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Variants in activators and downstream targets of ATM, radiation exposure and contralateral breast cancer risk in the WECARE Study

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Abstract

Ionizing radiation is a breast carcinogen that induces DNA double strand breaks (DSBs), and variation in genes involved in the DNA DSB response has been implicated in radiation-induced breast cancer. The Women's Environmental, Cancer and Radiation Epidemiology (WECARE) Study is a population-based study of cases with contralateral breast cancer (CBC) and matched controls with unilateral breast cancer. The location-specific radiation dose received to the

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contralateral breast was estimated from radiotherapy records and mathematical models. 152 SNPs in six genes (*CHEK2*, *MRE11A*, *MDC1*, *NBN*, *RAD50*, *TP53BP1*) involved in the DNA DSBs response were genotyped. No variants or haplotypes were associated with CBC risk (649 cases, 1284 controls) and no variants were found to interact with radiation dose. Carriers of a *RAD50* haplotype exposed to ≥ 1 Gy had an increased risk of CBC compared with unexposed carriers (RR=4.31 (95% CI 1.93-9.62)); with an excess relative risk (ERR)/Gy = 2.13 (95% CI 0.61-5.33)). Although the results of this study were largely null, carriers of a haplotype in *RAD50* treated with radiation, had a greater CBC risk than unexposed carriers. This suggests that carriers of this haplotype may be susceptible to the DNA-damaging effects of radiation therapy associated with radiation-induced breast cancer.

Keywords

DNA repair; haplotypes; polymorphisms; radiation; contralateral breast cancer

Introduction

Many of the genes known to be associated with increased susceptibility to breast cancer function within a common biochemical pathway involved in signaling the presence of, and coordinating the response to, DNA double-strand breaks (DSBs) (i.e., *BRCA1* (MIM# 113705), *BRCA2* (MIM# 600185), *CHEK2* (MIM# 604373), *ATM* (MIM# 607585)) (Thompson and Easton, 2004). Ionizing radiation (IR) is a known breast carcinogen (Boice Jr, 2001; Boice Jr, et al., 1992; Hooning, et al., 2008; Land, et al., 2003; Preston, et al., 2002; Stovall, et al., 2008) and induces multiple types of DNA damage, most notably DSBs, that activate this signaling pathway.

The cellular response to the presence of DSBs begins with the recognition of damage sites. A major component of DNA DSB sensing is the MRE11A-RAD50-NBN (MRN) - complex which acts to stabilize the broken strands of DNA at the break and carry out initial processing of the free DNA ends (Dzikiewicz-Krawczyk; Jazayeri, et al., 2008; Lee and Paull, 2005). The MRN complex recruits ATM, a large serine-threonine kinase to the site of damage and facilitates its activation. Once activated, ATM phosphorylates a number of downstream targets including CHEK2, NBN, MDC1 and TP53BP1 (Dzikiewicz-Krawczyk; Lee, et al., 2010; Lee and Paull, 2005), amplifying the damage signal by stabilizing the presence of proteins at the DSB site, such as the MRN, and recruiting others, such as MDC1 and TP53BP1. Ultimately, multiple signaling cascades are activated by this process invoking cell cycle checkpoint arrest, DNA repair, and apoptosis (Lavin, 2008). Given the importance of DNA damage both in initiating carcinogenesis and in treating existing cancers, the key molecules in the pathway that signal the presence of DNA DSBs have become candidate risk-factors for a variety of cancers, including breast cancer.

Treatments received for a first breast cancer can influence a woman's risk of developing a second primary breast cancer in the contralateral breast (CBC) especially among long-term survivors treated with radiation (RT) at an early age (Boice Jr, et al., 1992; Hooning, et al., 2008; Stovall, et al., 2008). Chemotherapy and tamoxifen both can reduce CBC risk (Bertelsen, et al., 2008; Chen, et al., 1999). Additionally, mutations in *ATM*, *MRE11A* (MIM# 600814), *RAD50* (MIM# 604040) and *NBN* (MIM# 602667) lead to the syndromes (ataxia-telangiectasia (MIM# 208900), ataxia-telangiectasia-like disorder (MIM# 604391), *RAD50* deficiency (MIM# 613078) and Nijmegen breakage syndrome (MIM# 251260), respectively, that are associated with increased cellular sensitivity to ionizing radiation (Helleday, et al., 2008).

Previously we showed that CBC was not significantly associated with RT dose to the contralateral breast (RR=1.1 (95% CI 0.9-1.3) overall, but that women under age 40 exposed to >1Gy had higher risk of CBC than unexposed women (RR=2.5 (95% CI 1.4-4.5)) (Stovall, et al., 2008). We also showed that there are genetic variants that influence a woman's susceptibility to this radiation exposure. For example, rare variants in *ATM* predicted *in silico* to be deleterious, were associated with a non-significant increase in CBC risk in this study population (Concannon, et al., 2008). Conversely, some of the common *ATM* variants were found to be associated with a reduction in CBC risk (Concannon, et al., 2008). When missense variants in *ATM* (MAF<1%), predicted to be deleterious were examined in the presence of RT, a statistically significant increase in CBC risk was seen among carriers exposed to ≥ 1 Gy to the contralateral breast compared radiation-unexposed women with wild-type genotype (RR=2.0 (95% CI 1.1-3.9)). Additionally, an increased risk was seen among carriers exposed to RT compared with unexposed carriers with an excess relative risk (ERR) per Gy of 2.6 (95% CI 0.0-10.6), *p* for trend=0.04 (Bernstein, et al., 2010). CHEK2 is a downstream phosphorylation target of ATM and *CHEK2**1100delC is a rare variant that truncates the CHEK2 protein eliminating its kinase function. Previously we reported data suggestive of an increased risk of CBC among carriers of this mutation treated with radiation when compared with unexposed women with the wild-type genotype (RR=2.6 (95% CI 0.8-8.4)) (Mellemkjaer, et al., 2008). The current study seeks to expand on these research findings, identifying additional areas of variation that increase a woman's risk of radiation-induced breast cancer.

We hypothesized *a priori* that carriers of common low penetrance variants in genes that interact with or are phosphorylated by ATM could be more sensitive to radiation damage that increases the risk of radiation-induced CBC. The objective of this study was to evaluate the influence of variants in DNA-damage response genes in the presence and absence of radiation exposure on CBC risk. Using population-based case-control study data we examined the interaction of ionizing radiation exposure and variants in six genes coding for proteins that are central players in the cellular response to RT and act as activators and down-stream targets of ATM (*CHEK2*, *MRE11A*, *MDC1* (MIM# 607593), *NBN*, *RAD50*, *TP53BP1* (MIM# 605230)). We estimated associations with individual SNPs and with gene haplotypes.

Materials and Methods

Study Population

The Women's Environmental Cancer and Radiation Epidemiology (WECARE) Study is a multi-center, population-based, case-control study where cases are women with asynchronous CBC and controls are women with unilateral breast cancer (UBC) (Bernstein, et al., 2004). Participants were identified, recruited and interviewed through four population-based cancer registries in the United States that are part of the National Cancer Institute's Surveillance, Epidemiology and End Results program: the Los Angeles County Cancer Surveillance Program; Cancer Surveillance System of the Fred Hutchinson Cancer Research Center (Seattle); State Health Registry of Iowa; and Cancer Surveillance Program of Orange County / San Diego-Imperial Organization for Cancer Control (Orange County / San Diego). The fifth registry from which participants were recruited was the Danish Breast Cancer Cooperative Group Registry and the Danish Cancer Registry (Bernstein, et al., 2004).

Eligible women with CBC (cases) (n=708) were selected from a cohort of 52,536 women with histologically confirmed breast cancer reported to one of the five population-based cancer registries who met the following criteria: a) diagnosed between 1/1/1985 and 12/31/2000 with UBC followed by a second primary, *in situ* or invasive, breast cancer in the contralateral breast, diagnosed at least 1 year later (i.e., between 1/1/1986 and 12/31/2000);

b) resided in the same study reporting area for both diagnoses; c) had no previous or intervening cancer diagnosis; d) were under age 55 years at the time of diagnosis of the first primary breast cancer; e) were alive at the time of contact; and f) provided informed consent, completed an interview and provided a blood sample. The time between cases' two diagnoses defined the "at risk interval". A one year interval between first and second breast cancer diagnosis was used to rule out synchronous disease.

WECARE Study controls (n=1,399) were selected from the same five population-based cancer registries and met the following criteria: a) diagnosed between 1/1/1985 and 12/31/1999 with UBC while residing in one of the study reporting areas; b) resided on the reference date (date of first diagnosis plus at-risk interval of matched case) in the same cancer reporting area as when they were diagnosed with their breast cancer; c) never diagnosed with any other cancer; d) were under age 55 years at the time of UBC diagnosis; e) provided informed consent, completed an interview and provided a blood sample; and f) had not had prophylactic mastectomy of the contralateral breast during the at-risk interval. Two controls were individually matched to each case on year of birth (in 5-year strata), year of diagnosis (in 4-year strata), registry region and race/ethnicity. Additionally, to improve statistical efficiency, cases and controls were counter-matched on registry-reported radiation exposure so that each triplet contained two exposed women (Bernstein, et al., 2004). Thus, for each exposed case, one exposed and one unexposed control were selected from the relevant stratum and for each unexposed case, two exposed controls were selected. This ensured that each triplet contributed to the analysis, avoiding the situation where all members of a matched set had the same radiation status. Four participants, all of whom were controls, consented only to *ATM*, *BRCA1* and *BRCA2* genotyping and therefore were excluded from this analysis.

Data collection

The data collection protocol was approved by the institutional review board at each of the participating U.S. centers and the ethical committee system in Denmark, and each patient provided informed consent. All participants (708 cases and 1399 controls) were interviewed by telephone using a pre-tested, structured questionnaire. The questionnaire was designed to obtain information about events occurring before the diagnosis of the first primary as well as those occurring within the at-risk interval (prior to the date of second cancer diagnosis for cases and the corresponding reference date for controls). Medical records, pathology reports and hospital charts were used to collect detailed treatment information (chemotherapy, hormonal therapy, and radiotherapy) and tumor characteristics (location in the breast, stage at diagnosis, estrogen and progesterone receptor status and histology). DNA was prepared from blood samples by red cell lysis and standard methods of phenol/chloroform extraction.

Radiation therapy details were sought from the basic RT record, RT summary, RT notes, medical record notes, surgery reports (for brachytherapy) and physician correspondence. The absorbed radiation dose to the location in the contralateral breast where the second breast cancer arose (or to the equivalent breast location for UBC controls) was estimated for each woman, for each specific treatment regimen, using tissue-equivalent phantoms as previously described (Stovall, et al., 2008). Among women who had undergone RT, the mean dose received to the contralateral breast was 1.2 gray (Gy) (SD=0.7). Two RT variables were created: 1) RT (ever/never), which indicates if a woman received RT regardless of the dose received by the contralateral breast and 2) RT dose, which is a measure of the absorbed dose to the contralateral breast at the location of the second breast cancer in cases or corresponding location for the matched controls.

All analyses were restricted to Caucasian women (649 cases and 1284 controls) to minimize the potential influence of ancestral differences in genotype/haplotype frequencies. Analyses

using RT dose to the contralateral breast were restricted to women with, 1) complete RT records and 2) information on the location of the second primary CBC in cases (550 cases and 1096 controls).

SNP Selection and Genotyping

SNP lists from HapMap were imported into Tagger (in Haploview) (Barrett, et al., 2005) and haplotype tagging SNPs (tagSNPs) were selected based on patterns of linkage disequilibrium (LD) as determined by Gabriel et al (Gabriel, et al., 2002). tagSNPs were selected based on pairwise tagging with a minimum r^2 of 0.90. These were supplemented with non-synonymous coding SNPs identified in dbSNP. Where LD extended outside the gene, SNPs in these regions were also included. A total of 152 SNPs in six genes (*CHEK2* (NM_007194), *MRE11A* (NM_005590), *MDC1* (NM_014641.1), *NBN* (NM_001024688), *RAD50* (NM_133482), *TP53BP1* (NM_005657)) were selected. After genotyping, SNPs with >5% missing genotypes were excluded from analysis (4 from *CHEK2*, 3 from *MRE11A*, 3 from *MDC1*, 4 from *NBN*, 1 from *RAD50* and 0 from *TP53BP1*). A single monomorphic SNP in *RAD50* (rs28903087:G>A) and two variants that were found to deviate from Hardy-Weinberg Equilibrium (HWE) ($p < 0.001$): *CHEK2* rs6005834:A>G and *NBN* rs1881469:T>A, were also excluded. This left 134 SNPs for the current analysis, 32% of which had a minor allele frequency (MAF) <5% and 43% with a MAF <10% (Supp. Table S1). Using HapMap Phase II release 24, these remaining variants ($n=134$) captured 81% of the SNPs in *CHEK2*, 100% in *MRE11A*, 87% in *MDC1*, 88% in *NBN*, 97% in *RAD50* and 86% in *TP53BP1* ($r^2 > 0.90$). These values are likely underestimates of the actual coverage since not all genotyped variants can be found in HapMap and additional SNPs, outside the gene, were included based on patterns of LD.

All SNPs were genotyped in a custom oligonucleotide probe panel using the Illumina Golden Gate™ assay on the Sentrix Array Matrix and scanned with the Bead Array Reader (Illumina Inc., San Diego). WECARE Study laboratory personnel were unaware of the case/control status of the DNA samples. Additional quality control steps included 24% duplicate samples interspersed, matched case/control triplets assayed on the same plate and negative controls lacking DNA on each plate. Prior to the availability of the multiplex genotyping assays (Illumina, Inc.), individual SNPs in the *TP53BP1* gene region (8 SNPs) and in the *NBN* gene region (10 of 34 SNPs) were genotyped using the MGB Eclipse™ Probe System (Epoch Biosciences, Bothell, WA, USA). The Eclipse assay discriminates alleles based on temperature-induced annealing and dissociation of dye-labeled allele specific probes in 384-well plates. Results from the subset of SNPs genotyped by both methods were concordant.

Statistical Analysis

Single SNPs

Analysis of SNPs Without Interaction Terms—Rate ratios (RR) and 95% confidence intervals (CI) were estimated and represent the association between individual variants and CBC risk. SNP analysis was conducted using a log-additive model estimating the per-allele RR. This analysis used conditional logistic regression for matched case-control studies, incorporating the logarithm of the control counter-matching sampling probabilities as an “offset term” (Bernstein, et al., 2004), and adjusting for exact age of first breast cancer diagnosis. A conservative Bonferroni correction was used to determine the multiple comparison cut-point ($\alpha=0.0004$, obtained from $(0.05/134 \text{ SNPs})$) at which results were considered statistically significant. The P_{ACT} method of adjusting for multiple comparisons, which takes into account linkage disequilibrium between nearby markers, was also applied to this analysis (Conneely and Boehnke, 2007).

Interaction with Radiation Dose to the Contralateral Breast—For each individual variant we examined the interaction with RT (ever/never) using a model that included parameters for the individual effects of the SNP (dominant coding) and RT, age at diagnosis, and a SNP x RT interaction term. Radiation dose analysis was also conducted for all SNPs using the dominant genotype coding model, where unexposed women with a wild-type genotype were the reference group.

Haplotypes

Analysis of Haplotypes Without the Interaction Term—Haplotypes (frequency >0.01) were estimated using PLINK (version 1.06) (Purcell, et al., 2007), a program that utilizes the Expectation-Maximization (EM) algorithm (Excoffier and Slatkin, 1995) to estimate the phase and overall frequency of each haplotype. Seventeen haplotypes were identified for *CHEK2*, 12 for *MRE11A*, 9 for *MDC1*, 18 for *NBN*, 7 for *RAD50* and 4 for *TP53BP1* (Supp. Table S2). Haplotype analysis was conducted using the expected haplotype (EHAP) method by assigning each participant her expected haplotype score (dose) (Kraft, et al., 2005). Conditional logistic regression models were fit to the data adjusting for age at diagnosis and the counter-matching variable as described above. These models were fit without any interaction terms. The Bonferroni correction was used to take into account multiple comparisons, giving a corrected $\alpha = 0.0007$ (0.05/67 haplotypes).

Interaction with Radiation Dose to the Contralateral Breast—Similar to the single SNP analysis, haplotype interactions with RT (ever/never) were examined by fitting conditional logistic regression models that included parameters for the individual effects of the haplotype (dose) and RT, age at diagnosis, and a haplotype x RT interaction term. Radiation dose effects were modeled in two ways; first, a model was examined that included an interaction term between each haplotype and RT dose (both continuous variables). Second, the expected (most likely) haplotype was assigned to each individual to allow for a categorical analysis that modeled the risk associated with having at least one copy of a haplotype in the presence or absence of RT. P-values for trend across RT dose categories and the excess relative risk (ERR)/Gy in haplotype carriers were also calculated. The Bonferroni correction was applied to these analyses giving an $\alpha = 0.0007$ (0.05/67 haplotypes), at which results were considered statistically significant.

Statistical analyses were conducted using SAS 9.2 (SAS Institute Inc., Cary NC), and ERR/Gy calculated using the Epicure module in Pecan (HiroSoft International, Seattle WA).

Results

Cases and controls were similar for all matching characteristics with an average age at diagnosis of 45.5 years and an average age at reference date (age at second breast cancer diagnosis in cases) of 50.6 years (Table 1). The average number of years between the two diagnoses in cases (first diagnosis and reference date in controls) was 5.1 years. The six variants that had an uncorrected p-value <0.05 for the main-effect analysis (without interaction terms) are shown in Table 2. The most striking relationship was seen for *MDC1* rs4713354 (per allele RR=1.53 (95% CI 1.20-1.96), p for trend = 0.0007). None of the associations for these six variants however, met the Bonferroni or P_{ACT} criterion for significance. Further adjustment for BRCA1/2 carrier status or for rs1800057:C>G carrier status, a variant in ATM we have previously showed is associated with a statistically significant reduction in CBC risk (Concannon, et al., 2008), did not alter the results (results not shown). None of the interactions examined between individual variants and RT ever/never or RT dose to the contralateral breast (0, <1, ≥ 1 Gy) were statistically significant (results not shown).

The results for the five haplotypes that were weakly associated with CBC risk (uncorrected p-value <0.05) are presented in Table 3. This analysis was based on individual probabilities of carrying a specific haplotype (haplotype dose) as a continuous variable in the model. None of these haplotypes were associated with CBC risk after taking into account multiple comparisons.

A single haplotype in RAD50 (HAP5) was found to have a statistically significant interaction with RT. In an analysis looking at the interaction with RAD50 HAP5 and RT ever/never, the interaction term had a p-value of 0.006. This result was not significant after adjustment for multiple comparisons (a QQ-plot for this analysis can be found in the Supporting Information (Supp. Figure S1)). However, in an analysis of RT dose, carriers of at least one copy of *RAD50* HAP5, treated with radiation, had an increased risk of CBC compared with unexposed carriers (RR=2.83 (95% CI 1.18-6.77) for women exposed to <1 Gy and RR=4.31 (95% CI 1.93-9.62) for women exposed to ≥1 Gy, p for trend=0.0003) (Table 4). The difference in radiation dose effects between *RAD50* HAP5 carriers and non-carriers was also statistically significant (p=0.01). Further, a notable dose-response was seen among carriers of this haplotype with an ERR/Gy of 2.13 (95% CI 0.61-5.33). The impact of this haplotype on radiation associated CBC risk did not vary by age at first diagnosis or length of the latency period.

Discussion

Ionizing radiation is a breast carcinogen known to cause DNA DSBs, and variation in genes involved in the DNA DSB response has been implicated in radiation-induced breast cancer (Bernstein, et al., 2010; Brooks, et al., 2007; Millikan, et al., 2005). The current study is to date, the most comprehensive analysis of common variants in activators and downstream targets of ATM, one of the key regulators of the DSB damage response pathway, and breast cancer risk. In single variant analysis no strong associations were found between CBC risk, or radiation-induced CBC risk. Similarly, no strong associations were observed between any haplotype and CBC risk in the analyses without the interaction terms. However, among women treated with radiation, a haplotype in RAD50 was identified as being associated with CBC risk after adjustment for multiple comparisons. Further, this haplotype showed a statistically significant dose-response relationship between radiation dose to the contralateral breast and CBC risk.

RAD50 is a highly conserved component of the MRN complex and is involved early in the detection of DSB and the initial processing of DNA ends. Within this complex, RAD50 is thought to hold the two DNA ends together to allow for further processing (Dzikiewicz-Krawczyk, 2008; Lee and Paull, 2004; Lee and Paull, 2005; Williams, et al., 2007). RAD50 is an essential gene. Knockout of the mouse homologue results in embryonic lethality and cellular sensitivity to ionizing radiation (Luo, et al., 1999). RAD50 deficiency as a result of hypomorphic mutations in humans is associated with a clinical phenotype that shares many similarities with Nijmegen Breakage Syndrome (Waltes, et al., 2009). Further, RAD50 deficiency is associated with decreased levels of MRE11 and NBN, leading to overall MRN deficiency, suggesting that RAD50 is required for the stability of the MRN complex (Luo, et al., 1999; Waltes, et al., 2009). Indeed it appears this may be a general rule that deficiency in any one component of MRN reduces the levels of the remaining components (Cerosaletti and Concannon, 2004). RAD50 deficiency, both *in vivo* and in *in vitro* models, is associated with a phenotype of hypersensitivity to DNA-damaging agents characterized by increased chromosomal instability and an altered intra-S phase checkpoint after exposure (Luo, et al., 1999; Waltes, et al., 2009; Zhong, et al., 2005). Disruption in the activity of RAD50 and thus the MRN complex could affect carcinogenesis through some or all of these mechanisms leading to a reduction in the overall efficiency of the DNA DSB repair process. Rare

mutations in *RAD50* have previously been found to be associated with increased breast cancer risk (Ripperger, et al., 2008).

RAD50 HAP5 was found to be associated with an increased risk of CBC among radiation-exposed HAP5-carriers, with an ERR/Gy of 2.13. Women carrying this haplotype exposed to ≥ 1 Gy to the contralateral breast, had more than a 4-fold greater CBC risk than unexposed carriers. This haplotype consists of the wild-type (most common) genotype for all genotyped variants, none of which were independently associated with CBC risk, and is the fifth most common haplotype in this study population, carried by 4.9% of participants. Of note, this haplotype was not associated with CBC risk in main-effect analysis indicating that its impact on risk may be modified and magnified by environmental factors, in this case radiation exposure.

A unique strength of this study is the detailed information on RT, including individualized estimated dose to the contralateral breast at the location where the second primary breast tumor arose. These measures of radiation dose and nearly complete gene coverage are unique features of this study and limit the comparison of these results to those of other studies. Thus, these results require replication. In addition, we had limited power to examine rare variants by RT status (ever/never or by dose to the contralateral breast). Accordingly, the impact of rare variants and haplotypes in these genes on radiation-induced CBC warrants further investigation.

Another limitation is the current analytic challenge to examining these data using a pathway-based approach. The ability of the DNA damage response pathway to adapt to changes in the amount or activity of a single player is an important consideration. For example, when the MRN complex is compromised, ATM can still be activated, albeit, with slower kinetics. This suggests that examining the joint impact of multiple variants in multiple genes taking a pathway-based approach (i.e., gene-gene interactions) might provide additional information about the relationship between variation in the DNA-damage response pathway, radiation exposure and subsequent breast cancer risk. Methods for this analysis are currently being developed and validated and will be applied to the DNA-damage response pathway using the detailed radiation treatment data available for this study population.

The results of this study are largely null, suggesting that overall, variations in the genes evaluated are not related to increased susceptibility to radiation-induced breast cancer; however, we found that carriers of a haplotype in *RAD50* who were exposed to ≥ 1 Gy to the contralateral breast had a 4-fold greater CBC risk than unexposed carriers. This relationship remained statistically significant after adjustment for multiple comparisons. Although this result requires replication, it suggests that carriers of this haplotype may have an increased susceptibility to the DNA-damaging effects of radiation therapy that leads to radiation-induced breast cancer. Given the widespread use of RT for breast cancer, identification of susceptible populations warrants further study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1
Characteristics of Caucasian cases (women with asynchronous contralateral breast cancer) and controls (women with unilateral breast cancer only) from the WECARE Study population

Variable	Level	Cases N (%)	Controls ^A N (%)
Center	Iowa	111 (17.1)	221 (17.2)
	UC Irvine	107 (16.5)	212 (16.5)
	Los Angeles	157 (24.2)	307 (23.9)
	Seattle	95 (14.6)	190 (14.8)
	Denmark	179 (27.6)	354 (27.6)
Year of First Diagnosis	1985 -1989	289 (44.5)	549 (42.8)
	1990 -1995	279 (43.0)	571 (44.5)
	1996 -1999	81 (12.5)	164 (12.8)
Radiation Therapy	Never	329 (50.7)	250 (50.6)
	Ever	320 (49.3)	1034 (49.4)
Radiation Therapy Dose ^B	0 Gy	266 (40.9)	217 (16.9)
	< 1 Gy	155 (23.9)	486 (37.9)
	1+ Gy	129 (19.9)	389 (30.3)
	Unknown	99 (15.3)	192 (14.9)
Chemotherapy	No	363 (55.9)	594 (43.4)
	Yes	286 (44.1)	690 (56.6)
Radiation Therapy and Chemotherapy	No	497 (76.6)	737 (72.3)
	Yes	152 (23.4)	547 (27.7)
Hormone Therapy ^C	No	479 (73.8)	850 (66.9)
	Yes	170 (26.2)	432 (32.8)
	Unknown	0 (0.0)	2 (0.3)
Histology	Ductal	465 (71.6)	963 (76.3)
	Lobular	84 (12.9)	123 (8.7)
	Medullary	30 (4.6)	45 (3.0)
	Other	70 (10.8)	149 (11.5)
	Unknown	0 (0.0)	4 (0.5)
Stage	Localized	469 (72.3)	839 (64.1)
	Regional	180 (27.7)	445 (35.9)
ER Status ^D	Positive	308 (47.5)	686 (54.1)
	Negative	170 (26.2)	301 (23.1)
	Other	68 (10.5)	142 (11.8)

Variable	Level	Cases N (%)	Controls ^A N (%)
	Unknown	103 (15.9)	155 (11.0)
PR Status ^D	Positive	259 (39.9)	563 (43.3)
	Negative	149 (23.0)	281 (23.1)
	Other	73 (11.2)	158 (12.6)
	Unknown	168 (25.9)	282 (21.1)

Variable	Cases Mean (SD)	Controls Mean (SD)	Cases Median (Range)	Controls Median (Range)
Age at First Diagnosis	45.5 (6.4)	45.5 (6.2)	46 (24-55)	46 (23-55)
Age at Reference Date	50.6 (7.3)	50.6 (7.1)	51 (27-71)	51 (27-69)
At-risk Period ^E	5.1 (3.2)	5.1 (3.2)	4.3 (1.0-15.6)	4.3 (1.0-15.6)

Abbreviations: CBC=asynchronous contralateral breast cancer; UBC=unilateral breast cancer; SD=standard deviations, ER=estrogen receptor, PR=progesterone receptor

^A Weighted percentages (with the exception of matching variables.)

^B Detailed dose estimation data was available for 550 Caucasian cases and 1096 Caucasian controls (i.e., Women for whom estimates of RT dose to the contralateral breast in the location of the second primary tumor in cases (and the corresponding location in controls) was available.

^C Hormone therapy includes all hormonal breast cancer treatments including: Tamoxifen, Raloxifene, Toremifene, Anastrozole, Letrozole, Aromasin, Aminoglutethimide, Gosereline, Leuprolide, Faslodex and Megestrol Acetate

^D The 'Other' category consists of women where no lab test was given, the test was given and the results are unknown and the test was given and the results were borderline

^E Interval between first diagnosis and second diagnosis (cases) or reference date (controls)

Table 2
Association between selected variants in DNA-damage response genes and CBC risk^A

SNP	Allele	Cases (CBC) ^B	Controls (UBC) ^B	Per Allele RR ^C (95% CI)	p-value for trend
CHEK2					
rs6005861	AA	624	1205	0.59 (0.36-0.98)	0.04
	AG	22	73		
	GG	1	2		
MRE11A					
rs13447682	CC	628	1206	0.55 (0.31-0.97)	0.04
	CT	18	54		
	TT	0	2		
MDC1					
rs4713354	TT	503	1056	1.53 (1.20-1.96)	0.0007
	TG	139	219		
	GG	7	8		
NBN					
rs14448	TT	602	1131	0.68 (0.47-0.97)	0.03
	TC	43	141		
	CC	3	5		
rs9297757					
	GG	614	1168	0.59 (0.39-0.89)	0.01
	GT	35	109		
	TT	0	5		
rs3736640					
	TT	621	1185	0.60 (0.38-0.95)	0.03
	AT	28	93		
	AA	0	3		

Abbreviations: CBC=asynchronous contralateral breast cancer; UBC=unilateral breast cancer; RR=rate ratio; CI=confidence interval; RT=radiation therapy

^AResults are presented only for those variants with a p<0.05 in main-effect analysis (no interaction term). None of these associations remained significant after adjustment for multiple comparisons (both Bonferroni correction and PACT methods were applied)..

^B The number of cases and controls varies for each SNP due to missing genotype data.

^C Per allele RR (log-additive model), adjusted for age at first diagnosis and countermatching weight.

Table 3
Association between selected haplotypes in DNA-damage response genes and CBC risk^A

	Cases (CBC) ^B	Controls (UBC) ^B	RR (95% CI) ^C	p-value
CHEK2				
HAP10	32	43	1.72 (1.03-2.88)	0.04
MDC1				
HAP7	51	72	1.59 (1.05-2.41)	0.03
MRE11A				
HAP7	18	59	0.53 (0.30-0.94)	0.03
HAP9	12	40	0.37 (0.18-0.77)	0.01
NBN				
HAP10	27	94	0.60 (0.37-0.96)	0.03

Abbreviations: CBC=asynchronous contralateral breast cancer; UBC=unilateral breast cancer; RR=rate ratio; CI=confidence interval; RT=radiation therapy

^A Results are presented only for those haplotypes with a $p < 0.05$ in main-effect analysis (no interaction term). None of these associations remained significant after adjustment for multiple comparisons.

^B Haplotype frequencies were determined by assigning the most likely haplotype to each individual.

^C RR adjusted for age at diagnosis of first primary breast tumor and counter-matching weight. RRs are based on individual probabilities of carrying a specific haplotype (haplotype dose) as a continuous variable in the model.

Table 4
Interaction between a RAD50 haplotype and radiation treatment dose to the contralateral breast

Gene/Radiation Dose	Cases (CBC)	Controls (UBC)	RR (95% CI) ^A	p for trend ^B	Cases (CBC)	Controls (UBC)	RR (95% CI) ^A	p for trend ^B	p for heterogeneity
RAD50			No HAP5				HAP5		
0 Gy	247	191	1.00	0.53	22	28	1.00	0.0003	0.01
<1 Gy	140	451	1.10 (0.85-1.41)		14	35	2.83 (1.18-6.77)		
≥1 Gy	107	355	1.05 (0.79-1.41)		22	33	4.31 (1.93-9.62)		

Abbreviations: CBC=asynchronous contralateral breast cancer; UBC=unilateral breast cancer; RR=rate ratio; CI=confidence interval; RT=radiation therapy; Gy=Gray

^A Adjusted for exact age at diagnosis of first primary and counter-matching weight. Haplotype status was determined by assigning the most likely haplotype to each individual. To be classified as having a given haplotype an individual must carry at least one copy. Numbers are provided for comparison purposes only.

^B p for trend across RT dose categories