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No increased susceptibility to breast cancer from combined *CHEK2* 1100delC genotype and the HLA class III region risk factors

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Abstract

CHEK2 is low-penetrance breast cancer susceptibility gene. The 1100delC mutation may interact with variants/mutations in other breast cancer susceptibility loci. We identified a risk haplotype in the HLA class III region in breast cancer patients [de Jong MM, Nolte IM, de Vries EGE, *et al*. The HLA class III subregion is responsible for an increased breast cancer risk. *Hum Mol Genet* 2003, **12**, 2311–2319] and tested whether it interacted with 1100delC mutation.

The *CHEK2* 1100delC mutation was analysed in the same series of patients and controls as in the HLA breast cancer study.

In 962 unselected breast cancer patients, the 1100delC mutation was observed in 2.9% and in 367 controls in 1.4% (NS). The highest 1100delC frequency occurred in high-risk (4.4%), followed by moderate-risk (3.8%), and lowest in low genetic risk patients (2.4%, P_{trend} 0.029). In HLA risk haplotype carriers no increased breast cancer risk was observed in the presence of 1100delC mutation. Patients more often had one than both genetic risk factors.

The 1100delC mutation and the HLA risk haplotype confer increased breast cancer risks, but an interactive effect on breast cancer between both factors is unlikely. In contrast, the effect of 1100delC mutation on breast cancer risk was limited to individuals without HLA risk haplotype, suggesting a mutual excluding effect between these risk factors.

Keywords: Breast cancer; *CHEK2*; 1100delC mutation; HLA class III region; Sporadic

1. Introduction

Breast cancer is the most common cancer in women in the Western world [1]. It is a heterogeneous disease, both clinically and genetically [2]. At this moment,

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genetic testing in breast cancer patients is offered for high-penetrance genes, *i.e. BRCA1* [MIM 113705] and *BRCA2* [MIM 600185]. Other genes associated with breast cancer have been identified, but are not (yet) applicable for individual risk assessments [3]. Identification of more genetic risk factors and the understanding of interactions between different breast cancer susceptibility genes (with either high- or low-penetrance) as well as their interactions with dietary, lifestyle and hormonal factors, will be important steps towards more specific risk assessments for individual women.

The *CHEK2* gene [MIM 604373] encodes the human homologue of the Cd1 and RAD53 checkpoint kinases. The CHEK2 protein plays a crucial role in the DNA damage response pathway mediating cell cycle arrest and apoptosis [4]. In response to DNA damage, CHEK2 stabilises Tp53 by phosphorylation, which leads to cell cycle arrest and prevents cellular entry into mitosis [5–7]. The CHEK2 protein is activated in an ataxia telangiectasia mutated (ATM) dependent manner in response to ionising radiation and in an ATM-independent manner in response to UV light [7,8]. CHEK2 also regulates the BRCA1 protein function after DNA damage [9].

CHEK2 was identified by two groups as a low-penetrance breast cancer susceptibility gene in high-risk familial breast cancer cases without *BRCA1* and *BRCA2* mutations [10,11]. Recently, a large study showed that the *CHEK2* 1100delC genotype frequency is also increased in unselected breast cancer cases [12]. In total, 10,860 breast cancer cases and 9065 controls from 10 case–control studies in five countries, including the Netherlands, were genotyped. Although there was a substantial variation in carrier frequency by study, there was no evidence for heterogeneity in the odds ratio among studies or among countries [12]. Other mutations in *CHEK2* did not occur at elevated frequency in 605 familial and in 1786 sporadic breast cancer cases (<65 years of age) as compared to controls [13–15].

It was hypothesised that the 1100delC mutation multiplied the risks associated with variants of other susceptibility genes, thereby displaying only a modifying effect on breast cancer risk. With an estimated relative breast cancer risk of 2.34, the recent large multicenter study is most consistent with a polygenic model, *i.e.* several low-penetrance alleles with additive or multiplicative effects on breast cancer risk [10,12].

Recently, we reported a strong association between sporadic breast cancer and the HLA class III region [16]. The highest risk was observed for the haplotype consisting of alleles 110 and 184 at marker loci D6S2672 and MICA, respectively. Considering a possible interaction with *CHEK2*, we decided to analyse the *CHEK2* 1100delC mutation in the same series of breast cancer patients and controls in which the HLA breast cancer study was performed. This permits to test whether there is an interaction between both risk factors. First, we analysed the *CHEK2* 1100delC incidence in our series to confirm the association found by others. In addition, we studied the correlation between *CHEK2*, genetic risk stratification and age at diagnosis. Finally, we analysed the interaction between the 1100delC genotype and the HLA class III risk haplotype, with respect to breast cancer risk.

2. Materials and methods

2.1. Breast cancer patients and controls

The breast cancer patients ($n = 962$) and controls (spouses, $n = 367$) participated in a population-based study that aims to detect breast cancer susceptibility genes other than *BRCA1* and *BRCA2* [16]. All participants were Caucasian and were living in the northern part of the Netherlands. Patients were accrued from six hospitals (the University Medical Centre Groningen, the Medical Centres in Leeuwarden and Harlingen, Ny Smellinghe in Drachten, Talma Sionsberg in Dokkum, and the Antonius Hospital in Sneek, The Netherlands) [16]. No selection was performed, cases were included between July 1998 and January 2002, irrespective of family history. Patients completed a health questionnaire including their family history for cancer. Only those patients were excluded in whom the presence of a *BRCA1* or *BRCA2* mutation was known.

Twenty-two patients in the study were known to have undergone mutation analyses and tested negative for *BRCA1* and *BRCA2* (11 in the high-risk group, 4 in the moderate-risk group and 7 in the low-risk group). For this study, the unselected breast cancer patients were not screened for *BRCA1* and *BRCA2* mutations.

All DNA samples and data in this study were handled anonymously and individuals were aware that they would not be informed about individual test results. All included subjects gave written informed consent. The Medical Ethical Committees of the participating hospitals approved the study.

The breast cancer cases were assigned to 'high', 'moderate' or 'low' genetic risk groups as described earlier, based on age at diagnosis, bilaterality of breast cancer, number of first degree relatives with breast cancer, and co-existence of ovarian cancer and male breast cancer in the family (Table 1) [16].

2.2. Genotyping

DNA was extracted from 20-ml EDTA-blood following standard procedures and stored at −80 °C. The primers for the 1100delC mutation were developed based on the published gene sequence. A suitable primer

Table 1
Criteria used to define the different genetic risk groups for breast cancer susceptibility

High-risk
• Age at diagnosis under 35 years
• Fulfilling the moderate risk criteria and a positive family history of ovarian cancer or of male breast cancer
• Two or more first degree relatives with breast cancer,[a] at least one of them diagnosed before the age of 45 and another one before the age of 60
Moderate-risk
• Bilateral breast cancer
• Age at diagnosis under 45 years
• Two or more first degree relatives with breast cancer,[a] at least one of them diagnosed before the age of 60
Low-risk
• All women with breast cancer not fulfilling the high- or moderate-risk criteria

[a] A second degree female relative via a male is in this study considered a first degree relative.

pair was developed using Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).

Primer sequences were checked for specificity using BLAST (http://www.ncbi.nlm.nih.gov/). The forward primer was ATCACCTCCTACCAGTCTGTGC and the reverse primer GCAAGTTCAACATTATTCCC-TTT.

Polymerase chain reactions (PCR) were performed in a volume of 10 μl, which contained ~25 ng DNA. For each PCR, 0.5 U *Taq* DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden) were used to amplify the fragments. Reaction mixtures contained 0.2 mM dNTP (Roche Diagnostics, Mannheim, Germany), 2.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 9.0), 50 mM KCl (Amersham Pharmacia Biotech) and 0.25 μM of each primer, with the 5′ primer labelled with fluorochrome 6-FAM (Sigma, Malden, The Netherlands). Cycling was performed on a PTC-225 thermal cycler (MJ Research, Waltham, MA, USA) and a PrimusHT (MWG Biotech, Ebersberg, Germany). A standard protocol was used for amplification. A 2.3-μl sample of the PCR product was mixed with 2.5 μl MilliQ and 0.2 μl ET-400R size standard (Amersham Pharmacia Biotech) and separated on a MegaBACE 1000 capillary sequencer (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Results were analysed using genetic profiler version 1.1 (Amersham Pharmacia Biotech). Scoring of the alleles was performed blinded for affection status.

In the HLA breast cancer study, the HLA region was genotyped with microsatellite markers in germline DNA from breast cancer patients and controls [16]. Association analyses and the haplotype sharing statistic (HSS) were used to search for differences in haplotype sharing between patients and controls. The HSS revealed a significant difference in mean haplotype sharing between patients and controls, and the results were confirmed with association analyses.

2.3. Statistical methods

For the *CHEK2* 1100delC association analysis, the frequencies of the genotypes were compared between patients and controls using a χ^2 test or, when one or more of the expected numbers were smaller than five, a Fisher's exact test. Furthermore, χ^2 trend analysis on the different genetic risk groups was used to test whether the frequency of *CHEK2* 1100delC heterozygotes correlated with the genetic risk categories. In addition, an ANOVA test was performed for the age at diagnosis, being one of the criteria for genetic risk stratification, as dependent variable with the *CHEK2* 1100delC genotype as factor. The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated, without adjustment for external variables [3].

In order to study the interaction between the HLA risk haplotype and the *CHEK2* 1100delC mutation, both patients and controls were stratified according to carriership of the HLA class III risk haplotype. The genotype frequency of the 1100delC mutation was determined in four subsets: for individuals with and without the HLA risk haplotype, analysing patients and controls separately.

Power of our statistical analysis for interaction was determined using Monte Carlo simulation on a χ^2 table. A preset number of individuals were randomly assigned a combination of risk factors according to the frequencies of the combinations of risk factors under the assumed alternative hypothesis, *i.e.* the observed frequencies in the current sample were used. The power was defined by the percentage of 1000 randomisations that gave a χ^2 value above the critical of 3.84 (at 0.05 significance level, 1 degree of freedom). The required sample size to obtain a power of 80% was determined by means of optimisation of the preset number of individuals.

3. Results

In 962 breast cancer patients, the *CHEK2* 1100delC mutation was detected 28 times (2.9%), which is not significantly different from that in controls (1.4%, Table 2). However, when the patients were stratified according to defined genetic risk groups (low, moderate or high), the

Table 2
The *CHEK2* 1100delC variant in breast cancer patients and controls

	Individuals positive for the 1100delC variant		
	Total number of individuals (%)	OR	95% CI
Controls	5/367[a] (1.4)	1.00	–
Breast cancer patients	28/962 (2.9)	2.17	0.83–5.66
Low-risk	16/662[a] (2.4)	1.79	0.65–4.94
Moderate-risk	8/209[a] (3.8)	2.88	0.93–8.93
High-risk	4/91[a] (4.4)	3.33	0.88–12.7

[a] *P*-value for trend 0.029 (controls, low-risk, moderate-risk, high-risk).

Table 3
CHEK2 1100delC mutation frequency stratified by carriership for the HLA risk haplotype

	Carriers of the HLA risk haplotype and the 1100delC mutation[a]		Carriers other HLA haplotypes and the 1100delC mutation		
	Total number of individuals	OR[b] (95% CI)	Total number of individuals	OR[b] (95% CI)	OR[c] (95% CI)
Controls	3/130 (2.3%)	1.00	2/218 (0.9%)	1.00	0.39 (0.06–2.38)
Patients					
All	8/340 (2.4%)	1.02 (0.27–3.91)	20/601 (3.3%)	3.72 (0.86–16.0)	1.43 (0.62–3.28)
Low-risk	4/217 (1.8%)	0.79 (0.18–3.61)	12/424 (2.8%)	3.15 (0.70–14.2)	1.55 (0.49–4.87)
Moderate-risk	3/96 (3.1%)	1.37 (0.27–6.92)	5/113 (4.4%)	5.00 (0.95–26.2)	1.44 (0.33–6.17)
High-risk	1/27 (3.7%)	1.63 (0.16–16.3)	3/64 (4.7%)	5.31 (0.87–32.5)	1.28 (0.13–12.9)

[a] Heterozygous and homozygous carriers of the HLA risk haplotype.
[b] *versus* controls.
[c] *versus* HLA risk haplotype carriers.

highest 1100delC frequency was in high-risk (4/91, 4.4%), followed by moderate-risk (8/209, 3.8%), with the lowest frequency in the low-risk group (16/662, 2.4%). This trend was significant ($P_{trend} = 0.029$). The mean age at diagnosis for patients with the wild-type *CHEK2* genotype was 55.2 years and for patients with the 1100delC genotype, 51.5 years (*P*-value 0.12).

In carriers of the HLA risk haplotype, 2.4% of the patients and 2.3% of the controls carried the *CHEK2* 1100delC mutation (OR = 1.02, NS, Table 3). In addition, stratifying the patients according to defined genetic risk did not reveal significant differences between patients and controls. By contrast, in patients without the HLA risk haplotype, the frequency of the 1100delC mutation was increased, although non-significant (OR = 3.72; 95% CI = 0.86–16.0). This frequency increased with defined genetic risk (NS, Table 3). In contrast with our hypothesis, the breast cancer risk for 1100delC carriers was slightly higher in carriers of other HLA haplotypes compared to carriers of the HLA class III risk haplotype (NS, Table 3).

4. Discussion

The present study showed that the 1100delC mutation conferred no additional increase in breast cancer risk in carriers of the HLA risk haplotype. In patients without the HLA risk haplotype, the 1100delC mutation conferred an increased, although statistically non-significant, breast cancer risk.

Patients more often had one risk factor, the HLA risk haplotype or the 1100delC mutation, than both risk factors. Furthermore, in 1100delC carriers, the breast cancer risk was slightly higher in carriers of other HLA haplotypes compared to carriers of the HLA class III risk haplotype. A multiplicative effect or an additive effect is therefore unlikely. In contrast, the effect of the 1100delC mutation on breast cancer risk seemed to be limited to individuals without the HLA risk haplotype, suggesting a mutual excluding effect between the two risk factors. This lack of interaction is also known to be present between *CHEK2* and *BRCA1* and *BRCA2*, since the 1100delC mutation is rare in carriers of *BRCA1* of *BRCA2* mutations [10].

Our sample suggests that there is a mutual excluding effect between the 1100delC mutation and the HLA class III risk haplotype. Since the frequency of the 1100delC mutation is relatively low, a sample of 10,000 breast cancer patients is required to prove this excluding effect with an 80% power.

In the present study, unselected breast cancer patients showed a non-significantly increased frequency of the *CHEK2* 1100delC genotype. The increased frequency was similar to those revealed by other studies [10–12]. Hence, our sample is adequate to study the possible interaction between the *CHEK2* 1100delC mutation and the HLA class III risk haplotype [16].

We observed a trend of an increasing frequency of the *CHEK2* 1100delC mutation from controls via low- and moderate-risk to high genetic risk individuals (P_{trend} 0.029), with the highest risk in *BRCA1/2*-negative

familial breast cancer cases. Thus, this study shows that not only the sample size is important for detection of an association, but also the genetic risk stratification.

The study performed by the *CHEK2* Breast Cancer Case–Control Consortium revealed a trend for an increased genotype frequency of the 1100delC mutation in breast cancer patients at younger ages at diagnosis ($P_{trend} = 0.002$) [12]. In our study however, the age at diagnosis as tested with ANOVA, did not reveal a statistically significant difference (*P*-value 0.12) between patients with the wild-type *CHEK2* genotype and those with the 1100delC genotype. Nevertheless, breast cancer occurred at an earlier age in patients with the 1100delC mutation (51.5 years) than in those with the wild type genotype (55.2 years). Hence, there may well be an effect of the 1100delC mutation on age at diagnosis of breast cancer, but our group of patients with the 1100delC genotype is too small to show significance.

In this study, all controls are men (*i.e.* spouses of the breast cancer patients). We assume that bias is unlikely, since the frequency of the 1100delC genotype in our study is similar to that found in controls in other Dutch studies [10,12]. The use of male controls would at most weaken the results, since males carrying the *CHEK2* 1100delC mutation likely do not get breast cancer. Therefore, the frequency of the *CHEK2* 1100delC mutation is probably higher among males than among unaffected females.

In conclusion, our data show that an interactive effect on breast cancer between the *CHEK2* 1100delC mutation and the HLA class III haplotype is unlikely. In contrast, the effect of the 1100delC mutation on breast cancer risk seemed to be limited to individuals without the HLA risk haplotype, suggesting a mutual excluding effect between these two risk factors.

Conflict of interest

There are no financial and personal relationships with other people or organisations that could inappropriately influence our work. This statement is on behalf of all co-authors.

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