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The major source of estrogen in postmenopausal women is conversion of androstenedione in adipose tissue to estrone by the enzyme aromatase. In adipose tissue, the aromatase gene, CYP19, is regulated by the cytokines TNF- α and IL-6. The objective of this study was to determine whether polymorphism in the TNF- α gene, in the IL-6 gene, or in the regulatory region of the CYP19 gene is associated with the plasma estrone (E1) to androstenedione (A) ratio, a measure of peripheral estrogen production, or with risk of post-menopausal breast cancer. This investigation was carried out using DNA and serum samples from women enrolled in the Hawaii-Los Angeles Multiethnic cohort study.

We found that among African-American women, but not among other women, polymorphisms in both the TNF- α and IL-6 genes were associated with increased breast cancer risk after stratification on obesity. Increasing weight also appeared to be associated with increased peripheral estrogen production among women with high-inducibility TNF α genotypes but not among other women. Because our findings were not consistent across ethnic groups and because they were based on relatively small numbers of women within each ethnic group, we are attempting to confirm these results in a second multi-ethnic population.

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INTRODUCTION

The major source of estrogen in postmenopausal women is conversion of androstenedione in adipose tissue to estrone by the enzyme aromatase. In adipose tissue, the aromatase gene, CYP19, is regulated by the cytokines TNF- α and IL-6, which act on a regulatory region of the CYP19 gene called the I.4 promoter. The objective of this study was to determine whether polymorphism in the TNF- α gene, in the IL-6 gene, or in the CYP19 gene I.4 promoter is associated with (1) the plasma estrone (E1) to androstenedione (A) ratio, a phenotypic measure of aromatase expression, or with (2) risk of post-menopausal breast cancer. This investigation was carried out using DNA and serum samples from women enrolled in the Hawaii-Los Angeles Multiethnic cohort study.

BODY

Task 1: Set-up and optimize TNF- α assay. Identify genotypic controls and confirm genotypes by sequencing.

Task 1 has been completed. PCR amplification conditions were optimized for the genomic region containing the TNF α polymorphism at nucleotide position -308. By cutting PCR products with the NcoI restriction enzyme, control samples were identified for each of the three genotypes, GG, GA, and AA. Genotypes of control samples were confirmed by sequencing using manual methods. In addition to the expected polymorphism at position -308, apparent polymorphisms were identified at positions -244 and -241; however, resequencing both DNA strands of the DNA samples in question on an ABI sequencer revealed that these were in fact artifacts.

Task 2: Set-up and optimize IL-6 assay.

Task 2 has been completed. A genotyping assay was designed for the TaqMan system. Two oligonucleotide probes, one specific for the G allele and one for the C allele, were designed and labeled with two different fluorophores. PCR primers were designed to amplify a 100 bp region surrounding the polymorphism. A series of control samples, to be included in each TaqMan run, were identified by direct sequencing.

Task 3: As samples accrue, genotype TNF- α polymorphism.

Task 3 has been completed. We have exceeded our goal of genotyping 1360 women (680 cases and 680 controls). TNF- α genotyping has been completed on 791 women with incident post-menopausal breast cancer and 1383 post-menopausal women with no history of breast cancer. We have found that the frequency of the -308 A allele varies by ethnicity. Frequency of the A allele among controls was 2% in Japanese-Americans, 8% in Latinas, 12% in African-Americans, and 17% in whites. Genotype frequencies were in Hardy-Weinberg equilibrium within each ethnic group.

Task 4: As samples accrue, genotype IL-6 polymorphism

Task 4 has been completed. We have exceeded our goal of genotyping 1360 women (680 cases and 680 controls). IL-6 genotyping has been completed on 791 women with incident post-menopausal breast cancer and 1383 post-menopausal women with no history of breast cancer. We have found that the frequency of the -174 C allele also varies by ethnicity. Frequency of the C allele among controls was 1% in Japanese-Americans, 10% in African-Americans, 19% in

Latinas, and 37% in whites. Genotype frequencies were in Hardy-Weinberg equilibrium within each ethnic group.

Task 5: Design sequencing primers for CYP19 I.4 promoter

Task 5 has been completed. Five overlapping primer pairs were designed to amplify 1148 basepairs of the CYP19 I.4 promoter region (from position 18 to 1166, according to Genbank L21982). Positions of the primers are given below. Annealing temperature for all primer pairs is 51°C.

| Forward primer | Reverse primer | PCR product length | MgCl ₂ concentration |
|----------------|----------------|--------------------|---------------------------------|
| 18-39 | 283-302 | 285 bp | 2.5 mM |
| 197-223 | 582-600 | 404 bp | 1.5 mM |
| 542-562 | 882-901 | 360 bp | 2.0 mM |
| 793-811 | 996-1016 | 224 bp | 2.0 mM |
| 882-901 | 1145-1166 | 285 bp | 2.5 mM |

Task 6: Identify 3 control subjects with lowest and 3 with highest E1/A ratio in each ethnic group (N=24)

Task 6 has been completed. Serum androstenedione (A) and estrone (E1) levels were available for 235 postmenopausal women who were not on hormone therapy. Serum E1/A ratios were calculated as a phenotypic measure of aromatase activity. Three subjects with low and three with high E1/A ratios were identified in each ethnic group (totally, 12 subjects with low and 12 with high ratios).

Task 7: Sequence CYP19 I.4 promoter for 24 subjects.

Task 7 has been completed. For the 24 women identified in task 6, the genomic region containing the CYP19 promoter I.4 was amplified in five overlapping fragments, and these PCR fragments were sequenced using manual methods. While four of the samples appeared to be polymorphic at one or more positions, resequencing both strands on an ABI sequencer revealed these to be artifacts. To verify that there are no common polymorphisms in this region, we also sequenced samples from 60 additional cohort members (randomly chosen from each of the 4 ethnic groups), and found no polymorphisms in the I.4 promoter.

Tasks 8: If CYP19 I.4 polymorphisms are found, genotype for N-1360 subjects.

Since no polymorphisms were found, no genotyping was necessary.

Task 9: Analyze associations between genotypes and plasma E1/A ratio among the 680 controls (objective #1).

This task has been completed. Overall, we found no association between serum hormone levels (androstenedione, estrone, or androstenedione:estrone ratio) and IL6 or TNF α genotype. Because of the low minor allele frequencies, heterozygotes and homozygotes for the minor allele were combined into one group for analysis (Table 1). P-values were obtained from regression models, adjusting for weight, race, and age.

Table 1: Serum hormone levels by genotype

| | Androstenedione | Estrone (E1) | Ratio (E1:A) |
|---------------|-----------------|--------------|--------------|
| IL-6 | mean (SD) | mean (SD) | mean (SD) |
| GG | 523 (279) | 41 (29) | 0.10 (0.14) |
| CG/CC | 511 (300) | 46 (47) | 0.12 (0.16) |
| p-value | 0.59 | 0.76 | 0.99 |
| TNF- α | mean (SD) | mean (SD) | mean (SD) |
| GG | 506 (262) | 42 (30) | 0.11 (0.14) |
| AG/AA | 575 (357) | 47 (52) | 0.12 (0.18) |
| p-value | 0.21 | 0.31 | 0.68 |

Because aromatase is expressed in adipose tissue, weight was expected to be positively correlated with aromatase expression. Overall, we found that weight was not significantly related to serum hormone levels; however, TNF α genotype appeared to be an effect modifier of the relationship between weight and hormone levels (test of interaction $p=.12$ for E1 and $p=0.11$ for E1:A ratio). Weight was positively correlated with E1 and E1:A ratio among those carrying a TNF α A allele (genotypes AA and AG), but not among those with genotype GG (Table 2). There was no evidence of effect modification by IL-6 genotype.

Table 2: Correlation between serum hormones and weight

| | Estrone (E1) | Ratio (E1:A) |
|---------------|--------------|--------------|
| | R (p-value) | R (p-value) |
| TNF- α | | |
| GG | 0.06 (0.36) | 0.00 (0.97) |
| AG/AA | 0.20 (0.14) | 0.20 (0.16) |

We also observed evidence of gene-gene interaction. After adjusting for weight, estrone levels (adjusted for androstenedione) and E1:A ratio was higher among those carrying both an IL6 C allele and a TNF α A allele (p for interaction = 0.048 for E1 and 0.087 for A:E1 ratio) compared to women with other genotype combinations.

Table 3: Hormone levels by TNF α / IL-6 genotype combinations

| IL-6 | TNF- α | Estrone (E1) | Ratio (E1:A) |
|-------|---------------|--------------|--------------|
| | | mean (SD) | mean (SD) |
| CG/CC | AG/AA | 63 (89) | 0.18 (0.32) |
| CG/CC | GG | 42 (31) | 0.11 (0.10) |
| GG | AG/AA | 41 (29) | 0.09 (0.06) |
| GG | GG | 41 (29) | 0.11 (0.15) |

Task 10: Analyze relationship between genotypes and breast cancer risk (objective #2).

Overall, in the Multi-ethnic Cohort Study, we found no significant association between IL6 or TNF α genotype and risk of post-menopausal breast cancer. Because minor allele frequencies were low, data were collapsed (heterozygotes plus homozygotes for the minor allele) before calculating odds ratios. Odds ratios were adjusted for age, age at menopause (categories: <45, 45-49, 50+) and HRT use (no HRT, past estrogen, current estrogen, current estrogen plus progesterone), and for genotype at the other locus.

Table 4: Odds ratios for IL-6 and TNF α genotypes, by ethnicity

| | White | African-American | Latina | Japanese-American | All groups (ethnicity-adjusted) |
|--------------------------------|-----------|------------------|-----------|-------------------|---------------------------------|
| | Ca/Co | Ca/Co | Ca/Co | Ca/Co | Ca/Co |
| IL-6 | | | | | |
| GG | 86/118 | 116/315 | 116/284 | 229/270 | 547/987 |
| CG | 114/131 | 37/68 | 55/134 | 1/3 | 207/336 |
| CC | 35/42 | 0/4 | 2/4 | 0/0 | 37/50 |
| Total | 235/291 | 153/387 | 173/432 | 230/273 | 791/1383 |
| OR | 1.12 | 1.36 | 0.89 | 0.33 | 1.09 |
| 95% CI | 0.78-1.60 | 0.86-2.17 | 0.60-1.31 | 0.03-3.38 | 0.87-1.37 |
| TNF-α | | | | | |
| GG | 174/202 | 117/301 | 142/362 | 221/262 | 654/1127 |
| AG | 54/80 | 30/82 | 30/67 | 9/11 | 123/240 |
| AA | 7/9 | 6/4 | 1/3 | 0/0 | 14/16 |
| Total | 235/291 | 153/387 | 173/432 | 230/273 | 791/1383 |
| OR | 0.79 | 1.01 | 1.09 | 1.08 | 0.95 |
| 95% CI | 0.53-1.15 | 0.63-1.61 | 0.67-1.75 | 0.43-2.71 | 0.74-1.21 |

To determine whether genotype might play different roles in obese vs. non-obese women, data were also stratified on BMI (<30 vs. 30+) (Table 5). Significant effect modification was not observed when all ethnic groups were combined. However, among African-Americans, obesity was an effect modifier of the genotype-disease relationship for both the IL-6 (interaction $p=0.05$) and the TNF- α (interaction $p=0.04$) genotypes. Among African-Americans, the C allele (minor allele) of the IL-6 locus was associated with significantly increased TNF α risk among non-obese, but not among obese, women. The A allele (minor allele) of the TNF α locus was associated with a more than two-fold (but not statistically-significant) increase in risk only among obese African-American women.

Table 5: Odds ratios, stratified on BMI (non-obese / obese)

| | White | African-American | Latina | Japanese-American | All groups (ethnicity-adjusted) |
|-------------------------|-----------|----------------------------|-----------|-------------------|---------------------------------|
| | Ca/Co | Ca/Co | Ca/Co | Ca/Co | Ca/Co |
| IL-6, BMI <30 | | | | | |
| GG | 68/99 | 70/201 | 74/199 | 210/258 | 422/757 |
| CG/CC | 128/149 | 29/44 | 42/106 | 1/3 | 200/302 |
| OR | 1.15 | 1.85 | 0.95 | 0.34 | 1.22 |
| 95% CI | 0.78-1.72 | 1.05-3.25 | 0.59-1.53 | 0.03-3.39 | 0.93-1.59 |
| IL-6, BMI 30+ | | | | | |
| GG | 18/19 | 43/109 | 42/81 | 16/11 | 119/220 |
| CG/CC | 21/24 | 7/28 | 15/40 | 0/0 | 43/92 |
| OR | 0.77 | 0.58 | 0.82 | NA | 0.75 |
| 95% CI | 0.30-1.97 | 0.23-1.51 | 0.38-1.76 | NA | 0.46-1.21 |
| interaction | $p=0.62$ | $p=0.05$ | $p=0.55$ | NA | $p=0.14$ |

Table 5 continued on next page.

Table 5: Continued

| TNF- α , BMI < 30 | | | | | |
|--------------------------|-----------|---------------|-----------|-----------|-----------|
| GG | 143/172 | 79/182 | 94/254 | 203/250 | 519/858 |
| AG/AA | 53/76 | 20/63 | 22/51 | 8/11 | 103/201 |
| OR | 0.82 | 0.71 | 1.13 | 0.93 | 0.87 |
| 95% CI | 0.54-1.26 | 0.39-1.28 | 0.64-2.00 | 0.36-2.37 | 0.65-1.17 |
| TNF- α , BMI 30+ | | | | | |
| GG | 31/30 | 37/116 | 48/102 | 15/11 | 131/259 |
| AG/AA | 8/13 | 13/21 | 9/19 | 1/0 | 31/53 |
| OR | 0.46 | 2.05 | 1.13 | NA | 1.11 |
| 95% CI | 0.15-1.47 | 0.91-4.64 | 0.45-2.84 | NA | 0.66-1.87 |
| interaction | p=0.46 | p=0.04 | p0.92 | NA | p=0.35 |

Additional work in progress: Because the above findings were not consistent across ethnic groups, and because sample size within each ethnic group was limited, we are attempting to replicate these findings in a second population. We have genotyped the same TNF- α and IL-6 polymorphisms using DNA samples from 1719 women (646 breast cancer cases and 1073 controls) from a population-based case-control study conducted by Dr. Esther John at the Northern California Cancer Center. Approximately equal numbers of African-Americans, non-Hispanic whites, and Hispanics were included.

As we found in the Multi-ethnic Cohort population, there was no overall main effect of genotype with respect to breast cancer risk, although again, we found some statistically significant effects among African-American women. Among African-American women there was a statistically significant interaction between age & IL6 genotype with respect to breast cancer risk (p=0.046). Among older women (>55) IL6 genotype GG was associated with increased risk (similar to what was seen for obese African American women in the multi-ethnic cohort). Among younger women, genotype GG was protective (similar to what was seen for non-obese women in the multi-ethnic cohort). There was no evidence of effect modification between TNF α genotype and age.

Table 6: Odds ratios from the Northern California Case-control study (Esther John, P.I.)

| | White | African-American | Latina | All groups (ethnicity-adjusted) |
|----------------|-----------|------------------|-----------|------------------------------------|
| IL-6; age < 56 | | | | |
| OR | 0.70 | 1.99 | 0.74 | 0.89 |
| 95% CI | 0.42-1.16 | 1.01-3.94 | 0.48-1.16 | 0.66-1.20 |
| IL-6; age > 55 | | | | |
| OR | 0.88 | 0.49 | 1.32 | 0.92 |
| 95% CI | 0.56-1.37 | 0.24-1.01 | 0.80-2.16 | 0.68-1.24 |
| Interaction | p=0.57 | p=0.05 | p=0.19 | p=0.93 |

Further analysis of this data set will be carried out as soon as data are available on menopausal status, weight, BMI, and other covariates. Serum hormone levels will also be available.

KEY RESEARCH ACCOMPLISHMENTS

1. We have provided some preliminary evidence that in women with the high-inducibility TNF α genotypes (AA and AG), increasing weight is related to increased peripheral production of estrogen. This effect was not observed in women with the low-inducibility genotype (GG).

2. We have provided preliminary evidence of gene-gene interaction with respect to peripheral estrogen production. Women having minor alleles of both TNF α (allele A) and IL-6 (allele C) appear to have markedly higher peripheral estrogen production than do women with other genotypes.

3. We have provided evidence that obesity is an effect modifier of the relationship between TNF α and IL-6 genotypes and breast cancer risk in African-American women. High-inducibility TNF α genotypes were associated with increased risk of breast cancer among obese, but not among non-obese, African-American women. For IL-6, genotypes containing a minor allele (CG and GG) were associated with increased risk among non-obese, but decreased risk among obese African-American women.

REPORTABLE OUTCOMES

There have been no reportable outcomes to date. We anticipate that at least one manuscript will be submitted, pending analysis of the additional data from Dr. John's case-control study.

CONCLUSIONS

We have provided evidence that polymorphisms in the TNF- α and IL-6 genes, whose products regulate aromatase expression in adipose tissue, are related to peripheral estrogen production and possibly to breast cancer risk. Because these results were not consistent across ethnic groups and because they were based on relatively small numbers of women within each ethnic group, we are attempting to confirm these results in a second multi-ethnic population. If we are able to confirm these findings, they would suggest that proinflammatory cytokines, which are produced in adipose tissue, contribute to breast cancer risk, at least in women with at-risk genotypes.

REFERENCES

None.

APPENDIX

List of Personnel paid from this project:

Sue Ingles
Rowena Bada
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