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A specific RAD51 haplotype increases breast cancer risk in Jewish non-Ashkenazi high-risk women

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ABSTRACT

While the precise genes involved in determining familial breast cancer risk in addition to BRCA1/2 are mostly unknown, one strong candidate is RAD51. Jewish non-Ashkenazi women at high-risk for breast/ovarian cancer and ethnically matched controls were genotyped using four single nucleotide polymorphisms spanning the RAD51 genomic region, and the resulting haplotypes were constructed using the GERBIL algorithm. A total of 314 individuals were genotyped: 184 non-Ashkenazi high-risk women (119 with breast cancer), and 130 unaffected, average-risk ethnically matched controls. Using GERBIL, three frequent haplotypes were constructed. One of the haplotypes (TGTA – coined haplotype 3) was present in 7.3% (19/260 haplotypes) of controls ($n = 130$) and in 16.8% (40/238 haplotypes) of high-risk breast cancer patients ($n = 119$, $P = 0.001$). A specific RAD51 haplotype is more prevalent among non-Ashkenazi Jewish high-risk women than in average-risk population.

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1. Introduction

Genetic factors play a pivotal role in breast and ovarian cancer susceptibility. In sporadic, non-familial cases, multiple genes combined with environmental exposures, converge to result in the tumourous phenotype.¹ The exact identity of the genes involved as well as their relative contribution to disease phenotype remains elusive, but they are generally referred to as “high prevalence low penetrance genes”, and mutations in individual genes only marginally increase the risk for devel-

oping cancer.^{1,2} In familial breast cancer, encountered in about 10% of incident cases, germline mutations in the BRCA1 and BRCA2 genes can be detected in some families, with an autosomal dominant mode of transmission.³ These germline mutations substantially increase breast and ovarian cancer risk, yet penetrance is incomplete.^{3–5} Estimates of penetrance have varied widely, ranging from 36% to 85% life-time risk for breast and 16–63% for ovarian cancer, depending on mutation location, mode of ascertainment and ethnic origin.^{4–6} In Jewish Ashkenazi high-risk women, two mutations in BRCA1

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(185delAG and 5382InsC) and one in BRCA2 (6174delT) account for a substantial proportion of germline mutations in both genes in high-risk families, in incident cases as well as in up to 2.5% of the general Ashkenazi population.⁶ King⁵ estimated penetrance rates of these predominant mutations: life-time risk for breast cancer was 82%, and life-time risk for ovarian cancer was 54% for BRCA1 and 23% for BRCA2 mutation carriers. Incomplete penetrance suggests that genetic and/or environmental factors modify cancer risk in BRCA mutation carriers. Several genes have been proposed and tested as modifiers of cancer risk in BRCA mutation carriers, among them the RAD51 gene reviewed in.¹ The physical and functional interactions between the RAD51 protein and BRCA1 and BRCA2 proteins in double strand DNA breaks repair pathways⁷ make it a likely candidate modifier gene. Indeed, a missense mutation in RAD51 (Arg-150-Glu) has been described in two Japanese patients with bilateral breast cancer.⁸ In three studies, a single nucleotide polymorphism (SNP) in the 5' untranslated region of RAD51 (135G/C) was reported to be associated with an increase in breast cancer risk in BRCA2 mutation carriers.^{9–11} Wang¹¹ reported an association between breast cancer and the presence of C allele in the 135G/C SNP in BRCA2 mutation carriers (odds ratio 3.2; 95%CI [1.4–4.0]). Two subsequent studies of Ashkenazi Israeli women^{9,10} confirmed this increased breast cancer risk in BRCA2 mutation carriers.

In addition to being a putative modifier of mutant BRCA alleles, the RAD51 gene seems to be a likely candidate to be involved in breast cancer pathogenesis in general. Increased RAD51 expression was reported somatically in sporadic ductal breast cancer, and expression levels were also correlated with tumour grade,^{12,13} whereas in another study, about 30% of breast carcinomas displayed decreased RAD51 levels.¹⁴ Radiation resistance of breast cancer cell lines correlated with increased RAD51 expression,^{12,15} and in vitro reduction of RAD51 levels decreased radiation resistance.^{16,17} Yet, Blasiak and coworkers¹⁸ failed to show any differences in the rate of the 135G/C SNP both somatically in node-negative and node-positive breast carcinoma, and in germline DNA in affected and ethnically matched (Polish non-Jewish women) controls. Similarly, a previous case control study of RAD51 haplotypes in Jewish Ashkenazi women with and without breast cancer¹⁹ did not demonstrate an effect of RAD51 haplotypes on breast cancer morbidity in this ethnic group. Thus, RAD51 may be involved in modifying the effects of mutant BRCA2 alleles, and may also be somatically involved in breast cancer pathogenesis.

To shed light on the contribution of RAD51 to breast cancer phenotype in Jewish non-Ashkenazi women, we genotyped RAD51 SNPs and constructed the resulting haplotypes in Jewish non-Ashkenazim at high-risk for breast and ovarian cancer, who were non-carriers of any of the predominant Jewish BRCA gene mutations.

2. Patients and methods

2.1. Study population and data collection

Study participants were ascertained through familial cancer genetics clinics at two institutions in Israel: Sheba Medical

Center in Tel Hashomer and Rambam Medical Center in Haifa. All women who were counselled and genetically tested in these institutions from January 1 1997 to December 31 2002 were eligible for participation. From each counselled individual, the data collected via a personal interview included demographic data, ethnic origin, use of hormones and oral contraceptives, reproductive history, personal and familial history of cancer including type of malignancy (based on pathology reports), and age at diagnosis. Ethnic origin was assigned by the country of birth of both grandparents and parents as Ashkenazi (East Europe) or non-Ashkenazi. Counselling individuals were offered testing if they were deemed "high-risk" for breast and or ovarian cancer by standard definitions.²⁰ The institutional review boards in both participating medical centers approved the study, and each individual signed a written informed consent. Based on the results of genetic testing the study population was divided into two main subsets: high-risk non-Ashkenazi non-carriers, that was further subdivided into affected (with breast cancer) and unaffected; and BRCA2 6174delT mutation carriers, including all Ashkenazim with either breast cancer or unaffected mutation carriers.

2.2. Control population

Samples from healthy, non-Ashkenazi controls were taken from DNA samples from unrelated individuals with no personal or familial history of cancer who gave their consent for anonymous testing. These individuals were recruited during oncogenetic counselling from amongst female escorts to high-risk counselled individuals, who were unrelated to the counselled individual (e.g., a close friend). These controls were also interviewed and counselled as to the aims of the study.

2.3. DNA isolation

Genomic DNA was prepared from anticoagulated venous blood samples using the PUREGene DNA isolation kit (Gentra systems Inc., Minneapolis, MN) using the manufacturer's recommended protocol.

2.4. Detection of the recurring Jewish BRCA1/BRCA2 mutations

Each participant was initially tested for being a carrier of one of the four recurring mutations in Jewish individuals in BRCA1 (185delAG, 5382InsC, Tyr978X) and BRCA2 (6174delT). Detection of these mutations was carried out by employing modified restriction enzyme digest, that distinguishes the mutant from the wild type allele, using primer sequences, cycling profiles, PCR conditions, and gel electrophoresis as previously described.^{21,22}

2.5. RAD51 genotyping

RAD51 genotyping was performed by PCR amplification of four SNPs spanning the genomic length of the gene (about 38 Kb). The SNPs were chosen from three databases: www.ensembl.org, www.genome.ucsc.edu and www.ncbi.nlm.nih.gov:

SNP1 T/C rs3092983 (11717536), SNP2 C/G rs3092982 (11747028), SNP3 T/G rs1051482 (position 11795379) and SNP4 A/G rs3092978 (position 11822332).

2.6. PCR amplification

PCR amplification was performed in a total volume of 25 μ l, using 10 ng human genomic DNA, 10 pmol of each primer and ready-to-go PCR beads (Amersham Pharmacia Biotech AB, Uppsala, Sweden) containing \sim 1.5 U Taq DNA polymerase, 10 nM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM MgCl₂, and 200 μ M of each dNTP according to the company's instructions. After an initial denaturation at 95 °C for 5 min, 35 cycles of 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s were carried out with a final extension period of 5 min.

2.7. Pyrosequencing analysis

PCR products were prepared using the manufacturer's recommended protocol.²³ The polymorphic positions were analyzed using a PSQ 96 System together with SNP Software and SNP Reagent Kits (Pyrosequencing AB). After the run, the peaks were evaluated according to the expected pattern by referring to the dispensation order.

2.8. Haplotype reconstruction

The novel software GERBIL²⁴ performed the process of phasing the genotypes. GERBIL's algorithm is based on a new model for genotype generation. In this model, frequent haplotypes are redefined in a probabilistic setting, where each has a particular frequency, and each individual allele in it has an assigned error probability. The program seeks a set of parameters and an accompanying haplotype reconstruction that has maximum likelihood, using an EM algorithm. The model allows both errors and rare haplotypes, and the algorithm is particularly tailored to the common situation in which the number of frequent haplotypes is small. When applied to the RAD51 genotypes, up to five frequent haplotypes were allowed. The running time for resolving all genotypes on Pentium 4 2000 MHz PC was 15 s.

2.9. Comparison of haplotype frequencies between cases and controls

Odds ratios (OR) and 95% confidence interval (CI) were estimated for each haplotype using the frequent haplotype observed among all ethnic groups combined. Analyses were stratified by ethnicity and a summary OR was estimated controlling for age and ethnicity.

2.10. Statistical methods and association analyses

Tests for association between sequence variants and breast cancer were performed by comparing the allele frequencies between cases and controls using the χ^2 test with 1 degree of freedom. Estimates of relative risk and odds ratio were calculated and adjusted for potential confounders. Association between the haplotypes of the four SNPs and breast cancer

risk was also evaluated using the results of the GERBIL algorithm.²⁴

3. Results

3.1. Characteristics of study participants: high-risk non-carrier group

A total of 184 unrelated non-Ashkenazi high-risk women participated in the study and, in addition, 130 unaffected, average-risk population controls. The 184 high-risk group included non-Ashkenazi non-carriers of any of the predominant Jewish mutations in the BRCA1/2 genes, of whom 119 (64.7%) are affected with breast cancer, and 65 (35.3%) asymptomatic, non-carriers with family history of breast and ovarian cancer. The ethnic distribution of this high-risk, non-carrier group was as follows: Iraqi origin 60 (32.6%), North African (mostly Moroccan) 44 (23.9%), Balkan origin 33 (17.9%) Yemenite origin 32 (17.4%), and the rest 15 (8.2%) were of mixed non-Ashkenazi origins. The ethnic distribution of breast cancer patients among this high-risk group was as follows: Iraqi origin 34 (28.6%), North African 38 (32%), Balkan origin 16 (13.4%) Yemenite origin 17 (14.3%), and the rest 14 (11.7%) were of mixed non-Ashkenazi origins. The mean age of all high-risk cases ($n = 184$) at counselling was 50.4 ± 11.3 years (average \pm SD); mean age at diagnosis of breast cancer ($n = 119$) was 48.1 ± 10.1 years. The mean age at counselling for high-risk asymptomatic women ($n = 65$) was 50.4 ± 11.3 years. Among the high-risk non-carriers, 134 of 184 (72.8%) had a family history of cancer. All non-carriers with no ascertainable family history of cancer were personally affected with breast cancer. Women with breast cancer and no family history were classified as "high-risk" if breast cancer was diagnosed under the age of 40 years, or had bilateral breast cancer with the first one diagnosed at less than 45 years of age, or had both breast and ovarian cancer at any age. The reasons for non-ascertainment were mostly truncated pedigrees (as a result of the holocaust) or migration to Israel with loss of contact with other relatives.

3.2. Control group

The ethnic distribution of the 130 healthy, unrelated average-risk non-Ashkenazi controls was as follows: 46 (35.4%) Iraqis, 40 (30.7%) North African, 36 (27.7%) Yemenites, and 8 (6.2%) of Balkan origin. The mean age at recruitment for the control group was 50.8 ± 12.1 years. None among the control group had a personal or family history of cancer in first or second-degree relatives.

3.3. Genotyping the control group and haplotype construction

Initially, SNPs were genotyped in the control group. The observed genotype distributions based on allele frequencies for all 4 SNPs were consistent with Hardy-Weinberg equilibrium using the χ^2 test with 1 degree of freedom for all ethnic groups. When applying the GERBIL algorithm for block partitioning and phasing the genotypes from all groups together, only one block with low haplotype diversity was found, and

three frequent haplotypes were constructed: haplotype 1-CCTG (40.1%), haplotype 2-TGGG (36.2%) and haplotype 3-TGTA (16.7%). The three frequent haplotypes could be predicted by 2 tag SNPs. In addition, all three frequent haplotypes were shared across the different ethnic groups (Table 1).

3.4. Linkage disequilibrium tests

We measured the linkage disequilibrium (r^2) between each pair of SNPs in the data set. The most correlated SNPs are the first and the second with $r^2 = 0.75$, which corresponds to P -value = 0 for linkage equilibrium in our data set (a number lower than a normal computer accuracy, corresponding to obtaining a value of 106.74 for a χ^2 statistic with one degree of freedom). The least correlated pair is the third and the fourth SNP with $r^2 = 0.06$, which corresponds to P -value of 0.00021 for linkage equilibrium.

3.5. Haplotype-phenotype analyses

None of the haplotypes in the single block showed significant differences between the various ethnic groups among controls ($P = 0.65$). Comparison of haplotype frequencies between all high-risk cases (regardless of disease status) and controls showed that RAD51 haplotype 3 (TGTA) was present in 19 of 260 haplotypes of controls (7.3%, $n = 130$) and in 60 of 384 haplotypes of all cases (16.8%, $n = 184$) ($P = 0.00105$). Among high-risk, non-carriers breast cancer patients diagnosed under age 50 years ($n = 40$), 16 of 80 haplotypes displayed haplotype 3 (20%) compared with 30 of 148 control haplotypes (7.5%, $n = 74$) ($P = 0.034$, OR = 3.14 [95%CI (1.24-7.9)] RR = 1.36 [95% CI (1.13-1.63)]) (Table 2). The remaining two haplotype combination frequencies (1 + 2 vs. 3 and 3 + 2 vs. 1) were not statistically significant (data not shown).

3.6. BRCA2 6174delT mutation carriers

A total of 59 BRCA2 6174delT mutation carriers were analyzed for a single RAD51 SNP (see below), all were unrelated Jewish Ashkenazi women. Of these, 42 (71.2%) were asymptomatic mutation carriers (mean age at counselling 44.3 ± 5.6 years), 17 (28.8%) had breast cancer (mean age at diagnosis 42.4 ± 7 years).

3.7. Analysis of the RAD51 135C/G SNP

The occurrence of 135G/C SNP was evaluated in 59 BRCA2 6174delT carriers. In total, 7.1% (3/42) of the asymptomatic carriers had GC genotype, compared with 17.6% (3/17) of the carriers with breast cancer ($P = 0.226$). The occurrence of GG genotype was 92.9% (39/42) of asymptomatic car-

Table 1 - Haplotype frequency among healthy controls

Haplotype#	Common haplotypes	Haplotype frequency
1	CCTG	0.40
2	TGGG	0.36
3	TGTA	0.16

Table 2 - Haplotype and phenotype

Cases high-risk	Haplotype frequency N (%) (cases)		Haplotype frequency N (%) (controls)		χ^2 test P-value	Odds Ratio [95% CI]	Relative Risk [95% CI]
	3	1+2	3	1+2			
Affected n = 119	40 (16.8)	198 (83.2)	19 (7.3)	241 (92.7)	0.001	2.56 [1.43, 4.56]	1.5 [1.22, 1.84]
Asymptomatic n = 65	17 (13)	113 (87)	19 (7.3)	241 (92.7)	0.19	1.91 [0.095, 3.81]	1.48 [1.014, 2.158]
All n = 184	57 (15.5)	311 (84.5)	19 (7.3)	241 (92.7)	0.0047	2.35 [1.36, 4]	1.32 [1.15, 1.5]
Controls unaffected average-risk							
n = 130							
n = 130							
n = 130							

riers and 82.4% (14/17) of the carriers with breast cancer. No individual displayed the CC genotype.

4. Discussion

In the present study, the role of RAD51 in conferring breast cancer risk was assessed in several subsets of Jewish Israeli individuals. Initially, the occurrence rate of 135G/C SNP in 59 BRCA2'6174delT mutation carriers was determined. In total, 7.1% of the asymptomatic carriers had GC genotype, compared with 17.6% of the carriers with breast or ovarian cancer ($P = 0.226$). Although this result is not statistically significant, in all probability because of the small sample size, it indicates that this specific SNP occurs at a higher rate among affected carriers. This finding is consistent with three previously published studies reporting an association of this specific SNP with breast cancer risk in BRCA2 mutation carriers.^{9–11} We performed a combined statistical analysis of the results of the two prior studies that targeted Jewish Ashkenazi women^{9,10} and the present study, analysis encompassing a total of 232 BRCA2'6174delT Jewish mutation carriers: 21 of 121 (17.3%) carriers with breast or ovarian cancer had GC genotype, and 6 of 111 (5.4%) asymptomatic mutation carriers had GC genotype ($P = 0.0047$). Obviously a combined prospective multicenter study may help in resolving the issue and determine the feasibility of using this information in a clinical setting, to further define breast cancer risk among BRCA2 mutation carriers.

A novel aspect of the present study is the use of the GERBIL algorithm for haplotype inference,²⁴ an algorithm previously shown to be more efficient than three state-of-the-art phasing algorithms: HAP,²⁵ HaploBlock,²⁶ and PHASE²⁷ in analysis of real biological genotype data sets.²⁴ The statistical model employed by the GERBIL algorithm is particularly suited for use in association studies: all genotypes in the data are assigned to a few frequent haplotypes. Consequently, when testing association, only few hypotheses need to be tested. Using GERBIL, we have found that a specific RAD51 haplotype (Haplotype 3) is associated with an increased risk (of about 46%) for developing breast cancer in high-risk, non-Ashkenazi individuals. These results further establish RAD51 as a breast cancer susceptibility gene in high-risk non-Ashkenazi population, in addition to its putative role as a modifier of cancer risk in BRCA2 mutation carriers. It is that noteworthy one of the haplotypes (Haplotype 3) can be distinguished from the other haplotypes by a single allele. However, performing association tests between each allele and the phenotype separately revealed a lower association compared with association test for haplotypes, since the correction factor for multiple testing is 4 for the four SNPs compared with two tests for the three haplotypes.

The central role of RAD51 in homologous recombination and in maintaining genomic integrity is strongly supported by mouse RAD51 knockout models, which result in early embryonic lethality,²⁸ and by the inducible loss of RAD51 expression in chicken cells that leads to chromosome breaks, large genomic aberrations and cell lethality.²⁹ In this model, decreased RAD51 levels are predicted to lead to an abnormal tumorous phenotype, yet several authors described an increased RAD51 expression levels in tumour cell lines.³⁰ Thus, it has been proposed that the aberrant increase observed in

RAD51 expression in tumour cells may contribute to genomic instability by stimulating aberrant recombination between short repetitive elements and homologous sequences.^{31,32} In support of this notion, increased levels of RAD51 protein are observed in primary patient derived chronic myelogenous leukemia (CML) cell lines with higher expression in cells in blast crisis, a phase of the disease marked by increased genome instability and multiple chromosome rearrangements.³² Increased RAD51 expression also promoted aneuploidy and multiple chromosomal rearrangements. These data provide a link between elevated RAD51 protein levels, genome instability, and tumour progression.

Focusing on highly selected population, enriched for family history of cancer in order to define low penetrance genes, is recommended as a means to try and limit the sample size tested. Peto and Houlston,² have shown that genotyping such high-risk individuals would significantly decrease the sample size needed to achieve statistical significance by at least onefold. Hence, the present study that genotyped a sizeable number of high-risk women may provide the initial clue for a subsequent larger study that aims at determining the role of this haplotype in the average-risk, non-Ashkenazi population. In the broader perspective, these preliminary findings need to be confirmed in a larger, population based study, encompassing ethnically diverse, non-Jewish populations. Even though the majority of Jewish communities remained segregated for generations, some admixture with their non-Jewish neighbours did occur, especially among North African and Balkan Jews.³³ If these results are duplicated in any of the tested populations, then this would provide for an objective tool for assessing breast cancer risk in the general, average-risk population, and potentially target these individuals for early detection schemes or offer these higher than average-risk individuals prophylactic measures.

In conclusion, using a novel phasing tool, a specific RAD51 haplotype is seemingly more prevalent among Jewish Israeli high-risk non-Ashkenazi population of diverse ethnic origin. The applicability and the generalibility of these findings to ethnically diverse, non-Jewish populations and to the average-risk population remain to be established.

Conflict of Interest statement

None declared.

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