threonine kinase; Raptor, regulatory associated protein of mTOR; Rictor, rapamycin-insensitive companion of mTOR; S6, 40S ribosomal protein; S6K1, S6 kinase 1; STK11, serine/threonine kinase 11; TSC, tuberous sclerosis complex.
genes that had a nominal P value < 0.05 in relation to breast cancer in the gene-level analysis. Tuberous sclerosis 2 (TSC2) and B-Raf proto-oncogene, serine/threonine kinase (BRAF) were associated with all breast cancer; TSC2 with ER+ tumors; and BRAF, placental growth factor (PGF), and mitogen-activated protein kinase 3 (MAP3K) with ER– tumors. Comparing ER– cases with ER+ cases, MAP3K and ribosomal protein S6 kinase, 70kDa, polypeptide 2 (RPS6KB2) had a nominal P < 0.05. Top SNPs in non-significant genes are listed in Table 3, is available at Carcinogenesis Online, were statistically significant after a Bonferroni correction for total number of tested SNPs.

### Discussion

In the AMBER consortium study of breast cancer in AA women, several genes in the mTOR pathway, namely TSC2, BRAF, PGF, and MAPK3, were associated with overall risk of breast cancer and subtypes defined by ER status. In these genes, specific SNPs associated with breast cancer risk were identified after correcting for multiple comparisons at the gene level. To our knowledge, this is the first study that systematically examined the association of mTOR pathway genes with breast cancer risk in AA women, a population with higher proportions of obesity and ER– breast cancer than white women.

Among the significant SNPs in our results, several appear to be in regions with important regulatory functions (Supplementary Table 3, is available at Carcinogenesis Online). PGF rs11542848 is located in a region with transcriptional promoters for breast myoepithelial cells. In PGF, the other significant SNP rs61759375 maps to a region containing transcriptional enhancers, and its tagged SNP rs11542848 is located in the promoter region of 5′-untranslated region. In addition, MAPK3 rs78564187 also tags several SNPs that overlap enhancer binding sites. Also, our case–case analysis showed that a synonymous coding SNP rs35363135 in RPS6KB2 was associated with ER– breast cancer. The SNP is located in a region containing active promoters and likely
affecting transcription factor binding (RegulomeDB score = 2b). *RPS6KB2* encodes a member of the S6K1 family of serine/threonine kinases, which can modify ER expression (30).

Data on the mTOR pathway SNPs in relation to breast cancer risk or subtypes are very limited. One study examined three functional SNPs of the late endosomal LAMTOR complex (LAMTOR2 and LAMTOR3), which is a key protein for the crosstalk between the mTOR and the MAPK pathways, among European women (10). This study found that in a small case–control analysis (296 cases), variants in LAMTOR3 rs148972953 were associated with higher proportions of ER- and progesterone receptor-negative breast cancers. In a subsequent larger case–control analysis (2715 cases and 5216 controls), however, the SNP was not associated with breast cancer risk (10). Although LAMTOR3 was not included in our gene selection, we observed two SNPs in MAPK3 and BRAF, a member of the Raf family and main activator of the MAPK pathway, were associated with a modest increase in ER– breast cancer risk in AMBER. Another study examined six tag SNPs in TSC1 and TSC2 in 1137 breast cancer cases, with the majority being Caucasians (78%). Patients with the TSC1 rs1073123 variant were less likely to have ER– breast cancer than ER+ breast cancer (OR = 0.39, 95% CI = 0.14–0.80, P = 0.06; homozygous variant versus common allele) (11). In AMBER, we observed a non-significant inverse association for this SNP comparing ER– cases to ER+ cases (OR = 0.94 for each copy of the variant allele, 95% CI = 0.83–1.07, nominal P = 0.39; data not shown).

The mechanisms of TSC1 and TSC2 influencing ER expression may involve the inhibition of ER-α functions by tuberin, the protein product of TSC2 (31). The significant SNPs in our results have not been reported in Caucasian or Asian women for breast cancer risk or subtypes.

Current literature suggests that mTOR pathway genes involved in carcinogenesis may differ by cancer site. A number of SNPs in RPTOR and AKT3 have been linked to risk of bladder cancer and renal cell carcinoma, respectively, in non-Hispanic whites (32,33). The reported SNPs in these two genes were not significantly associated with breast cancer risk in AMBER (nominal P > 0.05; data not shown), although our exploratory analysis suggested that a number of SNPs in RPTOR may be potentially important for overall and ER+ breast cancer risks in AA women. However, for colorectal cancer, significant SNPs were observed in various genes including MTOR, PIK3CA, PRKAG2, PTEN, STK11, TSC1 and TSC2 (34). The variant in PRKAG2 rs4128396 was associated with an increased risk of rectal cancer (OR = 1.33, 95% CI = 1.09–1.63; AC/CC versus AA genotypes) (34). In AMBER, however, this SNP was associated with a decrease in ER– breast cancer risk (OR = 0.76 for each copy of the variant allele, 95% CI = 0.59–0.97, nominal P = 0.028; data not shown). These observations require validation using populations with the same ancestral backgrounds. From a biological point of view, the mTOR pathway can be signaled by multiple factors (growth factors, nutrients and energy), and all cells may not be equally responsive to these factors. Thus, cells in distinctive tissues or organs may have different requirement for mTOR (8).

The large sample size enabled analysis of risk for overall breast cancer, as well as for ER+ and ER– cancer separately. Furthermore, this was a more comprehensive evaluation of mTOR pathway SNPs than in most previous studies. However, several limitations should be noted. First, all the significant SNPs identified were imputed in either the CBCS/WCHS/BWHS combined genotyping project, the MEC genotyping project, or both. These imputed SNPs have high accuracy (imputation $r^2 \geq 0.9$, except for TSC2 rs181088346 and PFG rs61759375 ($r^2 = 0.802$ and 0.884, respectively, in the MEC genotyping project) and BRAF
rs114729114 ($r^2 = 0.728$ in the CBCS/WCHS/BWHS genotyping project). Table 3. To further validate our findings, we performed association analyses among the individuals with a posterior genotype probability ≥0.9 at the untyped SNPs in the TSC2/WCHS/BWHS project (35). There was no material difference in risk estimates between all individuals and those with high certainty of the imputed genotype (Supplementary Table 4, available at Carcinogenesis Online). Although the quality of these imputed SNPs was very high, results from these imputed SNPs warrant further confirmation by genotyping. Second, we did not have data on human epidermal growth factor receptor 2 in a sufficient number of cases for specific analyses of triple-negative breast cancer or other subtypes dependent on that molecular marker. Lastly, our findings require validation, as the gene-level associations were not significant after correction for multiple tests.

In conclusion, in this systematic assessment of genetic variation in the mTOR pathway, we identified several genes that are associated with risk of breast cancer overall (TSC2 and BRAF), ER+ tumors (TSC2) and ER− tumors (BRAF, PGF and MAPK3). Our findings suggest that the mTOR pathway may be important in breast carcinogenesis in AA women. Future studies on genetic variants in the mTOR pathway with breast cancer risk and subtypes should involve obesity phenotypes to reveal potential gene–environment interactions. In addition, direct assessment of mTOR activities, for example, mTOR protein expression in tumor tissues, can provide a better understanding of underlying mechanisms of obesity regarding energy imbalance in relation to breast cancer subtypes.

Supplementary material
Supplementary Tables 1–4 and Supplementary Material can be found at http://carcin.oxfordjournals.org/

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Conflict of Interest Statement: None declared.

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