In Situ Breast Cancer and Microchimerism

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Microchimeric cells of fetal origin persistent in the maternal circulation post-partum are associated with protection against invasive breast cancer. Here using quantitative genomic methods, we evaluated for the presence of male fetal microchimerism in buffy coat cells from women with a prior history of breast carcinomas in situ (CIS) and in healthy controls. Fetal microchimerism was detected in 75 of 88 controls (85%) and in 57 of 89 CIS patients (64%). The odds ratio for protection against non-invasive breast disease was 0.26 (95% confidence interval 0.12–0.56; p < 0.001 adjusted for age and body mass index). Similar to women with invasive breast cancer, women with CIS who are naturally at high risk for future invasive disease were deficient for fetal microchimerism. In addition to autologous anti-tumor immune responses, the maintenance of haploidentical microchimerism may impart an allogeneic edge in immunosurveillance.

Breast carcinomas in situ (CIS) are non-obligate precursors for invasive disease. Though no true prospective natural history studies of CIS are at this time ethnically feasible, a woman diagnosed with non-invasive breast carcinoma optimally managed with breast conserving surgery only is estimated to retain an 8–10 year risk of recurrent disease ranging from 26–31% in her remaining breast tissue1. Understudied compared to invasive breast cancers, the etiologic factors lending to development of in situ cancers can inform greatly about more aggressive forms of disease. Similar to invasive breast cancer, available evidence supports a protection against developing in situ breast cancer when women are parous2. In addition to autologous immune responses against neoantigens and direct hormonal changes to breast tissues originating during pregnancy that are known to afford an advantage against cancer3, we have been evaluating a new dimension to this protection, fetal microchimerism. Fetal microchimerism describes the small numbers of haploidentical cells that transit during pregnancy and persist in a woman’s circulation and tissues long-term. In prior studies published by our group, fetal microchimerism was both associated with freedom from breast cancer when present in the circulatory system4,5 and in breast tissue6. Moreover, when women are deficient in fetal microchimerism, they are at a higher risk for developing a future breast cancer7. Because pre-malignant or pre-invasive disease can be present years prior to developing an invasive cancer, we sought to determine if women with pure in situ breast cancers were deficient for fetal microchimerism. Specifically, if our hypothesis is correct, it suggests that there is likely a fundamental failure of acquiring or maintaining chimeric cells from the fetus in women with breast disease or cancer as opposed to a loss of it during progression towards overt disease.

Results
Peripheral blood cell buffy coat DNA from 100 women with a history of CIS and 100 healthy control women (also referred to as probands) were obtained from the Roswell Park Cancer Center Data Bank and BioRepository8. Probands included in our study were recruited to the biorepository over a 6-year span from 2004–2010 and donated their blood specimens a median of 34 days after diagnosis. Controls were matched to case probands on the basis of gender, age (in 5-year blocks), parity (yes vs. no), and race. Quantitative PCR was performed over a 7 month span from June 2011 to Jan 2012. Nine case and 12 control specimens were excluded from analysis because DNA quality (n = 9) or quantity (n = 12) was insufficient for PCR. We utilized a real-time quantitative PCR assay to detect a y-chromosome sequence of DYS14 to identify male DNA in probands’ buffy coat DNA. Following case status-blinded analysis of quantitative PCR results, data from 91 CIS and 88 control subjects were available for analysis. Two CIS probands were excluded from final analysis because male DNA quantities in these two women amplified substantially beyond the highest point on the calibration curve (500 DYS14 containing genome equivalents). Though precise estimates could not be ascertained, these patients’ peripheral blood cells were...
probands were included in the final analysis. The two cohorts were themselves in utero from a vanished twin. The remaining 89 CIS macrochimerism originating for these two women while they were composed of 27% and 80% male cells. We speculate hematopoietic

| Table 1 | Subject characteristics by disease status |
|---|---|
| **Cases (N = 89)** | **Controls (N = 88)** |
| **N (%)** | **N (%)** |
| **Age (years)** | | |
| 29–44 | 16 (18) | 16 (18) |
| 45–54 | 34 (38) | 28 (32) |
| 55–64 | 26 (29) | 30 (34) |
| ≥65 | 13 (15) | 14 (16) |
| **Education** | | |
| Below or at high school | 25 (28) | 18 (20) |
| Some college | 31 (35) | 34 (39) |
| College graduate | 14 (16) | 18 (20) |
| Advanced degree | 19 (21) | 18 (20) |
| **Body mass index (kg/m²)** | | |
| <25 | 35 (40) | 26 (30) |
| 25–29 | 33 (38) | 31 (36) |
| ≥30 | 19 (22) | 30 (34) |
| Unknown | 2 | 1 |
| **Smoking status** | | |
| Never | 48 (55) | 47 (53) |
| Current | 14 (16) | 10 (11) |
| Former | 25 (29) | 31 (35) |
| Unknown | 2 | 0 |
| **Moderate exercise** | | |
| No | 52 (60) | 49 (56) |
| Low | 16 (18) | 21 (24) |
| High | 19 (22) | 18 (20) |
| Unknown | 2 | 0 |
| **Family history of BRCA** | | |
| No | 71 (80) | 70 (80) |
| Yes | 18 (20) | 18 (20) |
| **Age at menarche (years)** | | |
| <11 | 13 (15) | 16 (18) |
| 12 | 27 (32) | 25 (29) |
| 13 | 27 (32) | 28 (32) |
| ≥14 | 18 (21) | 18 (21) |
| Unknown | 4 | 1 |
| **Number of births** | | |
| 0 | 23 (26) | 23 (26) |
| 1 | 11 (12) | 11 (12) |
| 2 | 22 (25) | 20 (23) |
| 3 | 18 (20) | 19 (22) |
| 4 or more | 15 (17) | 15 (17) |
| **Age at first birth (years)** | | |
| 19 or younger | 9 (14) | 12 (19) |
| 20–24 | 19 (29) | 27 (42) |
| 25–29 | 25 (38) | 15 (23) |
| 30 or older | 13 (20) | 10 (16) |
| Unknown | 0 | 1 |
| **Number of children breastfed** | | |
| No children | 23 | 23 |
| **Menopausal status** | | |
| Premenopausal | 43 (48) | 37 (42) |
| Postmenopausal | 46 (52) | 51 (58) |
| **Menopausal hormone use** | | |
| No | 66 (74) | 64 (73) |
| Yes | 23 (26) | 23 (26) |
| Unknown | 0 | 1 |

Median concentrations were 0.29 versus 0.07 male chimeric cells per 10⁷ host cells in control and case subjects, respectively (Figure 1) and differed substantially at all percentile ranks. Logistic regression models used to model the primary outcome of CIS with presence of fetal microchimerism may substantially underestimate the impact of any association because it does not account for quantitative differences between groups. We therefore applied Poisson modeling (because of the rightward skew of the data) to better capture quantitative differences between groups. Using this approach, the rate of microchimerism detection was significantly lower in women with a history of CIS than in the healthy women (p < 0.001) in a model adjusted for age and BMI. In subset analysis by number of children, the association of microchimerism and risk of CIS was stronger in women with two or more children than in nulliparous or uniparous women (Table 2). A test for interaction across subgroups according to number of children (0, 1, 2, 3 or more children) showed a decreasing trend in the odds ratios (p = 0.07). Table 3 shows subset analysis by age at first birth among women with at least one child, where the association of microchimerism and risk of CIS was significantly stronger in women who first gave birth before age 30 years compared to those over 30 (p = 0.04 for interaction).

Table 2: Odds ratios (OR) of CIS by microchimerism prevalence, for all subjects and by number of children.

| Parity | Presence of microchimerism | Disease Status (%) | OR (95% CI) | p-value |
|---|---|---|---|---|
| All subjects | No | 32 (36) | 13 (15) | 1.0 |
| | Yes | 57 (64) | 75 (85) | 0.26 (0.12–0.56) |
| No children | No | 6 (26) | 5 (22) | 1.0 |
| | Yes | 17 (74) | 18 (78) | 0.79 (0.20–3.06) |
| 1 child | No | 3 (27) | 3 (27) | 1.0 |
| | Yes | 8 (73) | 6 (73) | 1.00 (0.10–9.94) |
| 2 children | No | 10 (45) | 1 (5) | 0.06 (0.01–0.58) |
| | Yes | 12 (55) | 19 (95) | 0.06 (0.01–0.58) |
| ≥3 children | No | 13 (39) | 4 (12) | 1.0 |
| | Yes | 20 (61) | 30 (88) | 0.21 (0.04–0.81) |

*for all subjects, adjusted for age and BMI; in subset analysis, unadjusted test for trend.
Table 3 | Unadjusted OR of CIS by microchimerism prevalence, according to age at first birth in parous women

| Age at 1st birth | Presence of microchimerism | Proportions by Disease Status (%) | OR (95% CI) |
|------------------|-----------------------------|----------------------------------|------------|
|                  | Cases                       | Controls                         |            |
| <30 years        | No                          | 21 (40)                          | 4 (7)      | 1.0        |
|                  | Yes                         | 32 (60)                          | 50 (93)    | 0.12 (0.04–0.39) |
| ≥30 years        | No                          | 5 (38)                           | 4 (40)     | 1.0        |
|                  | Yes                         | 8 (62)                           | 6 (60)     | 1.07 (0.20–5.77) |

The prevalence of microchimerism and its association with characteristics of the CIS cases is shown in Table 5. There was no indication of variation in microchimerism prevalence according to these characteristics. Progesterone receptor results are not shown because they were identical to those for estrogen receptor.

**Discussion**

We report that women with in situ breast cancer are deficient in carriage of male microchimerism of presumed pregnancy origin at rates comparable to those observed in women with invasive breast cancer. Combined with our prior prospective study of microchimerism in healthy women who later develop invasive breast cancer, the current study also serves as evidence that the absence of microchimerism is not a result of having developed breast cancer but more likely a predisposing condition towards it. The data collectively indicate a primary failure of microchimerism acquisition during pregnancy or alternatively a loss well prior to developing non-invasive or invasive disease. The ideal study design to verify the temporality of the microchimerism loss with respect to cancer development would be a longitudinal serial study beginning with pregnancy completion; unfortunately, to the best of our knowledge a mature resource of this kind does not yet exist. In considering fetal microchimerism as a biomarker, this report is now the 6th study available demonstrating this consistent observation, all of roughly similar magnitude.

Though contamination of specimens with male DNA cannot be absolutely excluded, strengths of the study are that laboratory hygiene practices, a single female operator, and blinding insure non-differential uncontrolled effects among the groups and further bolsters confidence in the findings. Moreover, the presence of potential contamination would bias against our study because it would likely dilute differences between groups. It is noteworthy that the rate of microchimerism detection in controls was higher than observed in prior invasive cancer association studies. We attribute the higher detection rate in our prospective study and the current report to the use of more modern PCR rigs with known higher sensitivity and improved performance characteristics. Another consideration is that gravidity was not recorded for participants in the registry. Thus, we are not sure of the impact, if any, of miscarriages and abortions on the microchimerism prevalence in the cohort. The present study was powered to primarily identify an association of microchimerism with CIS versus matched controls based on assumptions derived from prior studies already published for invasive disease. However, there was insufficient power to draw firm conclusions regarding CIS specific characteristics such as laterality or tumor subtype, but generally no striking differences emerged in these analyses. Unfortunately, to the authors’ knowledge no other bio-repositories with appropriate specimens for microchimerism testing in CIS are available.
other than the Roswell Park resource used here, which we nearly exhausted for unique case specimens.

The mechanism by which microchimerism might protect against breast malignancy remains undefined but a leading hypothesis is that such cells are involved in allogeneic immune surveillance. In a sense, haploidentical fetal cells crossing over during pregnancy might serve as a natural version of microtransplant. As support that microchimeric populations of cells can be immunologically active participants in graft-versus-tumor effects absent of any concomitant graft-versus-host disease, Guo, et al recently reported that haploidentical related donor hematopoietic cell microtransplantation without an adequate conditioning regimen or immunophrophylaxis was an effective form of consolidative therapy for acute myelogenous leukemia with clear demonstration of graft-versus-tumor and host-versus-graft effects10. Moving forward and with additional studies, we pose the question whether primed or unaltered haploidentical cell infusions, possibly using child-origin cells could be considered as form of immunotherapy for women at high risk of breast cancer recurrence following otherwise definitive therapy for in situ or invasive disease.

Methods

Ethical considerations. Research subjects originally signed consent forms approved by the Roswell Park Institutional Review Board at the time of enrollment into the Data Bank and BioRepository8. Because only non-identifiable specimens were provided to investigators, the current research is considered non-human subjects research.

Fetal microchimerism testing. Each genomic DNA specimen was estimated for total DNA content and purity by spectrophotometry. Presence and quantity of fetal microchimerism was determined by targeting the y-chromosome sequence DYS14 by PCR8. Briefly, and with attention to modifications of the original technique, aliquots of genomic DNA (2–3.5 × 107), were tested using TaqMan chemistry performed on an ABI PRISM 7900 PCR rig for beta-globin (2 aliquots) to determine total proband genomes and for DYS14 (6 aliquots) to determine total microchimeric genomes present. Amplifications for total and microchimeric genomic DNA were plotted against calibration curves for both the beta-globin and DYS14 assays to determine quantities and final results were expressed as a ratio of microchimeric cells per 1.0 × 107 maternal genomic equivalents. All genomics workflow was performed by a single female operator (JKE) blinded to case-control status of specimens to prevent laboratory contamination of male DNA sequences and bias, respectively.

Table 4 | Rate ratios (RR) of CIS by microchimerism detection, for all subjects and by number of children

| Parity          | Rate Ratio (95% CI) | p-value |
|-----------------|--------------------|---------|
| All subjects    | 0.47 (0.31–0.71)   | <0.001* |
| No children     | 0.28 (0.12–0.64)   |         |
| 1 child         | 0.47 (0.20–1.11)   |         |
| 2 children      | 0.22 (0.10–0.49)   |         |
| ≥3 children     | 0.97 (0.53–1.76)   | 0.02    |

*For all subjects, adjusted for age, education level (college or more vs. less than college) and moderate exercise; in subset analysis, unadjusted test for trend.

Table 5 | Among CIS cases, unadjusted OR of microchimerism prevalence according to disease characteristics

| Laterality               | No microchimerism (N = 32) | Microchimerism (N = 57) | OR (95% CI) |
|--------------------------|----------------------------|-------------------------|-------------|
| Left origin of primary   | 19 (59)                    | 31 (54)                 | 1.0         |
| Right origin of primary  | 13 (41)                    | 26 (46)                 | 1.23 (0.51–2.95) |
| Estrogen receptor        |                            |                         |             |
| Positive                 | 12 (75)                    | 26 (72)                 | 1.0         |
| Negative                 | 4 (25)                     | 10 (28)                 | 0.87 (0.23–3.33) |
| Test not done            | 14                         | 20                      | –*          |
| Unknown                  | 2                          | 1                       | –*          |
| Grade                    |                            |                         |             |
| Well differentiated      | 2 (18)                     | 3 (27)                  | 1.0         |
| Moderately differentiated | 3 (27)                    | 4 (36)                  | 0.89 (0.09–9.16) |
| Poorly differentiated    | 6 (55)                     | 4 (36)                  | 0.44 (0.05–3.98) |
| Unknown                  | 21                         | 46                      | –*          |
| Histologic Subtype       |                            |                         |             |
| Ductal Carcinoma In Situ | 22 (69)                    | 43 (73)                 | 1.0         |
| Lobular Carcinoma In Situ | 3 (9)                     | 6 (10)                  | 0.97 (0.17–5.04) |
| Other*                   | 7 (22)                     | 8 (14)                  | 1.71 (0.48–6.12) |

*Patients with unknown values are excluded from analysis. *includes in situ subtypes of comedo, papillary, intracytic, and cribriform.

Statistical analysis. Differences in subject characteristics between CIS cases and controls were assessed via t-test for continuous variables and Chi-squared test for categorical factors. Pre-study power calculations for the primary analysis of presence of microchimerism informed that 100 case and control specimens each provided 83% power to detect a 0.2 prevalence difference from the assumed-true rate of 0.5 in controls, based on a chi-squared test with 2-sided type 1 error level of 5%. Logistic regression models were used to estimate the association between the primary outcome of disease status and the presence of fetal microchimerism. Subset analyses were performed according to number of children and age at first birth; exact logistic regression was used for analysis in small samples.

Fetal microchimerism concentrations were also analyzed by disease status. By definition, microchimeric cells occur at low concentrations; therefore the data distribution is skewed to the right and approximates a Poisson distribution. For this reason, we analyzed the concentrations as the outcome in log-linear regression models, estimating a rate of microchimerism detection as the number of genome equivalents of fetal DNA as a proportion of the number of maternal cells tested. Negative-binomial models were fit to account for the higher level of variability in the data than expected in a Poisson model; interpretation of the resulting estimates is identical to those of a Poisson model.

Factors examined as potential confounders or effect modifiers included age, body mass index (BMI), education, moderate exercise (none, low, and high, defined as at least 30 minutes per session on 3 or more days/week), 1st degree family history of breast cancer, age at menarche, age at first birth, number of births, history of breastfeeding, menopausal status, and menopausal female hormone use. Covariates were selected a priori based on having an established or suspected causal association with breast cancer incidence. We also considered the number of cell equivalents tested for detection of fetal microchimerism as a potential confounder. A factor was defined as a confounder if there was a discrepancy of 10% or more in the estimated coefficient of interest between the multivariable model including the factor and the model without it.

Additional analyses were conducted to examine whether CIS-specific features were associated with fetal microchimerism prevalence among the cases. Presence of fetal microchimerism was treated as a binary outcome in logistic regression models, with various disease characteristics as the predictors.

P-values from regression models were derived from the Wald test or an exact binomial test for small sample sizes in prevalence analysis. No adjustments were made for multiple comparisons. Analyses were performed on SAS software version 9 (SAS Institute, Inc., Cary, NC).
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**Acknowledgments**

This study was funded by grants from the NIH (P30 CA016056-27, P50 CA138293), the Prevent Cancer Foundation, and a gift from Nitza and Gideon Yuval.

**Author contributions**

J.K.E., K.A.G., G.Z. and V.K.G. participated in the design of the experiments, analysis of results, and writing of the manuscript. J.K.E. performed all bench experiments.

**Additional information**

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Eun, J.K., Guthrie, K.A., Zirpoli, G. & Gadi, V.K. *In Situ Breast Cancer and Microchimerism*. *Sci. Rep.* **3**, 2192; DOI:10.1038/srep02192 (2013).

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