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13. ABSTRACT (Maximum 200 Words)

Aims: The specific hypothesis was that women who have selected polymorphic variants in genes that predispose them to poorer repair of DNA damage will have greater breast cancer risk. Polymorphisms in base excision repair (XRCC1), nucleotide excision repair (XPD, XPC), double-strand break repair (XRCC3, Rad51, LIG4) and checkpoint-control (CDKN2A) genes were investigated. A secondary hypothesis was that risk may be further accentuated by lifestyle-related exposures to carcinogens or oxidants. Methods: A nested case control study of 612 matched pairs was conducted within the NYU Women's Health Study, a prospective study of over 14,000 healthy women aged 35-65 who were enrolled in the study during 1985-91. Genotyping was performed using RFLP. Statistical analyses were controlled for ethnicity, age, and various risk factors for breast cancer. Results: There was a statistically significant increase in breast cancer risk for women who had the homozygous variant genotype for the XPC-PAT+ polymorphism (OR=1.41, 95% CI: 1.04-1.91). Further analyses suggested that women with the variant allele had an 80% greater (p=0.09) breast cancer risk associated with smoking and 70% greater (p=0.06) for alcohol consumption than those who were wild type. The XRCC3 Thr241-Met substitution also showed a suggestive association with breast cancer risk.

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Introduction

The primary aims of the study were to evaluate the role of genes that repair DNA damage in the etiology of breast cancer. The specific hypothesis was that women who have allelic variants that predispose them to poorer detoxification of carcinogenic or oxidant exposures, or to poorer repair of DNA damage will have greater breast cancer risk. A secondary hypothesis was that risk may be further accentuated by lifestyle-related exposures to carcinogens or oxidants.

The loci that were genotyped were chosen because of preliminary evidence suggestive of an association between a variant allele and breast or other cancer. But certain loci that were initially proposed were dropped because studies became available in the interim suggesting that those loci had little or no impact on breast cancer risk (e.g., GSTM1). An attempt to genotype one polymorphism (MnSOD A16V) was made, but could not be done reliably by the RFLP analysis we were using, so it was dropped. Other new, interesting leads were chosen to replace those dropped, since the main thrust of this proposal was to be innovative in exploring new candidate polymorphisms or ones for which an association with breast cancer risk is uncertain. The loci that were genotyped are briefly described below.

***XRCC1*, exon 6, Arg194→Trp**. Two small studies (30 cases and 103 cases respectively) failed to find an association between breast cancer risk and the codon 194 polymorphism (Amirimani et al., 2001; Tang et al., 2001). However, another study found elevated breast cancer risk among women with this variant and without a family history of breast cancer. (OR= 2.5, CI= 1.0-6.8). They also found a gene-gene interaction in breast cancer risk between the XRCC1 and the XRCC3 genes. A South Korean study reported a suggestive association between the codon 194 polymorphism and breast cancer risk (OR=1.4, CI= 0.9-2.3), and also reported an interaction with alcohol consumption such that women with the 194Trp variant who consumed alcohol had an OR=2.9 (CI=1.3-6.2) (Hirvonen et al., 2001).

***XPD*, exon 23, Lys751→Gln**. Four small studies of the codon 751Gln allele and breast cancer have been conducted. One (136 breast cancer cases) found an increased risk associated with the 751Gln allele among women with late/no parity (Cheng et al., 2002). Another (115 breast cancer cases) weakly suggested an increased risk (OR= 2.1, CI: 0.8-5.9, trend p=.13) (Brewster et al., 2001), while one (103 cases) was null (Tang et al., 2002) and one (103 cases) showed a decreased risk (OR= 0.4, CI: 0.2-1.0) (Amirimani et al., 2001) associated with the 751Gln allele. The present study is larger than all the other four put together.

***XPC*-PAT+**. A poly(AT) insertion/deletion polymorphism (XPC-PAT+) has been reported in intron 9 in the XPC gene. The XPC-PAT+ variant is associated with squamous cell carcinoma of the head and neck (OR=1.4, CI: 1.0-2.1 for PAT+/- and OR=1.9, CI: 1.1-3.1 for PAT+/+; trend test p=.007) (Shen et al., 2001) but not with non-melanoma skin cancer (Nelson et al., 2002). No studies of this polymorphism and breast cancer risk have been reported.

***XRCC3*, exon 7, Thr241-Met.** Studies have found that the 241Met allele is associated with bladder cancer (Matullo et al., 2001), squamous cell head-and-neck cancer among smokers (Shen et al., 2002), and cutaneous malignant melanoma (Winsey et al., 2000). A recent large case-control study reported that women with the XRCC3 241Met allele had a significantly elevated risk of breast cancer (Kuschel et al., 2002).

***Rad51*, 5' UTR (at nt 135 G>C).** Small studies have reported a suggestive association of this polymorphism with breast cancer risk among BRCA1 and/or BRCA2 mutation carriers (Levy-Lahad et al., 2001; Wang et al., 2001), but no study has been reported among unselected breast cancer cases and controls.

***LIG4* (1977 T>C), Asp501Asp.** Two studies have suggested it may be important in breast cancer risk/survival in spite of its being a synonymous variant. In particular, a large study of breast cancer risk recently reported that the variant allele may confer a protective effect (OR=0.7, p=0.09 for the homozygous variant) (Kuschel et al., 2002)

***CDKN2A* (aka: *p16*), 3' UTR (at nt 540 C>T).** This is a frequent polymorphism found in malignant melanoma cases (Kumar et al., 2001). Breast cancers have also been associated with CDKN2A mutations in melanoma families (Borg et al., 2000).

BODY

Study Design: A nested case-control study was conducted within the NYU Women's Health Study, a prospective study of over 14,000 healthy women aged 35-65 who were being screened for breast cancer at a mammographic screening center. They were enrolled in the study during 1985-91. Cohort members donated 30 ml of blood and completed self-administered questionnaires at enrollment. A standardized questionnaire included questions on reproductive and menstrual history, family breast cancer history, height, weight, physical activity, and medication use. Additional information on smoking and alcohol consumption was obtained from early follow-up questionnaires.

The study cohort composition reflected the characteristics of New York City women who would attend a low-cost screening clinic in the 1980's – well educated, mostly Caucasian and health conscious (Toniolo et al., 1991). Nearly half (44%) have a college degree, 44% are Jewish, only 19% were smokers at the time of study enrollment, and 49% were under age 50. Ten percent are African-American, 5% Hispanic, 82% non-Hispanic white, and 3% other.

Ten or more aliquots of serum have been stored at -80°C. from all the 14,000 women. For approximately 1/3 of the women, blood clots and/or cell precipitates (after centrifuging) also were stored, which are abundant sources of DNA. For the remaining 2/3, DNA has to be extracted from serum.

They have been followed up to the present to ascertain subsequent breast cancer and other cancers. Follow-up questionnaires have been obtained by mail or telephone about every two

years, and reported cases of breast cancer were confirmed by review of clinical and pathology records. A high follow-up rate of 90% has been maintained. The National Death Index and tumor registries in New York, New Jersey and Connecticut are also searched to detect additional breast cancers among subjects who cannot be contacted. We estimate that about 95% of diagnosed breast cancers in this cohort have been identified by the combined modes of ascertainment (Kato et al., 1999). A number of results regarding hormonal and dietary factors in breast cancer risk have been published (e.g., (Sonnenschein et al., 1999; Toniolo et al., 1995; Toniolo et al., 1994; Zeleniuch-Jacquotte et al., 1997; Zeleniuch-Jacquotte et al., 1995)).

Nested Case-Control Study: All 612 available incident invasive breast cancer cases (defined as of February 1998, the most recent date of complete follow-up) and an individually matched set of 612 controls were selected. Specifically, a control was selected at random for each case from the risk set of women who were alive and free of disease at the time of diagnosis of the case, and who matched the case on age at enrollment, date of initial blood donation and menopausal status at enrollment. A computer algorithm which we previously developed to optimize case-control matching was utilized for control selection.

The biospecimens were provided to the laboratory personnel with only a sample number, so that they could neither identify an individual nor determine whether the sample came from a case or control. As much as possible, case-control pairs were genotyped sequentially (in random order) so that the effects of any potential inter-batch or temporal changes in procedures or calibration would be nullified.

As the study progressed, two decisions were made to adjust the basic research plan in the light of new considerations. First, it was decided that the study would have more scientific merit if a larger number of cases and controls were included in the nested case-control study, so as to achieve better statistical power and precision in the study. Unfortunately, the literature is replete with small "exploratory" genotyping studies that ultimately provide little useful information, and it would not be meritorious to add another such study. We therefore switched from the initially proposed sample size to one that used all 612 cases available, as mentioned above. However, this meant that much more time and effort had to be devoted to extracting DNA from the 1,224 biospecimens, and that each genotype also required more resources, so that the number of genotypes that could be investigated was restricted.

The human subjects protocol of this study was approved each year by the NYU School of Medicine Institution Board of Research Associates.

Laboratory Methods: <u>DNA Isolation:</u> DNA was isolated from 200 µl of all samples (clots, RBC aggregates, and sera) using Qiagen QIAamp Blood Mini Kits essentially following the manufacturer's instructions (Qiagen Inc., Valencia, CA) with the following modifications. The liquid portion of clots were pretreated with 100 units of β-hemolytic *Streptococcus* (Lancefield Group C) streptokinase (Sigma, St. Louis, MO) overnight at 37° C. DNAs were eluted from spin columns with 200 µl of sterile $_{dd}H_2O$ and undiluted DNA stocks were stored until use at -20° C.

The DNAs were then diluted down with $_{dd}H_20$ to working concentrations in 96 well plates and stored at 4° C until use.

PCR amplifications and genotyping: PCR primer sets were selected either from published studies or by scanning GenBank for polymorphic sequences and use of the Oligo 4.04 Primer Analysis Software Program (National Biosciences). DNAs from clots and RBC aggregates were diluted 1:10 with $_{dd}H_20$ and sera were used undiluted in PCR amplifications. PCR reactions were in 20 µl (clots and RBC aggregates) or 15 µl (sera) total volumes containing 2 µl of template DNA, 2.0 µl or 1.5 µl of KlenTaq 10 x PC buffer (AB Peptides, St. Louis, MO), 0.16 µl or 0.12 µl of a mix of dNTPs (25 mM each dNTP stock), 0.8 µl or 0.6 µl of each primer , 0.03 µl of KlenTaq (25 units/µl) and 14.2 µl or 10.2 µl of $_{dd}$H20. Cycling conditions were initial denaturation at 95° C for 5 min, experimentally optimized cycle numbers of denaturation at 95° C for 30 sec, annealing at optimized temperatures for 30 sec, and extension at 72° C for 60 sec, followed by a final extension at 72° C for 7 min. The PCR products were digested with the restriction enzymes (Roche Applied Science, Indianapolis, IN or New England Biolabs, Boston, MA) for 2 hr, electrophoresed in agarose gels made in TBE buffer at appropriate concentrations, stained with ethidium bromide, and gel images were recorded with a MultiImager 4400 Low Light Imaging System (Alpha Innotech Corp., Alexandria, VA).

Quality Control: Before any locus was genotyped on the 1,200+ samples, the genotyping first had to pass a reliability test. A total of 48 pairs of samples from 48 women were genotyped with blinding as to which samples were pairs. Most of the pairs consisted of a blood clot and a serum sample, because the reliability/validity of serum-DNA genotyping was the greatest concern. The two samples had to agree on at least 96% of pairs to be considered reproducible. For one locus (XPC-PAT) the initial reproducibility test was unsatisfactory, but an adjustment of the PCR conditions for the serum samples provided better discrimination, and a second reliability test yielded good results. The genotyping of 6 of the 7 loci in this report showed 98-100% agreement between serum and clot specimens. Additional controls also were run during the course of genotyping a locus.

Key Research Accomplishments and Reportable Outcomes

The frequency distributions for cases and controls of sociodemographic and risk-factor variables are shown in Table 1. The two groups were comparable on most variables but differed on those variables that typically characterize breast cancer cases: reproductive history, family history of breast cancer, and obesity.

The frequency distributions of genotypes for the loci chosen are shown in Table 2. The main differences between cases and control appear to be in the XPC-PAT+ polymorphism and the XRCC3 Thr241→Met substitution.

The formal analyses shown in Table 3 confirm this. Under the recessive model analysis, there was a statistically significant increase in breast cancer risk for women who have the homozygous variant genotype for the XPC-PAT+ polymorphism (OR= 1.41, 95% CI: 1.04-1.91). There was

also a suggestive, but not statistically significant elevation in risk for the homozygous variant genotype with respect to the XRCC3, Thr241→Met locus (OR= 1.32, CI: 0.94-1.85, p= 0.11).

The XPC-PAT+ and breast cancer association was examined in more detail. When a history of diabetes, height, weight, Body Mass Index, age at menarche, parity, age at first full-term pregnancy, family history of cancer, oophorectomy, or use of hormone replacement therapy were controlled for, the association was essentially unchanged indicating that these were not confounding variables. We examined whether there was effect modification by age at breast cancer diagnosis (<55 vs. 55+ years old). There was no indication of greater genetic risk among those with breast cancer diagnosis at earlier ages (data not shown).

For the XPC-PAT+ locus, we examined whether there was any statistical interaction (i.e., effect modification) between having the variant and either smoking or alcohol consumption with respect to breast cancer risk. There were suggestions of interactions for both alcohol consumption and smoking. The odds ratio of XPC-PAT+/+ with breast cancer was about 80% greater among smokers than nonsmokers (interaction OR = 1.82, CI: 0.92-3.60, p= 0.09). This is biologically plausible, in that the XPC gene acts in the nucleotide excision repair pathway, which includes repair of bulky DNA adducts such as those produced by cigarette smoke. For alcohol consumption, the odds ratio of the XPC-PAT+ allele with breast cancer was 70% greater among drinkers than non-drinkers (interaction OR = 1.73, CI: 0.97-3.09, p=0.06). This finding is biologically plausible because alcohol and its metabolite acetaldehyde cause the formation of DNA adducts (Fang and Vaca, 1997) and inhibit DNA repair (Espina et al., 1988). However, these suggestive findings of effect modification should be treated with caution until they are replicated.

Conclusions

Upon finding that several of the specific gene loci that we had intended to study were reported upon by others subsequent to our grant proposal, we changed course and sought, by and large, polymorphic loci in DNA repair pathways that had been studied little or none by others. A few of these -- in the Rad51, LIG4 and CDKN2 genes -- did not prove to have any identifiable impact on breast cancer, although laboratory studies have indicated that the variants have functional effects. However, we identified a modest association of breast cancer risk with the XPC-PAT+ polymorphism, and exploratory analyses also indicate a suggestive interaction of this variant with smoking and alcohol consumption with respect to breast cancer risk. Previously there has been no clear evidence from an adequate study of an association between a variant allele of a gene in the nucleotide excision pathway and breast cancer, so the result for the XPC-PAT+ locus is the first such finding. However, this requires replication before it can be considered conclusive. We also found a weaker suggestion of an association with the XRCC3 241Met allele. The XRCC3 finding was also reported in a large study within the last few months (Kuschel et al., 2002), so this study is confirmatory.

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Table 1. **Characteristics of the Breast Cancer Cases and Controls**

	Cases (%)	Controls (%)
Ethnicity		
Caucasian	77	71
African-American	8	10
Other	6	8
Unknown	9	11
Age first pregnancy / Parity		
Age <30 y	51	59
Age 30+ y	17	11
Nulliparous	33	29
Age at menarche		
Age 13+	49	53
No. of full-term births		
0	37	32
1-2	40	45
3+	23	23
First-degree family history of breast cancer		
None	76	78
One affected relative, age 45+	15	16
>1 affected relative, or age <45	9	6
Diabetes	6	3
Bilateral oophorectomy	14	12
History of oral contraceptive use	37	35
History of hormone replacement therapy	23	23
Smoking		
Never	49	48
Current / Past	51	52
Percent of ever smokers who smoked 15+ cigarettes per day	63	58
Height (cm) – Mean ± Standard Deviation	162.4 ± 6.6	162.2 ± 6.3
Body mass index – Mean ± Standard Dev.	25.2 ± 4.5	24.9 ± 4.5

Table 2. **Distribution of Genotypes for Matched Cases and Controls**

	Cases	Controls
XRCC1, Arg194→Trp		
CC	532 (87%)	537 (88%)
CT	74 (12%)	67 (11%)
TT	5 (1%)	7 (1%)
XPD, Lys751→Gln		
CC	264 (44%)	258 (42%)
AC	276 (45%)	283 (46%)
AA	69 (11%)	71 (12%)
XPC-PAT		
-/-	212 (36%)	225 (37%)
-/+	249 (42%)	275 (46%)
+/+	129 (22%)	103 (17%)
XRCC3, Thr241→Met		
CC	255 (42%)	249 (41%)
CT	259 (42%)	286 (47%)
TT	98 (16%)	76 (12%)
RAD51, 5' UTR, nt 135 G>C		
GG	516 (84%)	513 (84%)
GC	88 (14%)	89 (14%)
CC	7 (1%)	10 (2%)
Lig 4, nt 1977 T>C		
TT	425 (69%)	436 (71%)
TC	168 (27%)	154 (25%)
CC	19 (3%)	22 (4%)
CDKN2, 3' UTR, nt 540 C>T		
GG	469 (77%)	469 (77%)
GC	131 (21%)	132 (21%)
CC	12 (2%)	11 (2%)

Table 3. **Conditional Logistic Analyses of the Associations between Selected Polymorphisms and Breast Cancer Risk [1]**

Polymorphism	Model [2]		
Odds Ratio (OR)	**Dominant**	**Recessive**	**Log-additive**
XRCC1, Arg194→Trp OR (95% CI) p-value	1.10 (0.78 – 1.56) 0.59	0.74 (0.21 – 2.63) 0.64	1.13 (0.60 – 2.13) 0.71
XPD, Lys751→Gln OR (95% CI) p-value	0.91 (0.72 – 1.16) 0.46	0.96 (0.66 – 1.39) 0.83	0.89 (0.63 – 1.26) 0.52
XPC-PAT+ OR (95% CI) p-value	1.010.79 – 1.28) 0.95	1.41 (1.04 – 1.91) 0.02	1.23 (0.89 – 1.69) 0.21
XRCC3, Thr241→Met OR (95% CI) p-value	0.91 (0.72 – 1.17) 0.48	1.32 (0.94 – 1.85) 0.11	1.06 (0.75 – 1.49) 0.75
Rad51, 5' UTR OR (95% CI) p-value	1.02 (0.75 – 1.40) 0.88	0.71 (0.25 – 2.00) 0.51	0.99 (0.56 – 1.72) 0.96
Lig 4, nt 1977 T>C OR (95% CI) p-value	1.05 (0.81 – 1.35) 0.73	0.81 (0.43 – 1.54) 0.53	1.02(0.66 – 1.56) 0.94
CDKN2, 3' UTR OR (95% CI) p-value	1.04 (0.80 – 1.35) 0.79	1.18 (0.52 – 2.69) 0.70	1.09 (0.68 – 1.74) 0.73

[1] Controlling for race/ethnicity, and matched on age, date of blood donation and menopausal status at blood donation.

[2] The regressions were defined as: Dominant (AA=Aa=1, aa=0); Recessive (AA=1, Aa=aa=0); and Log-additive (aa=0, Aa=0.5, AA=1), where aa= wild type, Aa= heterozygous, AA= homozygous variant.

Papers Resulting from the Grant:

Polymorphisms in the DNA Nucleotide Excision Repair Pathway and Breast Cancer Risk. Roy E Shore, Isaac Wirgin, Anne Zeleniuch-Jacquotte, Harvey Mohrenweiser, Alan Arslan, Diane Currie, Yelena Afanasyeva, Karen Koenig, Paolo Toniolo. Paper in preparation, 2003.

Genetic Polymorphisms in Genes for Repair of DNA Double-Strand Breaks and Breast Cancer Risk. Roy E Shore, Isaac Wirgin, Anne Zeleniuch-Jacquotte, Harvey Mohrenweiser, Alan Arslan, Diane Currie, Yelena Afanasyeva, Karen Koenig, Paolo Toniolo. Paper in preparation, 2003.

Other papers will be prepared during the next 8-10 months.