The CHEK2*1100delC Variant Acts as a Breast Cancer Risk Modifier in Non-BRCA1/BRCA2 Multiple-Case Families

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ABSTRACT

The frame-shifting mutation 1100delC in the cell-cycle-checkpoint kinase 2 gene (CHEK2) has been reported to be associated with familial breast cancer in families in which mutations in BRCA1 and BRCA2 were excluded. To investigate the role of this variant as a candidate breast cancer susceptibility allele, we determined its prevalence in 237 breast cancer patients and 331 healthy relatives derived from 71 non-BRCA1/BRCA2 multiple-case early onset breast cancer families. Twenty-seven patients (11.4%) were carrying the CHEK2*1100delC variant. At least one carrier was found in 15 of the 71 families (21.1%). There was no evidence of cosegregation between the variant and breast cancer, but carrier patients developed breast cancer earlier than did noncarriers. We studied CHEK2 protein expression in 111, and loss of heterozygosity at CHEK2 in 88 breast tumors from these patients. Twelve of 15 tumors from carriers showed absent protein expression as opposed to 3 of 76 tumors from noncarriers (P < 0.001). CHEK2 loss of heterozygosity was associated with absence of protein expression but not with 1100delC carrier status. Thus, selecting breast cancer cases with a strong familial background not accounted for by BRCA1 or BRCA2 strongly enriches for carriers of CHEK2*1100delC. Our results support a model in which CHEK2*1100delC interacts with an as yet unknown gene (or genes) to increase breast cancer risk.

INTRODUCTION

First-degree female relatives of a breast cancer patient have an ~2-fold increased risk to develop breast cancer (1). Germ-line mutations in the BRCA1 and BRCA2 genes account for <5% of this familial risk (2, 3). To explain the remainder of familial risk, various genetic models have been proposed. Models incorporating a single third hypothetical gene, BRCA3, or a number of common low penetrance genes with additive effect seem to fit equally well, although the latter fitted best when the known effects of parity on breast cancer risk were included (3, 4). A mutation 1100delC in CHEK2 has been proposed recently to be a low-penetration breast cancer susceptibility allele (5, 6). CHEK2 is located on chromosome 22 and encodes the human orthologue of yeast Cds1 and Rad53, which are G2 checkpoint kinases (7). CHEK2 is involved in cell cycle control and DNA repair through its ability to phosphorylate p53, Cdc25c, and BRCA1. The CHEK2*1100delC variant is a protein-truncating mutation that abrogates the kinase activity of the protein. It occurs in 0.3–1.4% of healthy control individuals (5, 6, 8), but in about double that frequency among unselected cases of breast cancer. It is even further enriched among breast cancer cases with a positive family history in which BRCA1 and BRCA2 mutations have been excluded. Up to 5.5% of such cases may be carrying the CHEK2*1100delC variant, although it apparently incompletely segregates with breast cancer in the families of these cases (5). Other variants in CHEK2 seem to be very rare and are not enriched among familial breast cancer cases (9–11).

We have embarked recently on a genome-wide linkage search for new breast cancer susceptibility genes in a highly selected group of breast cancer families. Phenotypic and genotypic criteria (12) have minimized the probability that these families harbor mutations in BRCA1 or BRCA2, but have selected for families that are caused by other high penetrant genes. Here, we investigate the role of the CHEK2*1100delC variant as a cause of breast cancer in these families.

MATERIALS AND METHODS

Families. Families were ascertained through the Clinical Genetic Centres in Leiden, Rotterdam, and Nijmegen, as well as through the Netherlands Foundation for the Detection of Hereditary Tumors. Families were eligible if there were at least three cases of breast cancer diagnosed before the age of 60 from whom genotypes could be determined or inferred. DCIS or LCIS before the age of 60 as first primary cancer were also considered eligible diagnoses. Families with cases of ovarian cancer or male breast cancer were excluded, and occurrences of other cancer types were ignored. Seventeen of these 71 families were also part of the previous study identifying the 1100delC*CHEK2 variant as a low-penetration breast cancer susceptibility gene (5). The 71 families selected contained a total of 384 breast cancer patients, 297 of which diagnosed before the age of 60, 2 of which occurred in spouses (excluded from the statistical analysis), and 5 of which had in situ cancer (4 DCIS and 1 LCIS) only. There was one family where the third case diagnosed under 60 was an in situ cancer (combined DCIS/LCIS at age 53). Pathology reports were retrieved for 260 patients (68%). For another 84 patients, diagnoses were confirmed by medical records, and retrieval of pathology reports was still in progress at the time when this study was finalized. For the remaining 40 cases, breast cancer diagnoses were ascertained by family interview only. Blood samples and paraffin-embedded tumor tissues were collected after obtaining written informed consent. The institutional ethical committees of all of the hospitals involved approved this study.

BRCA1 and BRCA2 Mutation Testing. In each family, the youngest breast cancer patient from whom a blood sample was available was tested for mutations in the BRCA1 and BRCA2 genes (and for many families the next youngest as well). The different Clinical Genetic Centres applied a variety of methodologies. The large central exons (exon 11 in BRCA1 and 10 of BRCA2) were scanned by partial thromboplastin time (13). The small exons were scanned for mutations by denaturing gradient gel electrophoresis or direct sequencing. All of the laboratories specifically assayed the presence of large founder deletions in BRCA1 by deletion junction-PCR (14). For cases where scanning was still in progress at the time of sampling for the purpose of this research, we performed conformation-sensitive gel electrophoresis (15) covering all of the coding regions of both genes. This identified 10 different variants of uncertain clinical significance and 12 different polymorphisms. None of these were cosegregating with breast cancer or the CHEK2*1100delC variant.

Genotyping of the CHEK2*1100delC Variant. The DNA sequence of exon 10 of CHEK2, where the 1100delC resides, is present in multiple...
homologous copies in the genome. For PCR, we used oligonucleotides 10F (5′
TGT CTT GGA CTG GCA GA; Fam-labeled) and 10R (5′ TCC TAC CAG TCT GTG C), which specifically amplify the functional copy of CHEK2, relative to the nonfunctional pseudogenes (16). The reaction volume of 10 μl contained 20 ng of genomic DNA, 1 μl 10′ SuperTaq buffer
(HIT Biotechnology Ltd.), 1 μl dNTPs, 300 mM of each primer, and 0.1 units of Silverstar DNA polymerase (Eurogentec). Annealing temperature was 65°C, and the PCR ran for 38 cycles. The resulting PCR-products were analyzed on an ABI3700, in fragment analysis mode. The wild-type allele runs as a 291-bp fragment and the mutant allele as a 290-bp fragment, which are readily separated into two peaks of about equal signal intensity in this assay. All of the positive samples were confirmed by sequencing as described previously (5).

**LOH Analysis.** LOH at the CHEK2 locus was investigated by comparing the genotypes in normal and tumor DNA at four flanking markers, D22S240, D22S315, D22S280, and D22S283. CHEK2 maps between D22S315 and D22S280, which span an interval of ~7 Mb. Four punches (5 mm long and 0.6 mm in diameter) were taken from paraffin-embedded tumor tissues, in the area where the tumor was located. These punches generally contain >50% tumor cells. DNA was isolated from these punches as described previously (17). Allelic imbalance was defined as the ratio of allele intensities in the normal versus the tumor DNA. An AIF of ≥1.70 was scored positive (18). LOH at the CHEK2 locus was scored positive when the AIF- pattern was such that at least one proximal and one distal marker showed AIF ≥1.70 without interruption by a marker showing an AIF <1.70.

**Tissue Array and Immunohistochemical Analyses.** All of the tumor samples were embedded in standard paraffin blocks. On the respective H&E-stained sections, a representative tumor area was selected. Two to four tissue cores (0.6 mm in diameter; Beecher Instruments, Silver Spring, MD) were punched from the designated area using a biopsy needle and arrayed into the recipient blocks. Using a tape-transfer system (Instrumedics, Hackensack, NJ), 4-μm sections were transferred to glass slides. For antigen retrieval, the deparaffinized sections were boiled in a microwave for 15 min in citrate buffer (pH 6.0) before incubation with a mouse monoclonal antibody against the human CHEK2 protein. Examples of obtained staining patterns are shown in Fig. 2. As this indicates that the odds of detecting the variant is dependent on the probability curves were estimated and differences were tested by the log-rank test. To obtain an impression of the size of the effect of a CHEK2*1100delC mutation on age of onset, a Cox-regression analysis was performed.

**RESULTS**

We investigated 71 families with a phenotype of early onset breast cancer, defined as having at least 3 cases diagnosed before the age of 60, and no cases of ovarian or male breast cancer. Mutations in BRCA1 and BRCA2 were excluded in at least the youngest breast cancer case from which a blood sample was available. These families contained a total of 384 breast cancer patients. We collected DNA samples from 237 patients, including all of those with *in situ* cancer, as well as from 331 family members without breast cancer and 54 spouses. Of the 622 individuals we were thus able to assay for the presence of the CHEK2*1100delC variant, we found 41 (6.6%) to be carriers (Table 1). The prevalence among breast cancer patients was 11.4% (27 of 237), which was significantly higher than the prevalence of the variant in healthy female family members (6 of 212; χ2 = 12.047; df = 1; P < 0.001). Three carriers were known with *in situ* cancer (2 DCIS and 1 LCIS). Fifteen families (21.1%) had at least 1 positive individual for this variant. One of these was a family in which the only identifiable carrier was a woman with *in situ* cancer (DCIS; Fig. 1). The proportion of families in which at least 1 individual carried the CHEK2 variant increased to 31.8% in families with >5 breast cancer patients diagnosed under 60 (Table 1). However, this trend was not statistically significant (χ2 = 2.6; df = 2; P = 0.272).

In addition, CHEK2-positive families had on average slightly more blood-sampled cases than CHEK2-negative families (3.8 versus 3.2; data not shown). Although not a statistically significant difference, this indicates that the odds of detecting the variant is dependent on the number of blood-sampled breast cancer cases in a family.

In the 15 1100delC*CHEK2-positive families we defined the youngest carrier breast cancer case as the index patient. Under the null hypothesis of complete random Mendelian inheritance, we predicted that 12.9375 of the 54 affected relatives would be carrier of the variant. We observed 12 carriers, so that the null hypothesis could not be rejected.

We performed LOH analysis in 89 archival breast tumor tissues from 88 breast cancer cases from these 71 families, at four markers mapping to either side of CHEK2 (Table 2). LOH at CHEK2 was found in 11 tumors, 3 of which derived from 2 CHEK2*1100delC carriers. In all 3 of the tumors, we could demonstrate that the lost allele was derived from the nontransmitting parent (data not shown). Although the 1100delC variant occurred 2.7 times more frequently among cases showing LOH at CHEK2, this difference was not statistically significant (χ2 = 1.239; df = 2; P = 0.538).

A tissue microarray with 111 tumors from 111 cases was stained with a mouse monoclonal antibody against the human CHEK2 protein. Examples of obtained staining patterns are shown in Fig. 2.

### Table 1 CHEK2*1100delC prevalences

<table>
<thead>
<tr>
<th>Description</th>
<th>Total</th>
<th>CHEK2+</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>All sampled individuals</td>
<td>622</td>
<td>41</td>
<td>6.6</td>
</tr>
<tr>
<td>Male</td>
<td>154</td>
<td>8</td>
<td>5.2</td>
</tr>
<tr>
<td>Female</td>
<td>468</td>
<td>33</td>
<td>7</td>
</tr>
<tr>
<td>All sampled breast cancer cases</td>
<td>237</td>
<td>27</td>
<td>11.4</td>
</tr>
<tr>
<td>Cases diagnosed under 60</td>
<td>194</td>
<td>24</td>
<td>12.4</td>
</tr>
<tr>
<td>Cases diagnosed 60 or over</td>
<td>43</td>
<td>3</td>
<td>7.0</td>
</tr>
<tr>
<td>Cases with <em>in situ</em> cancer only</td>
<td>5</td>
<td>3</td>
<td>60.0</td>
</tr>
<tr>
<td>Healthy family members</td>
<td>331</td>
<td>14</td>
<td>4.2</td>
</tr>
<tr>
<td>Males</td>
<td>119</td>
<td>8</td>
<td>6.7</td>
</tr>
<tr>
<td>Females</td>
<td>212</td>
<td>6</td>
<td>2.8</td>
</tr>
<tr>
<td>Spouses*</td>
<td>54</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Male</td>
<td>35</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Female</td>
<td>19</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>All families</td>
<td>71</td>
<td>15</td>
<td>21.1</td>
</tr>
<tr>
<td>3 cases &lt; 60</td>
<td>30</td>
<td>4</td>
<td>13.3</td>
</tr>
<tr>
<td>4 cases &lt; 60</td>
<td>19</td>
<td>4</td>
<td>21.1</td>
</tr>
<tr>
<td>≥5 cases &lt; 60</td>
<td>22</td>
<td>7</td>
<td>31.8</td>
</tr>
</tbody>
</table>

* Two of these individuals were diagnosed with breast cancer.
noted in a previous study (19) there was considerable variability in the percentage of normal cells that were positive. CHEK2 protein expression was absent in 12 of 15 tumors from CHEK2*1100delC carriers (80.0%; Table 3). False-negative staining was considered unlikely, because in 6 of 12 tumors from CHEK2*1100delC carriers the stromal component stained normally. Notably, the one tumor showing moderate protein expression was an in situ carcinoma (DCIS) from a patient from family RUL154 (Fig. 1). In comparison, only 3 of 76 tumors (3.9%) from noncarriers showed an absent CHEK2 protein expression ($\chi^2 = 52.709; df = 3; P < 0.001$).

For 37 tumors, protein expression and LOH data were available. CHEK2 protein expression was absent in 3 of 10 tumors with CHEK2-LOH, 2 of which were from CHEK2*1100delC carriers. The other 7 tumors with CHEK2-LOH all showed a weak CHEK2 protein expression. In comparison, all 27 of the tumors, which retained heterozygosity at CHEK2, showed some degree of protein expression ($\chi^2 = 15.879; df = 6; P = 0.014$).

The mean age of diagnosis of the first primary tumor of CHEK2*1100delC carrier patients was not significantly different from that in noncarriers (48.3 versus 50.6 years; $P = 0.30$). However, any age difference may have been masked by our selection for early onset breast cancer. Indeed, in a Kaplan-Meier analysis the age of onset distribution between the two groups was different ($P < 0.0001$). It is unlikely that this effect is confounded by differences in tumor grade because the percentage of grade III tumors was higher in noncarriers than in carriers (22 of 81 versus 1 of 9). A Cox-regression analysis revealed an odds ratio of 2.1 (95% confidence interval, 1.393–3.166; $P < 0.001$) for carriers to develop breast cancer relative to noncarriers (derived from CHEK2*1100delC positive and CHEK2*1100delC negative families). Among the 237 genotyped breast cancer patients in our cohort, 35 (14.8%) were known to have had a second primary breast cancer. Five of these (14.3%) were positive for the CHEK2 variant. Of the 202 patients with one primary breast cancer, 22 tested positive (10.9%). This difference was not statistically significant.

<table>
<thead>
<tr>
<th>LOH at CHEK2</th>
<th>Number of cases</th>
<th>CHEK2 carriers</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive$^a$</td>
<td>11</td>
<td>3</td>
<td>27.3</td>
</tr>
<tr>
<td>Suspected$^b$</td>
<td>29</td>
<td>5</td>
<td>17.2</td>
</tr>
<tr>
<td>Negative</td>
<td>29</td>
<td>3</td>
<td>10.3</td>
</tr>
<tr>
<td>Unknown$^c$</td>
<td>20</td>
<td>3</td>
<td>15.0</td>
</tr>
<tr>
<td>Totals</td>
<td>89</td>
<td>14</td>
<td>14.0</td>
</tr>
</tbody>
</table>

$^a$ Cases in which at least one proximal and one distal marker showed AIF $\neq 1.70$ without interruption by a marker showing an AIF $< 1.70$.

$^b$ Cases in which LOH was found only proximal or distal of CHEK2.

$^c$ Cases in which one of the reactions failed.

![Fig. 2. Immunohistochemical staining of CHEK2 in human breast tumors on a tissue microarray. The samples shown are from four different tumors and represent the four different scoring categories used here. A and B, absent protein expression in a tumor from a CHEK2*1100delC carrier. Note the scattered strongly staining normal epithelial cells as positive internal control (B). C–F, represent tumors from noncarriers. C and D, weak protein expression. E, moderate protein expression. F, high expression. Magnification $\times 25$ in A, C, E, and F, and $\times 100$ in B and D.](/cancerres.aacrjournals/vol67/iss20/fig02.large)
Alternatively, breast cancer in these families has a polygenic basis involving multiple interacting low-penetrance alleles (26), one of which is the \( \text{CHEK2*1100delC} \) variant. The \( \text{CHEK2*1100delC} \) is approximately twice as prevalent among unselected breast cancer cases than among controls, suggesting it is a low-risk allele in its own right (5, 6). In keeping with this, we found that \( \text{CHEK2*1100delC} \) is associated with breast cancer, but it was unable to explain the majority of breast cancer cases in these families. A role for \text{CHEK2} inactivation in breast tumor development is nonetheless supported by the highly significant association we found between \( \text{CHEK2*1100delC} \) carrier status and an absence of protein expression in the breast tumors. This confirms results obtained by others (6, 19) irrespective of minor differences in interpretation of immunohistochemical staining patterns among these studies. It would also explain the slightly earlier age of onset of breast cancer in \( \text{1100delC} \) carriers, as these individuals only need to inactivate the wild-type allele whereas noncarriers would need to inactivate both copies of the gene. Paradoxically, the breast tumors of \text{CHEK2} carriers do not significantly more frequently show LOH at \text{CHEK2}. Hence, LOH may not be the only mechanism inactivating the wild-type allele, although the association between LOH and an absent protein expression we observed does indicate it is involved in some cases. Alternative mechanisms include promoter hypermethylation (27) and somatic mutations, but the roles of both appear to be marginal in breast cancer (19, 28). Conceivably, other components of the pathway(s) regulating the expression and/or stability of \text{CHEK2} protein are disturbed in these cases.

An association with bilateral disease, but only a marginal trend toward earlier age of diagnosis was reported in one study (6). In our cohort of cases we found an association between \text{CHEK2} carrier status and earlier age of diagnosis but not between carrier status and multiple primary tumors. This could be a peculiarity of the selected families. Conceivably, many cases not carrying the \text{CHEK2} variant are carriers of another gene defect that predisposes them strongly to develop breast cancer. In combination with a long retrospective follow-up time, this may have masked the subtle effect of \text{CHEK2} on risk.

In conclusion, we find a strong association between \( \text{CHEK2*1100delC} \) prevalence and breast cancer family history. Our results provide support for the hypothesis that this variant modifies the cancer risk conferred by an as yet unknown gene (or genes). Given the cancer occurrence in the families described here, this gene is expected to cause breast cancer risks comparable with those conferred by \text{BRCA1} and \text{BRCA2}. At this point it is in our opinion not appropriate to offer a predictive test for \text{CHEK2} in a clinical setting. The exact relative risk conferred by \( \text{CHEK2*1100delC} \) is not clear, but likely modest in comparison with \text{BRCA1} and \text{BRCA2}. In addition, estimates of breast cancer risk are difficult to make in these families, because the type of interaction (multiplicative or additive) and the role of other factors are presently unknown. Selecting for families with at least one carrier of the \( \text{CHEK2*1100delC} \) might reduce the genetic heterogeneity likely to exist among non-\text{BRCA1}/\text{BRCA2} families and facilitate the mapping of this breast cancer susceptibility gene by classical linkage analysis.

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