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14. ABSTRACT The purpose of this research is to investigate protein expression and promoter region DNA methylation status of six genes involved in epithelial-mesenchymal transition in relation to breast cancer lymph node metastasis, recurrence, and survival. Breast tumor tissue has been retrospectively identified from the Pathology Resource and immunohistochemical staining for all proteins has been completed. DNA from these tumor tissues has been obtained. Analyses of IHC-stained tissues and DNA methylation are ongoing.						
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Introduction: Breast cancer is incurable upon metastasis to distant organs, and metastasis to axillary lymph nodes is regarded as a critical prognostic factor for future recurrence and survival. Understanding the epidemiology and biology of metastasis could lead to better stratification of recurrence risk. We proposed to study genes related to epithelial-mesenchymal transition (EMT), invoking the hypotheses that EMT may explain the ability of tumor cells to form metastatic lesions and that these genes are regulated via DNA methylation. It is hypothesized that tumor cells co-opt the EMT program to transiently acquire properties generally reserved for mesenchymal cells, namely the ability to detach and migrate. The objectives of this project are to interrogate the protein expression and promoter methylation of six EMT-related genes: E-cadherin, N-cadherin, Vimentin, Twist1, RelB, and SATB1. Protein expression has been measured via immunohistochemistry (IHC) in breast tumor tissue and promoter methylation will be measured using DNA derived from these tumor samples. Protein expression and methylation status will be correlated with lymph node metastasis at diagnosis, time to metastatic recurrence, and disease-free survival. Effect modification by tumor grade, hormone receptor status, and HER2 status will also be investigated. This annual report describes the training and research accomplishments associated with the tasks outlined in the Statement of Work.

Training Plan, months 13-24

Tasks 1 and 5. All didactic predoctoral program requirements have been completed. Oral proposal defense was successfully completed 3/14/2012.

Task 2. Ongoing attendance in journal clubs for the Cancer Prevention research group and the Epigenetics research group at Roswell Park Cancer Institute (RPCI), works-in-progress meetings, weekly Institute-wide seminar series, and monthly Breast Disease Site Research Group meetings. Attendance and poster presentation at the 2012 American Association for Cancer Research (AACR) Annual Meeting (1).

Task 3. Continuation of research in molecular epidemiology focusing on molecular and genetic factors relating to lymph node metastasis, recurrences, and survival.

- A project investigating associations between polymorphisms *BRMS1* and *SIPA1* and lymph node status, tumor grade, tumor subtype, time to recurrence, overall survival, and disease-free survival will be submitted for publication, following extensive re-analyses with updated follow-up data obtained via linkage to the National Death Index.
- A second project aims to compare the effect of polymorphisms in metastasis-related genes on the risk of aggressive tumor characteristics and lymph node positive breast cancer at diagnosis between African-American and European-American women. Genotyping has been completed and data analysis is in progress. We expect to submit this manuscript for publication by early 2013.

- Findings from a third analysis of the relationship between tumor size and nodal metastases by tumor subtype were presented as a poster at the 2012 AACR Annual Meeting (Appendix 1). We expect to submit a manuscript for publication in early 2013.

Task 4. Analysis of IHC stains is ongoing and assays for methylation analysis are currently in the process of being designed (discussed in greater detail in the research plan update).

Research Plan, months 13-24

Task 1. The main focus of this task is to evaluate the associations between protein expression of genes involved in the EMT (E-cadherin, N-cadherin, Vimentin, Twist1, RelB, and SATB1) and lymph node metastases at the time of primary breast cancer diagnosis.

- IHC staining has been completed and interpretation of the stains is ongoing. To maintain blinding to study outcomes, associated data will be requested when interpretation of the stained tissue is nearly complete.

Task 2. Conduct the laboratory assays necessary for Specific Aim 3, which proposes to evaluate gene promoter methylation of the E-cadherin, N-cadherin, Vimentin, Twist1, RelB, and SATB1 genes.

- DNA samples have been acquired for 458 participants. We have requested DNA from additional participants as available, with emphasis on those participants with matching primary and metastatic tumor cores. This request is currently being processed by Pathology Resource staff.
- Preliminary testing of the DNA already acquired indicates that there may be issues with quality and quantity of material for downstream applications. We are currently investigating the scope of this potential problem. We have decided, based on extensive discussions of the merits and limitations of various methylation assays, that the best choice for this particular project is methylation-sensitive high resolution melting analysis. This technique is qPCR-based and is easy to implement as well as relatively inexpensive for screening of differentially methylated regions. Regions of interest can be further interrogated using pyrosequencing. We expect, pending results of DNA quality analyses and primer design and optimization, to begin the methylation assays in the next several weeks.

Key Research Accomplishments:

None in this reporting period.

Reportable Outcomes:

- Submission of an abstract to the 2012 AACR Annual Meeting, which was presented as a poster in April 2012 (Appendix 1).

Conclusion: There has been steady progress toward completed the tasks outlined in the Statement of Work. Interpretation of stained tumor tissue and preparation of DNA for methylation analyses is ongoing.

References:

1. Roberts MR, Bandera EV, Hwang H, Ciupak G, Zirpoli G, Yao S, Pawlish K, Davis W, Jandorf L, Bovbjerg DH, and Ambrosone CB. Tumor size and lymph node metastases in African-American and European-American women with breast cancer [abstract]. In: Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research; 2012 Mar 31-Apr 4; Chicago, IL. Philadelphia (PA): AACR; 2012. Abstract number 3593.

Appendix 1. Abstract presented at the 2012 AACR Annual Meeting.

Tumor size and lymph node metastases in African-American and European-American women with breast cancer

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Few studies have evaluated lymph node metastasis in African-American (AA) women with breast cancer, who are more likely to be diagnosed at an advanced stage and with lymph node positive tumors. Likelihood of nodal involvement increases with tumor size, although recent data have indicated that this may not be true for AA breast cancer patients and patients with basal-like tumors. Nodal metastases are also more likely in premenopausal AA patients compared to either premenopausal European-American (EA) patients or postmenopausal AA and EA patients. We examined risk factors for lymph node metastasis at breast cancer diagnosis in AA and EA women, and investigated the contributions of race, tumor subtype, and menopausal status to the tumor size-lymph node metastasis relationship. This analysis included 805 women diagnosed with primary, incident breast cancer enrolled in the Women's Circle of Health Study, a case-control study of AA and EA breast cancer patients and healthy women. Cases were identified using hospital-based ascertainment in New York City hospitals with high referral patterns for AA women and through population-based ascertainment in New Jersey using the State Cancer Registry. Eligible cases were self-identified AA and EA women age 20-75 with no previous history of cancer other than nonmelanoma skin cancer. In-person interviews were conducted and consent to review pathology reports was obtained. Tumor size was categorized as tumors 2 cm or less (small tumors) and tumors greater than 2 cm (large tumors). Unconditional logistic regression was used to estimate odds ratios (OR) and 95% confidence intervals (CI). AAs with small tumors were more likely to be node positive compared to EAs (OR=1.24, 95% CI 0.81-1.88) while among patients with large tumors, AAs were less likely to be node positive (OR=0.83, 95% CI 0.49-1.41). When grouped by race and tumor subtype, we found that the triple negative subtype was associated with a decreased risk of nodal metastases among EA women with small tumors (OR=0.17, 95% CI 0.04-0.79) and a nonsignificantly decreased risk among AA women with large tumors, using the luminal A subtype as the referent group. Associations were null in EA women with large tumors and AA women with small tumors. When grouped by race and ER status, ER negativity was associated with a decreased risk of nodal metastases among AA women with large tumors (OR=0.41, 95% CI 0.20-0.84), while AA women with small tumors were at increased risk (OR=1.90, 95% CI 0.92-3.91). Our data suggest an effect of race and tumor subtype on the relationship between tumor size and likelihood of lymph node metastases. Tumor size appears to affect lymph node metastasis differently by race, a mechanism that is modified by tumor biology. Our findings support the hypothesis that in AA breast cancer patients, large tumors may not be more likely to give rise to metastatic lymph nodes.

Tumor Size and Lymph Node Metastasis in African-American and European-American Women with Breast Cancer

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Background and Methods

- Lymph node status is an important predictor of prognosis, and likelihood of nodal involvement increases with tumor size.
- Previous data indicates that larger tumors may not predict risk of nodal metastases among African-American patients or those with basal-like tumors.
- The purpose of this analysis was to investigate the contributions of race, menopausal status, and tumor subtype to the tumor size-lymph node metastasis relationship.
- Women with breast cancer were enrolled in the Women's Circle of Health Study (WCHS), a case-control study recruiting hospital-based cases from New York City hospitals and population-based cases through the New Jersey State Cancer Registry.
- Self-identified African-American and European-American women age 20-75 with no previous history of cancer other than nonmelanoma skin cancer were eligible for enrollment.
- This analysis was limited to 721 cases diagnosed with stage I, 2, and 3 invasive breast cancer, who were enrolled between 2003 and 2011 and have available lymph node status and tumor size.
- Unconditional logistic regression was used to estimate age-adjusted odds ratios (OR) and 95% confidence intervals (CI) for the likelihood of having a node positive tumor at diagnosis.

Table 1. Characteristics of WCHS Participants

Characteristic	African-American (N=371)		European-American (N=350)	
	Node Positive (N=162)	Node Negative (N=209)	Node Positive (N=124)	Node Negative (N=226)
Age at diagnosis, mean (SD)	48.3 (9.8)	52.5 (10.4)†	50.1 (10.0)	54.3 (10.0)†
BMI, mean (SD)	31.3 (6.7)	31.4 (6.9)	27.0 (5.9)	26.8 (5.2)
Age at menarche, mean (SD)	12.6 (2.0)	12.5 (1.8)	12.4 (1.6)	12.4 (1.4)
Parity				
Nulliparous	33 (20.4)	30 (14.4)	38 (30.7)	66 (29.2)
Parous	129 (79.6)	179 (85.7)	86 (69.4)	160 (70.8)
Menopausal status				
Premenopausal	91 (56.2)	81 (38.9)†	74 (59.7)	104 (46.0)†
Postmenopausal	71 (43.8)	127 (61.1)	50 (40.3)	122 (54.0)
Family history				
Yes	18 (11.1)	34 (16.3)	32 (25.8)	51 (22.6)
No	144 (88.9)	175 (83.7)	92 (74.2)	175 (77.4)
ER status				
Positive	90 (64.8)	125 (65.1)	90 (82.6)	163 (78.7)
Negative	49 (35.3)	67 (34.9)	19 (17.4)	44 (21.3)
PR status				
Positive	73 (51.8)	104 (55.3)	81 (74.3)	143 (71.1)
Negative	68 (48.2)	84 (44.7)	28 (25.7)	58 (28.9)
HER2 status				
Positive	28 (21.2)	35 (19.9)	23 (23.2)	26 (13.8)
Negative	104 (78.8)	141 (80.1)	76 (76.8)	162 (86.2)
Tumor grade				
Low	13 (8.3)	28 (14.3)	18 (15.9)	66 (30.1)†
Moderate	59 (37.8)	75 (38.3)	54 (47.8)	87 (39.9)
High	84 (53.9)	93 (47.5)	41 (36.3)	65 (29.8)
Tumor size				
≤2 cm	68 (42.0)	133 (63.6)†	69 (55.7)	191 (84.5)†
>2 cm	94 (58.0)	76 (36.4)	55 (44.4)	35 (15.5)
Stage at diagnosis				
1	4 (2.5)	131 (63.0)†	11 (8.9)	187 (82.7)†
2	84 (51.9)	77 (37.0)	77 (62.1)	38 (16.8)
3	74 (45.7)	0 (0.0)	36 (29.0)	1 (0.4)

†p<0.001; †p<0.0001; †p-values from t-test, Chi-squared or Fisher's exact tests, as appropriate.

One stage 4 patient is included in the category for African-American women with stage 3, node positive tumors.

BMI=body mass index; ER=estrogen receptor; PR=progesterone receptor; AA=African-American; EA=European-American.

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Table 2. Race and Tumor Size are Associated with Increased Likelihood of Lymph Node Metastases

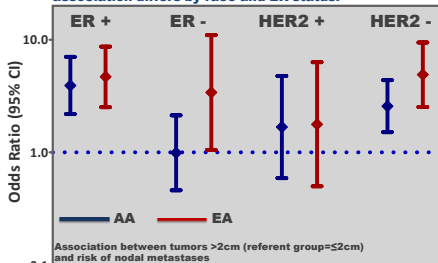
Characteristic		Node Positive (N=286)	Node Negative (N=435)	OR (95% CI)
Race	European-American	124 (43.3)	209 (48.0)	1.00
	African-American	162 (56.6)	226 (52.0)	1.31 (0.97-1.78)
Menopausal Status	Postmenopausal	121 (42.3)	249 (57.2)	1.00
	Premenopausal	165 (57.7)	185 (42.5)	0.97 (0.62-1.51)
Tumor Subtype	Luminal A	128 (58.2)	217 (62.2)	1.00
	Luminal B	32 (14.5)	35 (10.0)	1.29 (0.75-2.22)
	HER2-enriched	16 (7.3)	25 (7.2)	0.99 (0.50-1.96)
	Triple negative	44 (20.0)	72 (20.6)	0.88 (0.56-1.38)
Tumor Size	≤2cm	137 (47.9)	324 (74.5)	1.00
	>2cm	149 (52.1)	111 (25.5)	3.07 (2.22-4.24)

Unconditional logistic regression used to estimate age-adjusted odds ratios and 95% confidence intervals for the likelihood of node positive tumors (compared to node negative). Tumor subtypes were defined as follows: Luminal A: ER and/or PR positive, HER2 negative; Luminal B: ER and/or PR positive, HER2-enriched: ER and PR positive, HER2 positive; Triple negative: ER, PR, and HER2 negative. Receptor status was missing for 152 participants. OR=odds ratio; CI=confidence interval.

- African-American race and tumor size >2cm were associated with increased likelihood of lymph node metastases. High grade tumors were more likely to be node positive in European-American women only (Tables 1 and 2).

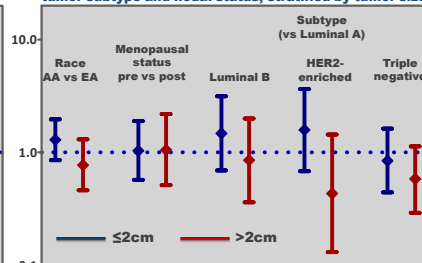
- Tumor size >2cm was significantly associated with increased likelihood of nodal metastases regardless of race or menopausal status. By subtype, tumor size was significantly associated with nodal metastases in the luminal A and triple negative subtypes. There was no effect of tumor size in the HER2-enriched subtype, however (Table 3).

Figure 1. The tumor size and lymph node status association differs by race and ER status.



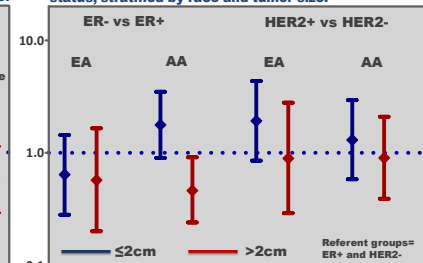
- In the ER negative subgroup, tumor size >2cm was associated with increased risk of nodal metastases among European-Americans, but not African-Americans.

Figure 2. Associations between race, menopausal status, tumor subtype and nodal status, stratified by tumor size.



- The relationships between race, menopausal status, and tumor subtype and nodal status did not differ between subgroups defined by tumors ≤2cm and >2cm.
- In HER2-enriched tumors >2cm, there was a suggestion of decreased risk of nodal metastases.

Figure 3. Effect of ER- and HER2+ status on lymph node status, stratified by race and tumor size.



- In African-American women, ER negative tumors ≤2cm were more likely to be node positive, but ER negative tumors >2cm were less likely to be node positive.
- In European-American women, HER2 positive tumors ≤2cm, but not >2cm, were more likely to be node positive.