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Association of germline microRNA SNPs in pre-miRNA flanking region and breast cancer risk and survival: the Carolina Breast Cancer Study

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Abstract

Purpose—Common germline variation in the 5′ region proximal to precursor (pre-) miRNA gene sequences is evaluated for association with breast cancer risk and survival among African Americans and Caucasians.

Methods—We genotyped 9 single nucleotide polymorphisms (SNPs) within 6 miRNA gene regions previously associated with breast cancer, in 1972 cases and 1776 controls. In a race-stratified analysis using unconditional logistic regression, odds ratios (OR) and 95% confidence intervals (CI) were calculated to evaluate SNP association with breast cancer risk. Additionally, hazard ratios (HR) for breast cancer-specific mortality were estimated.

Results—2 miR-185 SNPs provided suggestive evidence of an inverse association with breast cancer risk (rs2008591, OR = 0.72 (95% CI = 0.53 – 0.98, p-value = 0.04) and rs887205, OR = 0.71 (95% CI = 0.52 – 0.96, p-value = 0.03), respectively) among African Americans. Two SNPs, miR-34b/34c (rs4938723, HR = 0.57 (95% CI = 0.37 – 0.89, p-value = 0.01)) and miR-206 (rs6920648, HR = 0.77 (95% CI = 0.61 – 0.97, p-value = 0.02)), provided evidence of association with breast cancer survival. Further adjustment for stage resulted in more modest associations with

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CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

ETHICAL STANDARDS
All experiments comply with the current laws of the United States of America, where they were performed.

AUTHORS' CONTRIBUTIONS
JTB conceived the study and participated in its design, guided the analysis of data and drafted the manuscript.
CKT participated in the design of the study, performed the statistical analysis and participated in manuscript review and revision.
SJN participated in the design of the study, performed genotype data quality control analysis and participated in manuscript review and revision.
JSB-S participated in the design of the study, in particular the selection of the ancestry informative marker panel for genotyping and participated in manuscript review and revision.
SRC participated in the design of the survival analysis and participated in manuscript review and revision.
RCM contributed to the conception and design of the study, guided the analysis of data and participated in manuscript review and revision.
All authors read and approved the final manuscript.

survival (HR = 0.65 (95% CI = 0.42 – 1.02, p-value = 0.06 and HR = 0.79 (95% CI = 0.62 – 1.00, p-value = 0.05, respectively).

Conclusions—Our results suggest that germline variation in the 5' region proximal to pre-miRNA gene sequences may be associated with breast cancer risk among African Americans and breast cancer-specific survival generally, however further validation is needed to confirm these findings.

Keywords

microRNA; breast cancer; germline; single nucleotide polymorphism; risk; survival

INTRODUCTION

MicroRNAs (miRNAs), small non-coding RNAs, are one of the largest classes of gene regulators [1]. miRNAs regulate the stability or translational efficiency of targeted messenger RNAs (mRNAs) most commonly by binding to 3′-untranslated region (UTR) sequences to silence their target genes. miRNAs undergo a complex, multi-step process of biogenesis recently summarized by Schanen and Li [2]. Within the nucleus, a primary miRNA transcript (pri-miRNA), usually several hundred nucleotides (nt) to several kilobases (kb) in length, is cleaved to create a precursor miRNA (pre-miRNA) approximately 70 nt in length, which folds to form a stem-loop intermediate [2–4]. This intermediate is exported from the nucleus and further processed to form a mature single-stranded miRNA approximately 22 nt in length. Cleavage and processing of the pri- and pre-miRNA requires sequence and secondary structure recognition by RNA binding proteins and their partners [2]. Because mature miRNA seed sequences are small and only partial complimentary binding is required to the 3′-UTR, each miRNA may bind up to as many as 200 target genes [5]. Furthermore, target genes may contain binding sites for many different miRNAs, thus it is estimated that miRNAs may regulate the expression of as many as one-third of all human mRNAs [5].

miRNAs may be located within the introns of `host' protein coding genes (intragenic) and may be co-transcribed by the host's promoter or may lie between genes within the genome (intergenic) under other mechanisms of transcriptional control. Transcriptional regulation of miRNAs has recently been reviewed [2]. Direct experimental evidence has confirmed that miRNA genes can be transcribed by polymerase II and III promoters [6, 7] and recent evidence suggests that miRNAs can `self-transcript' in the absence of promoters [8]. Through computational methods and experimental verification, clustered polycistronic transcripts have also been observed [9, 10] providing evidence that miRNAs in clusters may be encoded by the same pri-miRNA transcript [2].

Recently a comprehensive survey of genomic variation associated with miRNAs and their predicted target sequences was performed [11]. This survey focused on variation within the pre- and highly conserved mature miRNA as well as complementary 3′-UTR sequences in predicated target genes that may impact miRNA processing or targeting. While information about the sequence and structure of pre- and mature miRNAs and associated variation is beginning to emerge, little is known about the genomic structure or sequence variation in the 5′ region proximal to the pre-miRNA gene sequence that includes the 5′ pri-miRNA and promoter regions. Sequence variation in this region may play an important role in influencing miRNA processing and transcription, especially since key RNA binding proteins and their partners require specific sequence and secondary structure for miRNA biogenesis.

miRNAs have been shown to influence numerous molecular pathways and pathological conditions including cancer [12–15] and may prove to be clinically valuable biomarkers.

Recently in breast cancer, miRNAs have been associated with genes involved in critical pathways including apoptosis, cell cycle checkpoints, and cell invasion [4, 16, 17]. Currently, germline mutations in genes including BRCA1 and 2 explain 20% of the familial aggregation of breast cancer; suggesting numerous other susceptibility gene variants have yet to be uncovered. Several molecular epidemiologic studies have assessed the association of common germline miRNA gene variation with disease risk including breast cancer [11, 18–24]. Of these studies, several show association between SNP variants in mature and precursor miRNAs and breast cancer risk in women of various racial-ethnic-religious background (US Caucasian, Chinese, and high-risk Jewish women in Israel). Some have evaluated the association of common germline miRNA variation with cancer survival [25–27] although none have reported on the association of miRNA germline variation with breast cancer risk and survival among African Americans. We sought to identify common variation in the 5′ region proximal to pre-miRNA gene sequences associated with breast cancer incidence and breast cancer-specific survival among African American and Caucasian women participating in the Carolina Breast Cancer Study (CBCS).

MATERIALS AND METHODS

Study Population

The CBCS is a population-based case-control study of breast cancer in North Carolina that has been previously described [28–30]. Briefly, eligible cases, were defined as women, ages 20–74, who were diagnosed with primary invasive breast cancer between 1993 and 2001 and resided within a 24 county area. African American cases and cases younger than 50 years old were oversampled using randomized recruitment [31]. Between 1996 and 2001, eligible women with breast carcinoma *in situ* (CIS) were also enrolled. Eligible controls were women residing in the same 24 county study area, aged 20 to 74 years, with no history of breast cancer. Controls were frequency-matched to cases by race and 5 year age categories.

Women who agreed to participate in the study provided informed consent and completed an in-home nurse-administered interview and were also asked to provide a 30 ml sample of blood for DNA. Overall, 2311 cases (894 African American/1417 non-African American) and 2022 controls (788 African American/1234 non-African American) enrolled in the study. Most (98%) non-African American participants were Caucasian. miRNA gene SNPs were genotyped in 1776 of 2022 controls and 1972 of 2311 cases in CBCS. This analysis was restricted to CBCS participants self-identifying as African American or Caucasian and included 1946 cases (742 African American/1204 Caucasian) and 1747 controls (658 African American/1089 Caucasian) (Table 1). All study procedures were approved by the University of North Carolina at Chapel Hill Institutional Review Board.

Gene Selection, SNP Identification and Genotyping

Seven human miRNA genes were selected for SNP analysis because previous reports demonstrate their association with breast cancer [32–35] (Online Resource 1). A region 1 kb in size, immediately 5′ to the pre-miRNA sequence was surveyed for common (MAF ≥ 0.05) SNPs. This size region was selected because the number of SNPs residing in this region would be feasible for inclusion on our custom genotyping array that included a larger survey of genetic variation related to breast cancer and because this size should include most if not all of the5′ pri-miRNA sequence and possibly a portion of the miRNA gene promoter sequence as well. Among the 7 miRNA genes surveyed in dbSNP (2 genes occur in miR gene clusters in close proximity to neighboring miRNA genes, specifically miR-16-1/15a and miR 34b/34c), 11 common SNPs (either or both HapMap YRI or CEPH populations) and 2 SNPs (miR-9-1 SNP rs12239077, miR-10b rs1867863) with no allele frequency data

reported in dbSNP for African Americans at the time of SNP selection were identified. Illumina pre- and post-genotyping quality control was applied to the selected SNPs (Online Resource 1). In summary, 9 polymorphic SNPs within 6 miRNA gene regions (containing 8 unique miRNA genes) were successfully genotyped, passed all quality measures and were evaluated for their association with breast cancer.

Ancestry informative markers (AIMs) were also genotyped in cases and controls to estimate African and European ancestry [36]. A set of 158 AIMs were selected from a panel that has been previously used to estimate ancestry in African Americans [37, 38].

Statistical analysis

Allele and genotype frequencies were calculated stratified by case and control status and African American or Caucasian race, Hardy Weinberg Equilibrium was assessed among controls within each race group using the chi-square test and pair-wise linkage disequilibrium (LD), r^2, was calculated using SAS Genetics (version 9.1.3) (SAS Institute, Cary, NC). Additionally, the chi-square test was used to compare differences in allele and genotype frequencies between cases and controls.

Odds ratios and 95% confidence intervals for the association between genotypes and breast cancer risk stratified by self -reported race or age at diagnosis (<50 or ≥50) were estimated among CBCS cases (invasive and CIS) and controls using unconditional logistic regression. Genotype associations were modeled using the general model (2 degrees of freedom), except where homozygote counts were too small (<30 cell size for any race-genotype category in either cases or controls) then a 2-level dominant model was used and rare homozygote and heterozygote categories were combined. The major allele (highest allele frequency) in Caucasians was selected as the referent allele for both race groups unless the two allele frequencies in Caucasians were the same; in this case the major allele in African Americans was selected as the referent allele for both race groups. No adjustment was made for multiple comparisons because all miRNA genes selected for this exploratory analysis had been previously associated with breast cancer and because several SNPs are either in linkage disequilibrium, in close genomic proximity or part of a gene family and thus may be processed or transcribed by common regulatory elements. All genotype regression models were adjusted for age, ancestry and offset term (defined as the natural log of (recruitment probability of cases/recruitment probability of controls)) to account for randomized recruitment sampling [31] and were run using SAS v9.1.3 (SAS, Cary, NC).

Haplotype analysis was performed using HAPSTAT [39, 40] to examine the association between miR-185 two-SNP haplotypes (rs887205 – rs2008591) and breast cancer risk, stratified by race or age at diagnosis in cases (invasive and CIS) and controls using a co-dominant model. HAPSTAT simultaneously uses maximum likelihood estimation and the EM algorithm to estimate OR parameters and haplotype distributions. Haplotype analyses were adjusted for age, race (as appropriate), ancestry, and study phase. Modification to HAPSTAT allowed for the inclusion of the offset term to account for randomized recruitment[41].

For the analysis of breast cancer-specific survival, only invasive breast cancer cases (654 African American/855 Caucasian) with available follow-up data were analyzed. Breast cancer specific deaths were identified using the National Death Index and additional details of cohort follow-up have been previously reported [42]. After censoring living individuals at December 31, 2006, we first examined breast cancer-specific survival curves by genotype using the Kaplan-Meier method (Online Resource 2). Violations of the proportional hazards assumption were assessed by visual inspection of log-log survival curves and a test of the SNP by time product term. To assess differences in survival log-rank tests were used. SNPs

met proportional hazards assumptions if they passed visual inspection (i.e., log-log survival curves did not cross) *or* the p-value for the interaction term with log time was >0.05. SNPs that met proportional hazards criteria were further evaluated to determine appropriate referent genotype(s) and for their association with breast cancer survival. Consistent with the risk analysis, genotypes previously collapsed due to small cell size (genotype category <30) remained collapsed in the survival analysis. After applying the small cell size rule, SNP genotype categories with nearly identical survival rates on visual inspection were also collapsed into a single group. The referent was kept consistent with the risk analysis and was based on the most common genotype in Caucasians. For SNPs that met our criteria (passed proportional hazards assumption and log rank p-value < 0.10), we estimated hazard ratios (HR) and 95% confidence intervals (CI) for associations with breast cancer-specific survival using multivariable Cox proportional hazard models adjusted for age, self-reported race, and ancestry. We further adjusted for stage among those cases (618 African Americans/804 Caucasians) with available information. Results are presented for the overall cohort and stratified by race. In light of the association between miR-206 whose target gene is the estrogen receptor, results were further stratified by estrogen receptor status (+/−).

RESULTS

miRNA SNP characteristics

This report provides allele and genotype frequency data for African Americans on several miRNA SNPs (rs12239077 [miR-9-1], rs1867863 [miR-10b]) for which dbSNP data was unavailable at the time of SNP selection. For those SNPs with dbSNP genotype data on African American or European sample groups, allele frequencies were generally consistent with those observed in African American and Caucasian CBCS controls. Our data provide additional accuracy for miRNA SNP allele and genotype frequency estimates for African Americans living in the southeastern US (Online Resource 3).

As expected, varying LD is present among SNPs genotyped within the same gene and is consistently higher among Caucasians compared to African Americans (Online Resource 4). For miR-185, LD between all SNPs is moderate to high among Caucasians; however for African Americans LD is low between rs2078749 and other miR-185 SNPs, but high between rs2008591 and rs887205. LD between SNPs in miR-206 is low for both Caucasians and African Americans.

Several of the SNPs genotyped and analyzed for their association with breast cancer are located within miRNA gene clusters and thus are close in proximity to more than one miRNA gene (Online Resource 5). Of the eight miRNA genes in close proximity to genotyped SNPs, 4 are intergenic, while 4 are within annotated genes (intragenic) either embedded within introns (N=3; miR-9-1, miR-34b, miR-185) or exons (N=1; miR-34c). This distribution is consistent with the genomic distribution of all miRNAs in miRBase as reported by Hinske et al. in 2010 [43].

miRNA SNP and haplotype associations with breast cancer risk

miRNA SNP associations with breast cancer overall were modest, with many effect estimates near the null (Table 2). ORs ranged from 0.71 to 1.16 (Table 2). A single gene emerged, miR-185, with 2 of the 3 SNPs (rs2008591 and rs887205) genotyped having ORs suggestive of an inverse association with breast cancer among African Americans. These SNPs are within 156 basepairs (bp) of each other, in high LD among African Americans (Online Resource 4) and 590 bp upstream of the pre-miRNA-185 sequence, which lies within an intron of the C22orf25 gene (Online Resource5). Results did not differ when cases with *in situ* (CIS) breast cancer were excluded (data not shown). No associations between

miRNA SNPs and breast cancer risk were identified when the analysis was stratified by age at diagnosis (<50 or ≥50) (data not shown).

There were two common miR-185 (rs887205 – rs2008591) haplotypes in the population. As expected, the race stratified associations were significant for African Americans where ORs were 0.71 (95% CI = 0.53 – 0.96) for two copies (vs. 0) of the G-T haplotype and 1.44 (95% CI = 1.07 – 1.94) for two copies (vs. 0) of the A-C haplotype (Table 3). No haplotype associations were observed among Caucasians or women age <50 or ≥50 at diagnosis (Table 3).

miRNA SNP breast cancer –specific survival analysis

None of the miRNA SNPs evaluated failed the proportional hazards assessment (SNPs met proportional hazard assumptions if they passed visual inspection (i.e., log-log survival curves did not cross) *or* the p-value for the interaction term with log time was >0.05) and therefore none of the SNP genotype HRs are time-stratified (Online Resource 6). Survival distributions by genotype of several miRNA genes were significantly different (unadjusted p-value ≤0.10) from one another based on the log-rank test (miR-16-1/15a rs9535416, miR34b/34c rs4938723 and miR-206 rs6920648).

The 3 SNPs further evaluated for association with breast cancer-specific survival also demonstrated modest effect sizes with one near the null. For the overall analysis, HRs (for Model 1) ranged from 0.57 to 1.05 (Table 4). Two miRNA gene regions, miR-34b/34c rs4938723 (HR = 0.57 (95% CI = 0.37 – 0.89 p-value = 0.01) and miR-206 rs6920648 (HR = 0.77 (95% CI = 0.61 – 0.97, p-value = 0.02) provided suggestive evidence of association with breast cancer survival following adjustment for age, self-reported race and ancestry. In the race and ER stratified analyses, associations were stronger among African Americans for miR-34b/34c, while Caucasians and women with ER+ tumors demonstrated stronger association for miR-206. No association was observed for women with ER- tumors. Further adjustment for stage resulted in somewhat more modest associations with survival (HR = 0.65 (95% CI = 0.42 – 1.02, p-value = 0.06 and HR = 0.79 (95% CI = 0.62 – 1.00, p-value = 0.05, respectively) (Table 4).

DISCUSSION

We analyzed candidate SNPs in the region 1 kb 5′ to the pre-miRNA sequence for 6 genomic regions containing 8 miRNAs to assess their effects on breast cancer risk and survival in a population-based study of African American and Caucasian women. We hypothesized that these SNPs would be within regions of the primary miRNA transcript that might control miRNA biogenesis or in further upstream promoter regions that might regulate miRNA transcription. With respect to breast cancer risk, our main finding was that 2 miR-185 SNPs in in linkage disequilibrium with one another, rs2008591 (T allele) and rs887205 (G allele), were inversely associated with breast cancer; associations between these variants and breast cancer were modest. In the case of these 2 SNPs the minor allele was flipped between African Americans and Caucasians, thus the alleles inversely associated with breast cancer are both the most common alleles in African Americans. Our study expands the set of SNPs and miRNA genes evaluated for association with breast cancer risk since no previous studies have examined variants in the region 5' to the pre-miRNA sequence. Additionally no other studies have reported on the association of miRNAs with breast cancer risk in African Americans.

The miR-185 gene is intragenic, located within the intron and on the same DNA strand as the gene C22orf25. miR-185 is involved in cell cycle regulation and functions as a tumor suppressor. Its expression has been shown to induce G1 cell cycle arrest in lung and

colorectal cancer cells, and induce apoptosis, inhibit the proliferation potential, and block invasion of colorectal cancer cells [44, 45]. An analysis of putative targets for mir-185 has implicated both RhoA and Cdc42, two key proteins involved in cellular proliferation [46]. For the 2 miR-185 SNPs associated with breast cancer in African Americans, LD was high (r^2=0.78), suggesting they may either be markers linked to a common breast cancer associated variant or may each play a role in breast cancer susceptibility as the result of their co-localization to a highly conserved regulatory region. Haplotype analysis further confirmed the association of the two SNP G-T haplotype with lower breast cancer risk in African Americans. While it is possible that these associations represent false positive findings several lines of evidence support this pre-miR-185 5′ flanking region as a potentially important breast cancer genetic risk factor for African Americans that deserve further follow-up. Specifically in this analysis the sample size for each genotype group are sufficient, the direction of the effect is consistent in these tightly linked SNPs, the association is biologically plausible given known function of this miR, and the association is consistent only among African Americans.

In addition to an evaluation of breast cancer risk, we assessed a subset of miRNA SNPs for their association with breast-cancer specific survival. SNPs rs4938723 (miR-34b/34c) and rs6920648 (miR-206) were significantly associated with breast cancer-specific survival prior to adjustment for stage and remained weakly associated with breast cancer-specific survival after adjustment for stage. A number of miRNAs have been associated with breast cancer prognosis and survival in previous studies including miR-34 and miR-206, both of which function as tumor suppressor miRNAs and have been shown to be lost in breast cancers [16, 47–49]. Specifically, the miR-34b gene resides in a CpG island within the intron of the BC021736 on the coding strand. Therefore, it is likely that miR-34b is co-transcribed by either the protein-coding gene's promoter or by its own transcription initiation region as is the case with most miRNAs whether they are intergenic or embedded within the introns of protein coding genes [50]. miR-34b (concomitantly with miR-34a and c) has been shown to be silenced in numerous cancers by DNA methylation of its own promoter [51]. All three genes are transactivated by the tumor suppressor, p53 [52–57]. One of the negatively regulated miR-34b target genes is receptor tyrosine kinase c-MET [58]. Increased levels of receptor tyrosine kinase c-MET have been shown to lead to enhanced invasion and metastasis in a number of cancers including breast [59] and have recently been associated with progression of basal-like breast cancer a subtype more common among younger African American women [60]. Thus miR-34b acts as a tumor suppressor gene by down-regulating receptor tyrosine kinase c-MET. In our analysis the miR-34b/34c rs4938723 CC genotype was associated with reduced breast cancer-specific mortality. Conversely women with one or two copies of the T allele (TC or TT) had poorer breast cancer-specific survival (Online Resource 2). Thus this SNP may contribute to alterations in miR-34b/34c promoter function or biogenesis. Specifically, the miR-34b/34c T allele may reduce activation of miR-34b/34c by p53, facilitate promoter methylation or decrease biogenesis. Any of these actions could lead to a reduction in miR-34b/34c levels that subsequently lead to up-regulation of c-MET, enhancing invasion and metastasis and leading to poorer survival. Given miR-34b tumor suppressor function and target gene, it is not surprising that this SNP is associated with breast cancer survival rather than breast cancer development.

The dysregulation of miR-206 also plays an important role in the molecular mechanisms of breast cancer risk and progression and its role has recently been reviewed by O'Day and Lal [16]. This intergenic miRNA functions as a tumor suppressor gene by inhibiting its target gene, the estrogen receptor gene ERα (ESR1) [61], with up-regulation observed in ERα-negative [33] and down-regulation in ERα-positive tumors [62]. miR-206 and several other miRNA genes have been shown to induce cell cycle arrest, inhibit estrogen-induced proliferation [62] and be down-regulated in metastatic breast cancer cells. Restoring the

expression of these miRNA genes has been demonstrated to reduce their invasive capability [35]. Specifically, restoring miR-206 in metastatic cancer cells alters their cellular morphology, potentially contributing to a decrease in cell motility and subsequent migration [35]. This evidence suggests that miR-206 could be a candidate for novel breast cancer therapies [16].

The strengths of this study include a systematic analysis of variation within a 1 kb 5′ region proximal to pre-miRNA sequences in a set of breast cancer candidate miRNAs in an African American and Caucasian cohort with comprehensive long-term follow-up data on breast cancer survival. While a portion of our selected SNPs failed pre-genotyping QC, this was not entirely unexpected. In particular, challenges arise in assay development when SNPs are in close proximity to one another and in genomic regions not well surveyed (introns and intragenic regions with potential duplication and repetitive regions). Generally, genotyped SNPs sufficiently covered gaps incurred by failed SNPs through their genomic location and LD. The study population was large enough that we were able to estimate race-stratified SNP associations with breast cancer risk. All analyses for associations with cancer risk and survival were adjusted for individual proportions of European ancestry, minimizing residual confounding due to population stratification within both self-reported race-stratified and race-combined groups.

While this study was exploratory in nature and evaluated only a 1 kb region immediately 5′ to the pre-miRNA sequence among a small set of genes associated with breast cancer – this report provides valuable new information regarding the possible association of miRNA sequence variation in breast cancer risk and survival in both African Americans and Caucasians for further validation in other cohorts. Additional research should not only include validation of the findings presented in this report, but should also include; 1) a survey of a larger region proximal to the 5′ pre-miRNA sequence to more fully capture pri-miRNA and promoter variation, and 2) a more comprehensive sequence assessment of all miRNAs genes related to breast cancers. Future studies such as these will begin to provide a more complete picture of the influence germline miRNA sequence variation on breast cancer risk and survival.

CONCLUSIONS

In conclusion, the consistency of the strength and direction of association with breast cancer among African Americans points to a tightly linked region proximal to pre-miR-185 as a promising candidate gene that may contribute to breast cancer risk among African Americans. These results also suggest that miR-34b/34c and miR-206 may be important genes influencing breast cancer-specific survival. To our knowledge this study is among the first to examine sequence variation flanking the premiRNA sequence region for its association with breast cancer risk and survival in both African Americans and Caucasians and points to the need for publicly available annotation of miRNA-specific primary transcripts and promoter regions that can be surveyed for sequence variation. Our results and those of others [18–27] also highlight the need to conduct comprehensive miRNA gene sequence assessment and validation in a variety of racial-ethnic populations in particular among African American women. Expanded knowledge of miRNA transcriptional regulation through comparative genomics using next-generation bioinformatics will allow the identification of pri-miRNA sequences, promoters, transcription start sites and many other regulatory features of miRNA transcription and biogenesis and will facilitate our comprehensive survey of disease-related variants in these regions in the future. In addition to comparative genomics, the integration of large datasets that include expression array and patient outcome data related to treatment and survival will enhance our ability to assess the

complex role of miRNAs and miRNA germline variation in breast cancer and the potential for miRNA-based therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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LIST OF ABBREVIATIONS

3′-UTR	3′- untranslated region
AIMs	ancestry informative markers
CBCS	Carolina Breast Cancer Study
cDNA	complementary DNA made from an mRNA template
CI	confidence interval
CIS	breast carcinoma *in situ*
HR	hazard ratio
kb	kilobase
LD	linkage disequilibrium
MAF	minor allele frequency
miRNA or miR	microRNA
mRNA	messenger RNA
nt	nucleotide
OR	odds ratio
Pol II	polymerase II
Pol III	polymerase III
pre-miRNA	precursor microRNA
pri-miRNA	primary miRNA transcript
SNP	single nucleotide polymorphism

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Table 1

Characteristics of CBCS participants with genotypmg data

Characteristic	Cases				Controls			
	African American		Caucasian		African American		Caucasian	
	N	%	N	%	N	%	N	%
N	742	100	1204	100	658	100	1089	100
Median age in years (range)	51 (23–74)		50 (24–74)		50 (26–74)		51 (21–74)	
Proportion of European ancestry Mean (range)	0.222 (0.054–0.962)		0.936 (0.110–1)		0.226 (0.045–1)		0.934 (0.083–1)	
Age (years)								
<50	355	47.8	592	49.2	314	47.7	491	45.1
>=50	387	52.2	612	50.8	344	52.3	598	54.9
Menopausal status								
Premenopausal	324	43.7	540	44.9	290	44.1	456	41.9
Postmenopausal	418	56.3	664	55.2	368	55.9	633	58.1
Stage								
CIS[a]	88	11.9	349	29.0				
1	216	29.1	393	32.6				
2	299	40.3	328	27.2				
3	76	10.2	68	5.7				
4	27	3.6	15	1.3				
Missing[b]	36	4.9	51	4.2				
Tumor Marker Status								
ER+	322	43.4	672	55.8				
ER−	341	46.0	344	28.6				
Missing	79	10.6	188	15.6				
ER−/PR−/HER2−	203	27.4	145	12.0				

[a] CIS carcinoma in situ

[b] Invasive breast cancer cases

Table 2

Association between miRNA SNPs and breast cancer in CBCS cases (invasive and CIS) and controls

		African Americans				Caucasians			
Locus/SNP	**Genotype**	**Cases (N)**	**Controls (N)**	**OR (95% CI)[a]**	**P-value[a,b]**	**Cases (N)**	**Controls (N)**	**OR (95% CI)[a]**	**P-value[a,b]**
mir-9-1									
rsl2239077	AA	707	631	Referent		1204	1087	Referent	
	AG+GG	35	27	1.14 (0.67 – 1.95)	0.63	0	2	na	na
mir-9-2									
rs 1501672	TT	481	415	Referent		914	841	Referent	
	CT+CC	261	243	0.91 (0.72 – 1.14)	0.39	290	248	1.05 (0.86 – 1.30)	0.61
mir-16-1/15a									
rs9535416	GG	445	397	Referent		281	259	Referent	
	AG+AA	297	261	1.03 (0.83 – 1.29)	0.78	923	830	1.02 (0.83 – 1.26)	0.82
mir-34b/34c									
rs4938723	TT	362	343	Referent		496	430	Referent	
	TC	317	257	1.16 (0.92 – 1.46)	0.21	563	503	0.93 (0.77 – 1.12)	0.46
	CC	63	58	1.08 (0.73 – 1.61)	0.69	144	155	0.80 (0.60 – 1.05)	0.10
mir-185									
rs2008591	CC	204	170	Referent		424	390	Referent	
	CT	383	306	1.11 (0.86 – 1.45)	0.42	586	527	1.01 (0.84 – 1.23)	0.90
	TT	155	182	0.72 (0.53 – 0.98)	**0.04**	193	172	1.05 (0.81 – 1.37)	0.71
rs887205	AA	173	132	Referent		397	365	Referent	
	AG	360	304	0.92 (0.69 – 1.21)	0.54	595	533	1.02 (0.84 – 1.24)	0.85
	GG	208	222	0.71 (0.52 – 0.96)	**0.03**	210	191	1.02 (0.79 – 1.32)	0.87
rs2078749	AA	447	412	Referent		299	270	Referent	
	AG+GG	294	246	1.08 (0.86 – 1.35)	0.50	904	819	1.00 (0.82 – 1.23)	0.97
mir-206									
rs6920648	AA	342	282	Referent		370	325	Referent	
	AG	308	296	0.84 (0.66 – 1.05)	0.13	564	554	0.85 (0.70 – 1.04)	0.11
	GG	91	80	0.97 (0.69 – 1.38)	0.88	269	209	1.15 (0.90 – 1.47)	0.28
rsl6882131	CC	365	342	Referent		671	592	Referent	

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		African Americans				Caucasians			
Locus/SNP	**Genotype**	**Cases (N)**	**Controls (N)**	**OR (95% CI)[a]**	**P-value[a,b]**	**Cases (N)**	**Controls (N)**	**OR (95% CI)[a]**	**P-value[a,b]**
	CT	308	254	1.15 (0.92 – 1.45)	0.22	458	419	0.94 (0.79 – 1.13)	0.53
	TT	69	62	1.00 (0.67 – 1.47)	0.98	75	78	0.82 (0.58 – 1.18)	0.29

[a]P-values and ORs adjusted for age, ancestry and offset term is for general or dominant model (in the case of small sample size for rare homozygote). The offset term is included in the model using the OFFSET option in SAS Proc Logistic.

[b]P-value is unadjusted for multiple comparisons

Table 3

Association between miR-185 two-SNP haplotypes (rs887205 – rs2008591) and breast cancer in CBCS cases (invasive and CIS) and controls

Haplotype	Estimated Haplotype Frequency*		Copies	OR (95% CI)[a]	P-value[b]
	Cases	Controls			
African American					
A-C	0.475	0.430			
			0	Referent	
			1	1.15 (0.94 – 1.41)	0.16
			2	1.44 (1.07 – 1.94)	**0.02**
G-T	0.466	0.508			
			0	Referent	
			1	0.90 (0.73 – 1.10)	0.29
			2	0.71 (0.53 – 0.96)	**0.02**
Caucasian					
A-C	0.578	0.580			
			0	Referent	
			1	1.03 (0.86 – 1.23)	0.78
			2	1.00 (0.78 – 1.26)	0.97
G-T	0.404	0.400			
			0	Referent	
			1	1.03 (0.89 – 1.20)	0.66
			2	1.02 (0.80 – 1.30)	0.85
Age at diagnosis <50					
A-C	0.549	0.525			
			0	Referent	
			1	1.03 (0.84 – 1.25)	0.80
			2	1.19 (0.91 – 1.56)	0.21
G-T	0.414	0.440			
			0	Referent	
			1	0.88 (0.74 – 1.05)	0.15
			2	0.82 (0.62 – 1.08)	0.15
Age at diagnosis ≥50					
A-C	0.530	0.522			
			0	Referent	
			1	1.07 (0.89 – 1.29)	0.47
			2	1.09 (0.84 – 1.40)	0.52
G-T	0.440	0.441			
			0	Referent	
			1	1.08 (0.91 – 1.28)	0.38
			2	0.96 (0.74 – 1.24)	0.74

[a]Haplotype analysis performed using HAPSTAT and co-dominant model.

[b] Adjusted for age, race (as appropriate), ancestry and study phase (phase 1, phase 2 invasive, CIS study). Modifications to HAPSTAT allowed for the inclusion of the offset term to account for randomized recruitment probabilities.

Table 4

Effect of germline miRNA SNP on breast cancer–specific mortality among invasive cases

		Model 1[a]		Model 2[b]	
SNP	**Genotype**	**HR (95% CI)**	**P[1]-value[c]**	**HR (95% CI)**	**P[2]-value[c]**
Overall					
miR-16-1/15a	GG	Referent			
rs9535416[d]	AG+AA	1.05 (0.82 – 1.33)	0.70	1.12 (0.87 – 1.44)	0.40
miR-34b/34c	TC+TT	Referent			
rs4938723[e]	CC	0.57 (0.37 – 0.89)	**0.01**	0.65 (0.42 – 1.02)	0.06
miR-206	AA	Referent			
rs6920648[e]	AG+GG	0.77 (0.61 – 0.97)	**0.02**	0.79 (0.62 – 1.00)	**0.05**
African American					
miR-16-1/15a	GG	Referent			
rs9535416[d]	AG+AA	1.05 (0.78 – 1.42)	0.74	1.16 (0.85 – 1.58)	0.35
miR-34b/34c	TC+TT	Referent			
rs4938723[e]	CC	0.49 (0.25 – 0.95)	**0.03**	0.63 (0.32 – 1.23)	0.18
miR-206	AA	Referent			
rs6920648[e]	AG+GG	0.85 (0.63–1.14)	0.28	0.93 (0.68 – 1.25)	0.62
Caucasian					
miR-16-1/15a	GG	Referent			
rs9535416[d]	AG+AA	1.04 (0.70 – 1.56)	0.84	1.03 (0.67 – 1.59)	0.89
miR-34b/34c	TC+TT	Referent			
rs4938723[e]	CC	0.66 (0.37 – 1.20)	0.17	0.65 (0.36 – 1.18)	0.16
miR-206	AA	Referent			
rs6920648[e]	AG+GG	0.66 (0.47–0.94)	**0.02**	0.62 (0.43 – 0.90)	**0.01**
ER+ tumor status					
miR-16-1/15a	GG	Referent			
rs9535416[d]	AG+AA	0.86 (0.59 – 1.27)	0.45	0.90 (0.60 – 1.35)	0.61
miR-34b/34c	TC+TT	Referent			
rs4938723[e]	CC	0.42 (0.20 – 0.90)	**0.03**	0.54 (0.25 – 1.17)	0.12
miR-206	AA	Referent			
rs6920648[e]	AG+GG	0.74 (0.52 – 1.07)	0.11	0.77 (0.53 – 1.12)	0.18
ER− tumor status					
miR-16-1/15a	GG	Referent			
rs9535416[d]	AG+AA	1.28 (0.92 – 1.77)	0.14	1.25 (0.90 – 1.73)	0.19
miR-34b/34c	TC+TT	Referent			
rs4938723[e]	CC	0.78 (0.45 – 1.36)	0.38	0.81 (0.47 – 1.40)	0.45

		Model 1[a]		Model 2[b]	
SNP	**Genotype**	**HR (95% CI)**	**P[1]-value[c]**	**HR (95% CI)**	**P[2]-value[c]**
miR-206	AA	Referent			
rs6920648[e]	AG+GG	0.81 (0.59 – 1.10)	0.18	0.80 (0.58 – 1.09)	0.16

[a] Model 1; Hazard Ratio adjusted for age, self-reported race (as appropriate) and ancestry

[b] Model 2; Hazard Ratio adjusted for age, self-reported race (as appropriate), stage (1,2,3+4), and ancestry

[c] P-value is unadjusted for multiple comparisons

[d] Genotype categories collapsed due to small cell size

[e] Genotype categories collapsed due to nearly identical survival rates