Functional and genomic approaches reveal an ancient CHEK2 allele associated with breast cancer in the Ashkenazi Jewish population

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Functional and genomic approaches can be integrated to screen efficiently for pathogenic alleles in founder populations. We applied such approaches to analysis of the cancer-associated cell cycle regulator CHEK2 in the Ashkenazi Jewish population. We first identified two extended haplotypes at CHEK2 that co-segregated with breast cancer in high-risk families. We sequenced CHEK2 in a case representing each haplotype and discovered two novel amino acid substitutions, CHEK2.S428F in the kinase domain and CHEK2.P85L in the N-terminal region. To assay these alleles for loss of CHEK2 function, we tested their capacity to complement Rad53 deletion in Saccharomyces cerevisiae. CHEK2.S428F failed to complement Rad53 and thus largely abrogates normal CHEK2 function, whereas CHEK2.P85L complemented Rad53 as well as did wild-type CHEK2. Epidemiologic analyses were concordant with the functional tests. Frequencies of CHEK2.S428F heterozygotes were 2.88% (47/1632) among female breast cancer patients not selected for family history or age at diagnosis and 1.37% (23/1673) among controls (OR = 2.13, 95% CI [1.26, 3.69], P = 0.004), whereas frequencies of CHEK2.P85L were 0.92% among cases and 0.83% among controls. On the basis of the experience of mothers, sisters and daughters of probands, breast cancer risk due to CHEK2.S428F was estimated as 0.17 (±0.08) by age 60. We conclude that CHEK2.S428F increases breast cancer risk ~2-fold among Ashkenazi Jewish women, whereas CHEK2.P85L is a neutral allele. In general, these results suggest that selecting probands with extended haplotypes that co-segregate with disease can improve the efficiency of resequencing efforts and that quantitative complementation tests in yeast can be used to evaluate variants in genes with highly conserved function.

INTRODUCTION

Breast cancer is the most common malignancy among women in the US, Canada, western Europe and Israel, with a lifetime risk to American women of >10% (1). Most breast and ovarian cancers are not inherited, but 5–10% are the result of inherited mutations in the tumor suppressor genes BRCA1 and BRCA2 (2,3). Among women with inherited mutations in these genes, lifetime risk of breast cancer can exceed 80% (3). However, most families with multiple cases of female breast cancer, but no cases of ovarian cancer, have no detectable inherited mutations in BRCA1 or BRCA2 (4,5). Statistical studies have suggested that genes harboring alleles of lower penetrance may explain breast cancer in such families (6,7).

One such gene is CHEK2, a serine/threonine protein kinase and the human homolog of Saccharomyces cerevisiae Rad53 and Schizosaccharomyces pombe Cds1 (8,9). In both yeast and mammals, this protein is a cell cycle checkpoint regulator that is rapidly phosphorylated in response to replication blocks and DNA damage (10). Activated CHEK2 inhibits CDC25C phosphatase, preventing entry into mitosis, and stabilizes p53, leading to cell cycle arrest in G1. CHEK2 and BRCA1
are linked functionally. CHEK2 interacts with and phosphorylates BRCA1, allowing BRCA1 to restore survival after DNA damage (11,12).

The inherited mutation CHEK2.1100delC truncates the kinase domain of the CHEK2 protein and is responsible for a 2-fold increase in breast cancer risk in families of northern and northwestern European ancestry (13–15). In the Ashkenazi Jewish population, CHEK2.1100delC confers similar risks but is very rare (3,16). Other individually rare cancer-associated CHEK2 mutations have been identified in European populations (17–22). The unique historical demography of the Ashkenazi Jewish population led us to hypothesize that a relatively common cancer-associated CHEK2 allele might exist in this community.

In order to identify candidate cancer-associated CHEK2 mutations in the Ashkenazi Jewish population, we first determined extended haplotypes at CHEK2 in Ashkenazi Jewish families multiply affected with breast or ovarian cancer but wild-type for BRCA1 and BRCA2. The purpose of this step was not to carry out a formal linkage analysis, but to select for sequencing those individuals most likely to carry a mutant CHEK2 allele. We identified two CHEK2 haplotypes segregating with breast cancer, in three families and one family, respectively. We sequenced an index case representing each CHEK2-associated haplotype. Sequencing revealed two previously undetected CHEK2 variants, 1283C > T in exon 11 leading to S428F (in three families) and 254C > T in exon 1 leading to P85L (in the fourth family) (Fig. 1A). In all families, the variant

**RESULTS**

**Identification of novel CHEK2 variants**

From 30 Ashkenazi Jewish families with high incidence of breast and/or ovarian cancer but with wild-type sequences of BRCA1 and BRCA2, we genotyped probands and relatives for seven markers spanning 645 kb at CHEK2 on chromosome 22q12. We determined haplotypes by direct analysis and identified pedigrees in which probands shared the same haplotype. In four families, a CHEK2 haplotype co-segregated with breast cancer. Nine affected relatives in three families shared the same haplotype and three sisters in a fourth family shared a different haplotype. Genomic DNA from the probands of these four families was fully sequenced for CHEK2 coding and regulatory regions.

Sequencing revealed two previously undetected CHEK2 variants, 1283C > T in exon 11 leading to S428F (in three families) and 254C > T in exon 1 leading to P85L (in the fourth family) (Fig. 1A). In all families, the variant
co-segregated with the shared haplotype. CHEK2 amino acid residues 85 and 428 differ in their evolutionary conservation and in the predicted impact of the observed substitutions. Residue 428 lies in the kinase domain, which is highly conserved from mammals to yeast, and phenylalanine is a non-conservative amino acid substitution of serine. In contrast, residue 85 is located in the much less conserved N-terminal region, and leucine is a more conservative amino acid substitution of proline (Fig. 1B).

To test the completeness of our haplotype-based approach, we subsequently sequenced all coding and regulatory regions of CHEK2 in genomic DNA from the probands of all 30 high-incidence families. No other cancer-associated CHEK2 variants were present. One proband was heterozygous for a previously unrecorded SNP (T6397 > C) in the CHEK2 3'UTR, which did not co-segregate with cancer in her family. Multiple probands were heterozygous for several intronic SNPs and for CHEK2.251A > G (E45E), a silent polymorphic substitution (rs1805129).

Complementation tests in yeast

To evaluate the effect of these mutations on normal function of CHEK2, we tested whether either could complement the lethality of a Rad53 deletion in the yeast S. cerevisiae. The identification of mammalian CHEK2 as the homolog of yeast Rad53 was based on the ability of the wild-type human CHEK2 to complement the deletion of Rad53 in yeast (8). We expanded this test to determine whether ability to complement the yeast deletion was lost by CHEK2 mutants. As Fig. 2 indicates, wild-type human CHEK2 complements the lethality of the Rad53 deletion in yeast, leading to exponential growth in selective media. Yeast carrying human CHEK2.P85L grew as well as those with the wild-type human CHEK2 gene, suggesting P85L does not abrogate normal CHEK2 function. Yeast carrying the silent polymorphism human CHEK2.E84E grew nearly as well. In contrast, yeast carrying human CHEK2.S428F or CHEK2.1100delC grew no better than the wild-type human CHEK2 complemented deletion of Rad53 lethality as well as did wild-type human CHEK2. These results suggest that CHEK2.S428F and CHEK2.1100delC have lost a critical normal function of CHEK2.

Genetic epidemiology of CHEK2 variants

In order to test whether CHEK2.S428F and/or CHEK2.P85L were associated with cancer risk in the Ashkenazi Jewish population, we compared carrier frequencies of these two alleles between 1848 Ashkenazi Jewish breast and ovarian cancer patients and 1673 controls. Patients were ascertained at cancer diagnosis regardless of family history or age at diagnosis. The case series did not include the breast cancer families in whom the CHEK2 mutations were originally identified. We also screened 768 breast cancer patients of various other ancestries for these two alleles. All breast cancer patients had wild-type alleles of the Ashkenazi Jewish founder mutations in BRCA1 and BRCA2. We also screened patients and controls for CHEK2.1100delC and CHEK2.ivs2(+1)G > A and excluded individuals heterozygous for these alleles from the case–control comparisons.

Results of the case–control comparisons were consistent with predictions of the functional tests. CHEK2.S428F carrier frequencies were 47/1632 (2.88%) of breast cancer patients and 23/1673 (1.37%) of controls (P = 0.004; Table 1). In contrast, frequencies of CHEK2.P85L did not differ between cases (0.92%) and controls (0.85%). Among 768 American breast cancer patients of various non-Jewish ancestries, none carried CHEK2.S428F or CHEK2.P85L.

The increased risk conferred by CHEK2.S428F was ~2-fold, similar to that of CHEK2.1100delC (14, 15). Odds ratios associated with CHEK2.S428F were higher among breast cancer patients with family history of breast cancer, ovarian cancer or male breast cancer (Table 1). However, CHEK2.S428F was not significantly more frequent among ovarian cancer patients (4/190, 2.11%) than among controls and none of 26 male breast cancer patients carried CHEK2.S428F. Nor was CHEK2.S428F more frequent among patients with lymphoma (9/415, 2.16%) or colon cancer (3/185, 1.62%) than among controls.

For two patients heterozygous for CHEK2.S428F, primary breast tumor tissue was available. To evaluate loss of heterozygosity in the breast tumors, we genotyped markers D22S275, D22S1169 and D22S689 spanning 645 kb at CHEK2 (Fig. 3). One patient was heterozygous at D22S689 and the other patient was heterozygous at D22S689 and D22S275. Breast tumor cells were hemizygous for markers linked to the mutant allele, indicating loss of the wild-type allele in each tumor.

Proportions of breast cancer patients by decade of diagnosis who carried mutations in BRCA1, BRCA2 or CHEK2 are
shown in Table 2. Inherited mutations in BRCA1, and to a lesser extent in BRCA2, are more frequent among patients with young breast cancer onset. However, among breast cancer patients heterozygous for CHEK2 mutations, ages at diagnosis were only slightly younger than ages at diagnosis among patients with no known mutation (51.5 years ± 8.5 SD versus 52.3 years ± 11.0 SD). CHEK2 mutations were most frequently associated with breast cancer in middle age. Among patients diagnosed at age 50 and older, mutations in CHEK2 were as frequent as mutations in BRCA1 or BRCA2, although the impact of CHEK2 mutations on risk was much less severe.

We also compared breast cancer incidence among mothers, sisters and daughters of patients with CHEK2.S428F mutations versus these relatives of patients heterozygous for CHEK2.P85L or with wild-type sequences at CHEK2 (Fig. 4). Because we did not know which relatives carried CHEK2 mutations, cumulative incidence is shown for carrier and non-carrier relatives combined, with the Mendelian expectation that 50% of mothers, sisters and daughters of probands with CHEK2 mutations would also be carriers. Risks of breast cancer among relatives of probands heterozygous for CHEK2.S428F were increased only after age 50. As expected, risks of breast cancer among relatives of probands heterozygous for CHEK2.P85L were not increased at any age. On the basis of the kin–cohort method (23), the risk of breast cancer due to CHEK2.S428F by age 60 can be estimated as 0.17 (± 0.08 SE).

**Table 1. CHEK2.S428F heterozygotes among Ashkenazi Jewish cases and controls**

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>CHEK2.S428F carriers (%)</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female breast cancer probands</td>
<td>1632</td>
<td>47 (2.88)</td>
<td>2.13 (1.25, 3.63)</td>
<td>0.004</td>
</tr>
<tr>
<td>Family history of breast cancer</td>
<td>766</td>
<td>25 (3.26)</td>
<td>2.42 (1.34, 4.51)</td>
<td>0.003</td>
</tr>
<tr>
<td>Family history of ovarian cancer</td>
<td>141</td>
<td>8 (5.67)</td>
<td>4.32 (1.74, 10.38)</td>
<td>0.002</td>
</tr>
<tr>
<td>Family history of male breast cancer</td>
<td>23</td>
<td>2 (8.70)</td>
<td>6.83 (0, 32.88)</td>
<td>0.044</td>
</tr>
<tr>
<td>Ovarian cancer probands</td>
<td>190</td>
<td>4 (2.11)</td>
<td>1.54 (0.45, 4.77)</td>
<td>0.62</td>
</tr>
<tr>
<td>Male breast cancer probands</td>
<td>26</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Lymphoma probands</td>
<td>415</td>
<td>9 (2.16)</td>
<td>1.59 (0.68, 3.46)</td>
<td>0.34</td>
</tr>
<tr>
<td>Colon cancer probands</td>
<td>185</td>
<td>3 (1.62)</td>
<td>1.18 (0.28, 4.19)</td>
<td>0.74</td>
</tr>
<tr>
<td>Ashkenazi Jewish population controls</td>
<td>1673</td>
<td>23 (1.37)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast cancer patients of other ancestries</td>
<td>768</td>
<td>0</td>
<td></td>
<td></td>
</tr>
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OR is odds ratio for each group of cases compared to population controls. CI is confidence interval.

*aFamily history is defined as ≥1 relative with cancer at the site indicated among parents, grandparents, siblings, children, aunts or uncles.*

DISCUSSION

Distinguishing disease-associated missense mutations from neutral polymorphisms in disease genes is an important problem in human genetics. For genes with highly conserved biological roles, complementation tests in yeast may serve as functional assays for mammalian genes. For the moderate-penetrance breast cancer gene CHEK2, conservation of gene function from yeast through mammals enabled us to develop an assay for naturally occurring CHEK2 variants on the basis of one of its defining features, the capacity to complement the loss of Rad53 in S. cerevisiae. We applied this functional assay to amino acid substitutions of CHEK2 identified among probands of high-risk Ashkenazi Jewish breast cancer families. Then in Ashkenazi Jewish breast cancer cases and controls, independent of the high-risk families, we confirmed epidemiologically the results predicted by the complementation tests. Biological assays of the consequences of amino acid substitutions are especially valuable for alleles that are individually too rare for epidemiologic analysis. However, the yeast complementation test measures an extreme phenotype, so the result is strong evidence of the functional importance of the CHEK2.S428F variant. Other CHEK2 alleles could have more subtle effects on protein function not measurable in this assay.

The distinctive demographic history of the Ashkenazi Jewish population has led to the discovery of ancient...
founder mutations responsible for many genetic disorders (24). The combination of a small number of founder mutations, subsequent rapid demographic expansion, selection and genetic drift resulted in reduced genetic heterogeneity and hence to fewer different pathogenic alleles at disease-associated genes in this population (25). The population genetics of BRCA1 and BRCA2 is a well-studied example of this phenomenon, with three ancient mutations at relatively high frequency (3,26–28) and other mutations extremely rare (29). However, most Ashkenazi Jewish breast cancer patients, even from high-risk families, do not carry one of these mutations (3). Therefore, we hypothesized that an ancient founder allele of CHEK2 might also exist in this population and be responsible for inherited predisposition to breast cancer among some Ashkenazi Jewish women without inherited mutations in BRCA1 or BRCA2. The identification and characterization of such a cancer-associated CHEK2 allele would be useful both to the Ashkenazi Jewish women at high risk of breast cancer and to the understanding of the impact of CHEK2 mutations on breast cancer risk among women generally.

**CHEK2.S428F** increases risk of breast cancer ~2-fold, with predicted breast cancer risk ~0.17 (±0.08 SE) by age 60. Breast cancer patients with a family history of female breast cancer, ovarian cancer and/or male breast cancer were more likely to carry CHEK2.S428F. The frequency of CHEK2.S428F carriers among ovarian cancer patients was higher than among controls (2.11% versus 1.37%), but the difference was not significant. It is not clear whether the association with family history of ovarian cancer reflects risk associated with ovarian cancer or family history as an indicator of inherited risk. Family history of male breast cancer appears likely to be simply an indicator of inherited risk in this population (30,31). Other European mutations in CHEK2 are associated with cancers of the thyroid, prostate, colon, and kidney, in addition to breast cancer (22). The CHEK2.S428F carriers in our series had more severe family histories only of breast cancer and ovarian cancer, not of cancers of other sites. Nor did we detect a significantly increased frequency of CHEK2.S428F among Ashkenazi Jewish patients with lymphoma or colon cancer. On the basis of a population prevalence of CHEK2.S428F heterozygotes of 1.37% and relative risk of 2.13, ~1% of breast cancer overall in the Ashkenazi Jewish population is attributable to this allele.

In a large series of Dutch patients, CHEK2.1100delC was associated with younger age at breast cancer diagnosis, but did not consistently co-segregate with breast cancer in families, leading to the suggestion that CHEK2 mutations may modify breast cancer risk due to unknown genes, rather than exerting a primary effect (32). In our study, CHEK2.S428F mutation carriers were diagnosed at only very slightly younger ages than patients without mutations, and the mutation segregated with disease in the three high-incidence families in which it first appeared. However, given a 2-fold relative risk associated with this allele in the population generally, and the high prevalence of breast cancer without any known inherited cause, we would expect many families with CHEK2.S428F mutations to include both phenocopies and carriers of who have remained cancer free. We did not evaluate persons with both CHEK2 and BRCA1 or BRCA2 mutations, so cannot exclude the possibility that CHEK2 interacts with BRCA1, BRCA2 or with other unknown breast cancer genes to influence risk. In practice, whatever mechanism is involved, the effect of carrying CHEK2.S428F is to increase breast cancer risk ~2-fold.
Haplotypes at CHEK2 in this population may be instructive in designing the efficient strategies for identifying novel breast cancer genes. In the Ashkenazi Jewish population, LD is significantly increased at markers flanking rare disease alleles (25). Not surprisingly, therefore, CHEK2.S428F is associated with an extended conserved haplotype. Also not surprisingly, the haplotype on which CHEK2.S428F arose is conserved over a shorter genomic distance than the haplotype with the mutation. However, in this population, the ancestral haplotype is nonetheless conserved over >600 kb, considerably longer than the CHEK2 locus itself (55 kb). Therefore, if CHEK2 were not already known to harbor cancer-associated mutations and were to be identified by an undirected LD strategy, LD differences between cases and controls could only be detected at a considerable distance from the CHEK2 locus. In other words, LD in the >600 kb region including CHEK2 is so highly conserved in this population that no differences would be detected between cases and controls, even with very large sample sizes. Instead, the genomic regions between 500 kb and 1 Mb from CHEK2 (Fig. 5) are most likely to differ in LD between cases and controls. Variation in haplotype block size on chromosome 22 is well characterized for a mixed European population (33). Our experience with one small region of chromosome 22 confirms previous predictions (25) that LD analyses in founder populations will encounter additional complexities in haplotype block size that will not always be known in advance. In founder populations, a search for disease-associated genes by LD may involve detecting LD signals in cases versus controls at sites hundreds of kilobase pair from an unknown critical gene rather than detecting LD signals within the candidate locus itself.

The identification of inherited breast cancer genes of lower penetrance than BRCA1 or BRCA2 raises new issues for clinical practice. In particular, should genetic testing for medium and low penetrance alleles be recommended for breast cancer patients or their families? The question is difficult because the lower penetrance of CHEK2 mutations leads to imperfect co-segregation of mutations with cancer in many families (32) and age of onset of breast cancer among carriers that is not particularly young. More genetic information will clarify risk in family members. Unaffected women heterozygous for breast cancer-associated CHEK2 mutations may be good candidates for research protocols of intensive screening strategies such as magnetic resonance imaging (34,35). Stratifying high-risk women by genotype in breast cancer screening and prevention trials will help reveal the utility of a given intervention in a specific-risk population. Until this type of information is available for CHEK2 mutations, it will be difficult to reach consensus on the usefulness of wide-scale testing. The identification of women with BRCA1 and BRCA2 mutations allowed retrospective analyses as well as prospective clinical trials targeted specifically for these women (34–38). Information generated from such studies has in turn increased the clinical utility of testing for BRCA1 and BRCA2 mutations. Testing for other breast cancer susceptibility genes will likely undergo a similar evolution.

**MATERIALS AND METHODS**

**Subjects**

Human subjects review committees of all participating institutions approved this study. Participants in this study, all of whom are Ashkenazi Jewish, included 30 families with at least four cases of breast cancer and/or ovarian cancer (the ‘discovery’ series); 1848 unrelated patients with female breast cancer, ovarian cancer or male breast cancer; 792 mothers, sisters and daughters of a subset of these patients (described subsequently) and 1673 controls. Cancer diagnoses were verified by pathology reports and/or hospital records. Probands of the 30 high-risk families had wild-type sequences of BRCA1 and BRCA2 as determined by complete sequencing of both genes. Cases in the subsequent case–control study were hospital registry-based Ashkenazi Jewish cancer patients ascertained regardless of family history or age at diagnosis and wild-type for the three BRCA1 and BRCA2 Ashkenazi founder mutations. The 2488 cases included 1632 patients with female breast cancer, 26 with male breast cancer, 190 with ovarian cancer, 415 with lymphoma and 185 with colon cancer. Patients were ascertained and enrolled, as previously described, by the New York Breast Cancer Study (3), Memorial Sloan Kettering Cancer Center (16) or Share Zedek Medical Center (39). The series of American breast cancer patients of other ancestries has also been previously described (40). Controls were 1673 cancer-free Ashkenazi Jewish adults from Israel or New York. One group of controls comprised 725 young adults who underwent pre-marriage counseling in Jerusalem. The second group of controls comprised 948 cancer-free adults who participated in the New York Cancer Project sponsored by the AMDeC Foundation (41), whose staff kindly made anonymized biological samples available.
Figure 5. Conserved haplotypes at the CHEK2 region of chromosome 22q12. The haplotype harboring CHEK2.S428F and the ancestral haplotype on which CHEK2.S128F arose are shown in green. The haplotype harboring CHEK2.P85L and the ancestral haplotype on which CHEK2.P85L arose are shown in yellow. Markers spanning 2.7 Mb flanking CHEK2 are indicated at the top of the figure and distances (kb) from CHEK2 are indicated at the bottom of the figure. Conserved alleles were defined by linkage disequilibrium (D) > 0.75 between adjacent markers and between each marker and D22S275. Blank entries for each haplotype indicate no conserved allele at that site. Haplotype blocks for a mixed European population (33) are shown in blue.

Sequencing and genotyping

Probands and all surviving relatives in the family study were genotyped by standard methods for haplotypes comprised of seven markers spanning 645 kb at the CHEK2 locus: microsatellites D22S1150, D22S275 and D22S689, and SNPs rs5762795, rs6005863, rs5762764 and rs743185. To determine extended haplotype structure, unrelated cases and controls were genotyped by standard methods for markers spanning 2.7 Mb at CHEK2. These haplotypes were defined by microsatellite markers D22S1150, D22S275, D22S689, D22S1163, and the following polymorphic simple tandem repeats developed as markers for this project: GCTC at 28626789, AC at 28544795, AC at 28538947, TG at 28391047, ATGG 28345343, ATT at 27958798, AAG at 26945911, AAAG at 26288824, ATGG at 26121818 and TCTT at 25930215. Positions refer to sequence of chromosome 22 on build 35 of the International Human Genome Sequencing Consortium as displayed on the May 2004 UCSC Genome Browser (http://genome.ucsc.edu/). Primer sequences for the simple tandem repeats are available on request.

For sequencing CHEK2, genomic DNA isolated from peripheral blood was amplified by PCR using intronic primers flanking exons of the gene. PCR products were then purified and sequenced in both directions with BigDye Terminator chemistry and electrophoresed on an ABI3100. This protocol was carried out for CHEK2 exons 1–2 and 4–9. In order to avoid slippage artifacts at poly-A/T tracts flanking exon 3, this exon with sequenced with a nested primer located 3′ of the poly-T tract in intron 2. CHEK2 exons 10–14 are wholly or partially duplicated as pseudogenes eight times in the human genome, with duplications retaining the CHEK2 intron structure (42). In order to carry out mutation analysis only of the functional copy of CHEK2 on chromosome 22q12, we amplified a long-range PCR product with primers in sequences unique to 22q12 in intron 8 (5′-GGTTGTGGTGTACCGCTTACGG-3′) and in the 3′-UTR (5′-ATCTAAATCCTCCTACCAGTCTG-3′). A 10 kb fragment was generated and used as template to amplify individual exons 10 through 14 using appropriate primers. These exons were sequenced using the procedure described for exons 1 through 9.

For each variant site in CHEK2, we developed genotyping assays based on differential restriction enzyme digestion. The CHEK2.P85L allele was genotyped by amplifying with primers 5′-ATTCCAGAGGACCAAGACGTTGAG-3′ and 5′-ACAACAAAGGGTGTTACCAAAG-3′, then digesting the amplified product with DdeI. Primer sequences were changed from wild-type to remove two additional DdeI digestion sites. The wild-type CHEK2 allele yields fragment lengths of 82 and 35 bp and the CHEK2.P85L allele yields a product of 117 bp. The CHEK2.S428F allele was genotyped by nested PCR. First primers 5′-GTGTGTATACCGCGTTACGG-3′ and 5′-AGTATGAGCAGACCGACCCAGGCC-3′ were used to amplify a 2170 bp product spanning exons 9 to 11 and unique to chromosome 22. This product was amplified with primers 5′-CTGATGATGCTGAATAATGC-3′ and 5′-CAGTGGAGATGACTGCTTACG-3′ in exon 11, then digesting the amplified product with Hpy188I. The wild-type allele yields fragment lengths of 82 and 25 bp and the CHEK2.S428F mutation yields a product of 107 bp.

Statistical analyses

Allele and genotype frequencies were compared by two-tailed χ²-tests. Cumulative risks of breast cancer among relatives were evaluated by standard Kaplan–Meier methods. LD was calculated as previously described (33,43). Risks due to CHEK2 mutation were estimated by a kin–cohort method, as previously described (23).

Yeast complementation assay

S. cerevisiae strain Y590 (delRad53::HIS3) and plasmid pMH267(2m LEU2 GAL-CHEK2) were kindly provided by S. J. Elledge (8). Site directed mutagenesis reactions were performed on the pMH267 plasmid using a QuikChange site directed mutagenesis kit (Stratagene, La Jolla, CA, USA) to create mutations S428F, P85L, 1100delC and E84I. The entire coding sequence of the CHEK2 gene in each of the
mutated plasmids was determined in order to ensure that only the desired mutation was present. Using the same procedure, reverse mutagenesis was performed on the S428F plasmid to recreate the wild-type sequence plasmid (this plasmid was named ‘F428S’). Expression of all alleles was confirmed by RT–PCR of the plasmids (Supplementary Material, Fig. S1). All the mutated plasmids and pMH267 (wild-type CHEK2) were transformed into the Y590 yeast strain using YEASTMAKER System 2 (CLONTECH, Palo Alto, CA, USA). Colonies of Y590 and of each transformed strain were chosen and re-plated on an appropriate selective plate; Y590 (His<sup>+</sup>) and CHEK2 variants (His<sup>−</sup> Leu<sup>+</sup>). Three colonies from each plate were used to inoculate three tubes with 2 ml of galactose His<sup>−</sup> liquid media and tubes were incubated 16 h at 30°C with shaking 250 rpm. Each culture was diluted to 0.5 OD<sub>600</sub> and 20 μl of the diluted cultures were used to inoculate 1 ml of fresh galactose His<sup>−</sup> liquid media in disposable 3 ml spectrophotometer cuvets. The cuvets were incubated at 30°C with shaking 200 rpm. OD<sub>600</sub> was measured approximately every 5 h between 6 and 24 h.

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REFERENCES


