ELSEVIER

European Journal of Cancer 41 (2005) 2896–2903

European Journal of Cancer

www.ejconline.com

German populations with infrequent *CHEK2**1100delC and minor associations with early-onset and familial breast cancer

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Received 17 January 2005; received in revised form 16 March 2005; accepted 12 April 2005
Available online 18 October 2005

Abstract

*CHEK2**1100delC is associated with a twofold increased breast cancer risk. This was shown in a collaborative analysis of European populations, but not in other populations from Europe and the US. Accordingly, there is a need to clarify the role of *CHEK2**1100delC in breast cancer.

We established its prevalence in two German populations GENICA (Northrhine-Westphalia, $n = 724$) and KORA (Bavaria, $n = 600$) and in women with breast cancer. The latter included cases ($n = 688$) from the GENICA breast cancer case-control study, patients with early-onset breast cancer ($n = 86$) and patients with familial breast cancer ($n = 71$). The latter patient groups were previously investigated for *BRCA1/2*-mutations and tested negative. Mutation analysis was performed by combined PCR/DHPLC methodology.

*CHEK2**1100delC was found in 0.9% of GENICA controls and was absent in the KORA controls indicating a significant difference between the two populations ($P = 0.03$). The frequency of *CHEK2**1100delC in age-matched cases of the GENICA collection was 0.8% and thus not different from controls (OR 0.88, 95% CI 0.21–3.50). In patients with early-onset disease *CHEK2**1100delC was found at a frequency of 2.3% referring to an increased breast cancer risk of 2.56 (95% CI 0.25–14.58). In patients with familial disease the frequency was 1.4% referring to an increased risk of 1.53 (95% CI 0.03–12.93).

Our data showed variations in *CHEK2**1100delC prevalence within German populations suggesting possible inaccuracies in breast cancer risk assessments from non population-based studies. In patients with a high-risk profile however, *CHEK2**1100delC was indicative for this risk and highest for early-onset breast cancer.

Keywords: *CHEK2**1100delC variant; Population-based study; Breast cancer patients selected for family history and age; *BRCA1/2*

1. Introduction

Cell cycle-checkpoint kinase 2 gene (*CHEK2*) [MIM 604373] also known as *Cds1* in *Schizosaccharomyces pombe* and *RAD53* in *Saccharomyces cerevisiae* encodes

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doi:10.1016/j.ejca.2005.04.049

a nuclear protein kinase that is a key mediator of cellular responses to DNA damage [1–4].

CHEK2 is polymorphic [5] and numerous reports addressed the possible role of the protein-truncating mutation *CHEK2**1100delC for familial breast cancers particularly those without mutations in the breast cancer predisposing genes *BRCA1* and *BRCA2* [6–8]. The 1100delC mutation is predicted to abolish the kinase activity of the CHEK2 protein [9] and may therefore be a likely candidate to impact DNA repair capacity and promote susceptibility to some breast cancers. A *CHEK2* associated breast cancer risk was observed for breast cancer patients of Northern European populations. Particularly, the prevalence of the *CHEK2**1100delC variant ranged from 1.1% to 1.4% in populations from Finland, The Netherlands and Great Britain and increased to 5.1–5.5% in women with familial breast cancer testing negative for *BRCA1/2* mutations [7,8]. In contrast, no such association was identified in other populations as it applies to a US population from New York [10] and populations from Spain [11], Germany [6], and Italy [12] where *CHEK2**1100delC frequencies ranged from 0% to 0.5% in controls and 0–1.6% in breast cancer patients.

Recently, a collaborative analysis of 10,860 breast cancer cases and 9065 controls reported a frequency of *CHEK2**1100delC of 1.9% in breast cancer cases and 0.7% in controls [13]. These data were based on the evaluation of 10 case-control studies from five countries but varied in study design.

To establish population specific prevalence and significance of *CHEK2**1100delC on the population level, stringent population specific case-control studies are now in demand. This will be important since changes in population frequencies of genome variations are well known for Caucasians [14,15] and must be implemented with respect to recommendations for the screening of at risk groups [16]. We recently established a population-based breast cancer case-control study GENICA in a German population to facilitate the identification of breast cancer susceptibility genes [17]. As a back-up for population controls we compared our data to those obtained from an age-matched subgroup of women from the KORA survey [18]. Here, we report population-based frequencies of the *CHEK2**1100delC allele in Germany and discuss these frequencies together with our findings in high-risk breast cancer patients including those with early-onset and hereditary disease.

2. Materials and methods

2.1. Study populations

2.1.1. The GENICA breast cancer case-control collection

Between August 2000 and October 2002 incident breast cancer cases and population-based controls have been recruited in the Greater Bonn Region in North Rhine-Westphalia, Germany. This is part of a wider effort of the Interdisciplinary Study Group on Gene Environment Interactions and Breast Cancer in Germany (GENICA) which is focused on the identification of breast cancer risks and susceptibility genes. There are 688 cases with a first time diagnosis of primary breast cancer that was histologically confirmed within six months of enrolment, and 724 population-based controls matched in 5-year age classes and matched for residency. Cases and controls were included when they were of Caucasian ethnicity, currently resided in the study region, and were below age 80 years. Clinical and histo-pathological data were collected from medical records and pathology reports. Information on potential risk factors was collected *via* in-person interviews using the core questionnaire of the German KORA survey [19]. The response rate was 88% for cases and 67% for controls. The GENICA study was approved by the Ethic's Committee of the University of Bonn and all study participants gave informed written consent.

2.1.2. KORA population control collection

Six-hundred female controls from a large population-based survey (KORA S2000) were included for comparison. The KORA survey was a cross-sectional population-based health survey carried out between October 1999 and April 2001 in the region of Augsburg, Bavaria, Germany [18]. KORA controls were matched to GENICA cases by age in five-year classes. The KORA S2000 sample serves as a population control group for case-control studies conducted by the German National Genome Research Network.

2.1.3. Early-onset breast cancer patients

Eighty-six women diagnosed with primary invasive or in situ breast cancer before the age of 41 years at the Städtisches Klinikum Karlsruhe and at the Women's Clinic, University of Heidelberg in South Germany, unselected for family history and negative for *BRCA1/2* mutations [20] were screened for *CHEK2**1100delC. It is important to note, that the recruitment of early-onset breast cancer patients preceded the population-based GENICA case-control study and KORA survey. These patient samples were initially included in the analysis of inherited breast cancer predisposition and subjected to *BRCA1* and *BRCA2* analyses. No healthy controls were obtained according to the case-only design. Due to the absence of age-matched and study region-matched controls we used the GENICA and KORA controls for a reference of *CHEK2**1100delC frequencies. We refrained from age-matching due to the limited number of early-onset breast cancer cases.

2.1.4. Hereditary breast cancer patients

Patients were originally recruited for the investigation of inherited breast and/or ovarian cancer predisposition with respect to *BRCA1* and *BRCA2* mutations. They included patients from all over Germany. Ninety-eight patients from families with at least three cases of breast cancer previously screened for *BRCA1* and *BRCA2* mutations [21,22] (U. Hamann, data not shown) were included in this study. Twenty-seven patients with deleterious *BRCA1/2* mutations and 71 patients without *BRCA1/2* mutations were screened for *CHEK2**1100delC. No healthy controls were obtained according to the case-only design. Due to the absence of matching controls we used the GENICA and KORA controls for a reference of *CHEK2**1100delC frequencies. We refrained from age-matching due to the limited number of familial breast cancer cases.

2.2. Isolation of DNA

Genomic DNA was isolated from blood samples according to standard procedures [23]. Isolation of genomic DNA from GENICA and KORA samples was performed using the PuregeneTM (Gentra Systems, Inc., Minneapolis, USA) according to the manufacturer's instructions. DNA samples were available for 630 of 688 (92%) breast cancer cases, 657 of 724 (91%) controls, 600 KORA controls, 86 early-onset breast cancer patients and 98 familial breast cancer patients.

2.3. Genotyping of CHEK2*1100delC

The 1100delC mutation is located within exon 10 of *CHEK2*. This exonic sequence is known to be present in multiple homologous copies in the human genome [24]. For the *CHEK2**1100delC (GenBank Accession No. NM_007194.2) genotyping allele-specific PCR amplification was performed. We used a newly designed forward primer CHEK2ex10f (5′-GCAAAATTAAATGTCCTAACTTGC-3′) primer in combination with previously described reverse primers CHEK2ex10r and CHEK2delCr primers [25]. The forward primer anneals to a region in intron 9 that is specific for the *CHEK2* gene. PCR reactions were carried out in 10 μl volumes containing 50 ng DNA, 1× PCR buffer (Qiagen, Hilden, Germany), 4 mM $MgCl_2$, 0.1 μM CHEK2ex10f primer, 0.2 μM of each reverse primer, 200 μM of each dNTP (Promega, Mannheim, Germany), 0.4 U HotStarTaq DNA polymerase (Qiagen). After an initial 15 min at 95 °C, DNA was amplified by 10 cycles of 20 s at 94 °C, 25 s at annealing temperature 68–55 °C touchdown (1.4 °C/cycle), and 30 s at 72 °C followed by 36 cycles of 20 s at 94 °C, 25 s at 55 °C, and 35 s at 72 °C, and then by a final extension step of 10 min at 72 °C. The PCR-products were separated on a 1% agarose gel (Sigma–Aldrich, Steinheim, Germany) containing ethidium bromide (Sigma–Aldrich). The fragment sizes of PCR products were 537 bp for the wild-type allele and 200 bp for the variant allele.

The reproducibility of the genotyping data were assessed by repeated analysis of 262 randomly selected DNA samples by DHPLC (WAVE system, Transgenomics, Omaha, NE). CHEK2 exon 10 primers were according to Sodha and colleagues [26]. PCR reactions were carried out in 25 μl volumes containing 50 ng DNA, 1× PCR buffer (Applied Biosystems (AB) Division, Foster City, CA), 3 mM $MgCl_2$, 0.1 μM of each primer, 200 μM of each dNTP (Promega), 0.5 U of a mixture (4 μl) Optimase (2.5 U/μl) (Transgenomics) and 18 μl AmpliTaq Gold (5 U/μl) (AB). After an initial 15 min at 95 °C, DNA was amplified by 3 cycles of 30 s at 94 °C, 45 s at annealing temperature 70–63 °C touchdown (1 °C/cycle; 3 cycles each), and 45 s at 72 °C followed by 20 cycles of 30 s at 94 °C, 45 s at 62 °C, and 45 s at 72 °C, and then by a final extension step of 10 min at 72 °C. DHPLC running conditions were established by S. Thorlacius (S. Thorlacius, Iceland Genomics Corporation). DHPLC temperature was 57 °C, DHPLC gradient was 55–60% buffer B [0.1 M triethylammonium acetate (Transgenomics), 25% acetonitrile (Fischer, Leicestershire, UK)]. A positive control was included in each set of agarose gel and DHPLC runs.

2.4. Statistical analysis

The null hypothesis of independence of rows and columns in two by two contingency tables was tested using Fisher's exact test. Two-sided P values of 0.05 or less were considered as statistically significant. Conditional Maximum Likelihood estimation was used to estimate odds ratios (OR). Estimation of the prevalence of *CHEK2**1100delC with exact 95% confidence limits (CI), computation of Fisher's exact test and OR with 95% CI for the risk of developing breast cancer were performed using R, version 1.9.1 [27]. Due to the absence of *CHEK2**1100delC variant in KORA controls resulting in infinite values of the OR, comparisons of familial and early-onset breast cancer patients were performed with GENICA controls.

3. Results

Constitutional C*HEK2**1100delC mutation analysis was performed for 2048 women including: (1) 613 patients and 651 controls of the GENICA breast cancer case-control study, (2) 600 women of the KORA survey, (3) 86 *BRCA1/2*-negative early-onset breast cancer patients, and (4) familial breast cancer patients, *i.e.*, 27 patients with *BRCA1/2* mutations, 71 patients without *BRCA1/2* mutations. Genotypes were established by

allele-specific amplification assay (Fig. 1(a)). Accuracy and reproducibility of genotyping data were 100% based on repeated analyses of 13% of DNA samples by DHPLC analysis (Fig. 1(b)).

3.1. Prevalence of CHEK2*1100delC in the German populations GENICA and KORA

3.1.1. GENICA population controls and cases

The frequency of *CHEK2**1100delC in women without breast cancer was 0.9% (6/651) and 0.8% (5/613) in women with breast cancer (Table 1). These frequencies were statistically not different ($P = 1.00$, OR 0.88, 95% CI 0.21–3.50) and therefore the overall frequency of *CHEK2**1100delC in GENICA women was 0.9% (11/1264). Among the five patients carrying *CHEK2**1100delC, two had a first-degree family history of breast cancer. All six carriers without breast cancer had a negative family history of breast cancer. Bilateral breast cancer was diagnosed in 17 out of 613 (2.8%) patients. None of these carried the *CHEK2**1100delC variant.

3.1.2. KORA controls

No *CHEK2**1100delC carriers were identified in 600 KORA women (Table 1). The difference of *CHEK2**1100delC prevalence between the GENICA controls (6/651) and KORA controls (0/600) was statistically significant ($P = 0.03$). Accordingly, we refrained from calculating a combined *CHEK2**1100delC frequency in controls from the two different German populations.

3.2. Prevalence of CHEK2*1100delC in breast cancer patients with hallmarks for non-sporadic breast cancer

3.2.1. Early-onset breast cancer patients without BRCA1/2 mutations

In this group patients were not selected on the basis of a family history of breast cancer but on the basis of

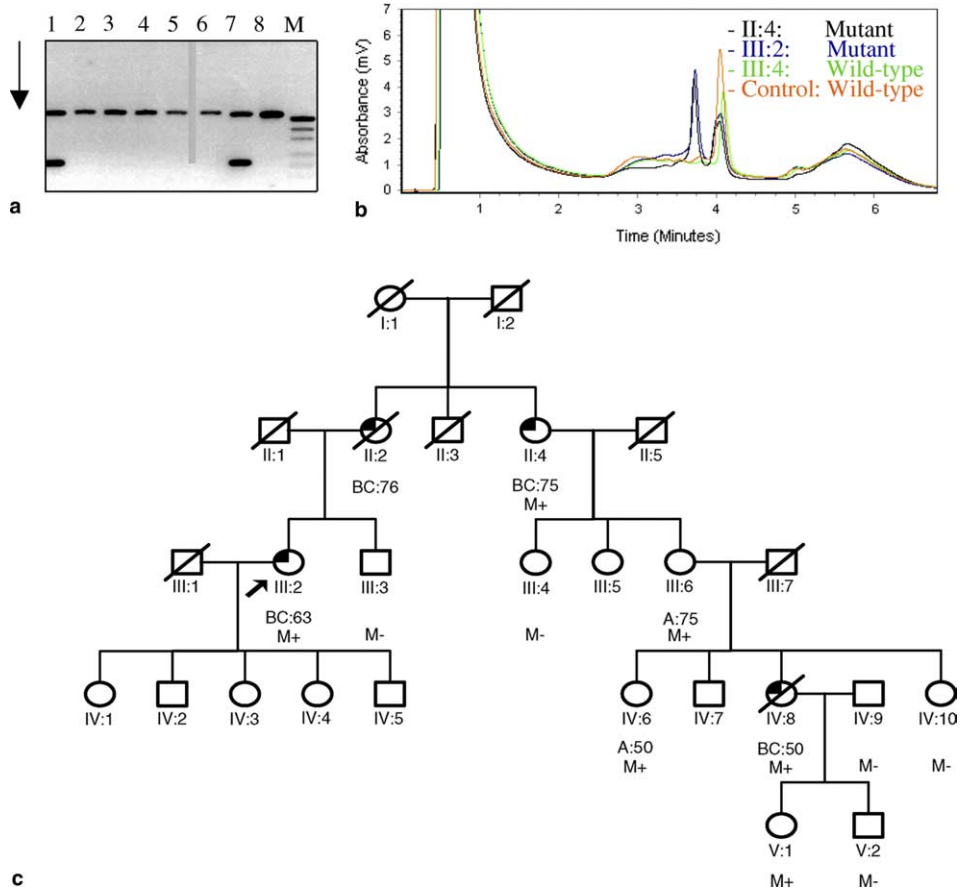

Fig. 1. (a) Ethidium bromide stained agarose gel. Lanes 2–6, 8: homozygous wild-type: 537 bp fragment; lanes 1, 7: heterozygous: 537 bp and 200 bp fragments; M: DNA marker (511 + 489, 404, 331, 242, 190, 147 bp). (b) DHPLC chromatograms of family members II:4, III:2, III:4 of Family 49 and a healthy control. Both the index patient (III:2) and the maternal aunt (II:4) with the *CHEK2**1100delC showed a double peak in the chromatogram whereas the *CHEK2**1100delC-negative cousin (III:4) and the control only showed a single wild-type peak. (c) Pedigree and *CHEK2**1100delC carriers of the German breast cancer Family 49. Circles are females, squares are males, and a diagonal slash indicates a deceased individual. Symbols with filled left upper quadrant: unilateral breast cancer. Identification numbers of individuals are below the symbols. The index patient is indicated by an arrow. BC: breast cancer; A: age. The numbers following these abbreviations indicate age at breast cancer diagnosis and age at recruitment. M+: mutation carriers; M−: non-carriers.

Table 1
Prevalence of *CHEK2**1100delC in German study groups

Study group	Number of subjects	Prevalence of *CHEK2**1100delC	
		N	% (exact 95% CI)
All German individuals	2048	14	0.68 (0.37–1.14)
Population controls			
GENICA	651	6	0.92 (0.34–2.00)
KORA	600	0	0.00 (0.00–0.61)
Breast cancer cases			
GENICA	613	5	0.82 (0.27–1.89)
Selected patient groups			
BRCA1/2 negative early–onset breast cancer	86	2	2.33 (0.28–8.15)
BRCA1/2 negative familial breast cancer	71	1	1.41 (0.04–7.60)
BRCA1/2 positive familial breast cancer	27	0	0.00 (0.00–12.77)

an early age at diagnosis (<41 years) and *BRCA1/2* negativity [20]. The prevalence of *CHEK2**1100delC in 86 patients was 2.3% (2/86) (Table 1). When we compared these data with those of GENICA controls *CHEK2** 1100delC carrier status was associated with a 2.56-fold increased early-onset breast cancer risk (OR 2.56, CI 0.25–14.58), which was not statistically significant ($P = 0.24$).

3.2.2. Familial breast cancer patients with and without BRCA1/2 mutations

Among the 27 *BRCA1/2*-positive familial breast cancer patients no *CHEK2**1100delC carrier was identified (Table 1). This result was not significantly different from that of GENICA controls (OR 0.00, 95% CI 0.00–21.27). The variant was found in 1.4% (1/71) of *BRCA1/2*-negative-familial breast cancer patients (Table 1). This frequency was not significantly different from that of the GENICA controls (OR 1.53, 95% CI 0.03–12.93). The incomplete penetrance of *CHEK2**1100delC in this family is shown in Fig. 1(c).

3.2.3. Breast cancer patients from families with bilateral breast cancer and with male breast cancer

Among 26 familial breast cancer patients from families with cases of bilateral breast cancer no *CHEK2** 1100delC was identified. This applied to families positive ($n = 14$) and families negative ($n = 12$) for *BRCA1/2* mutations. None of the breast cancer patients of three families with male breast cancer tested positive for *CHEK2**1100delC.

4. Discussion

*CHEK2**1100delC has recently attracted attention for its likely association with familial breast cancer particularly the disease phenotype that develops without predisposing mutations at *BRCA1* and *BRCA2* [7]. Of interest now is the relevance of this molecular marker as a screening target for an elevated breast cancer risk. Any recommendation will highly depend on the prevalence within a given population which may vary among different populations. Our contribution to this is to establish the prevalence of *CHEK2**1100delC in two German populations and high-risk breast cancer groups. While the frequency of the *CHEK2**1100delC variant has been reported from a collaborative analysis [13] and a population based case-control study in young women [28] our study may provide additional information since it was population-based and included incident breast cancer cases of all age classes.

First, we established the prevalence of the *CHEK2** 1100delC variant in study subjects of the GENICA breast cancer case-control collection which was less than 1% and similar for cases and controls. Although we expected this prevalence also to be low among KORA subjects originating from another German population, it is important to point out that the *CHEK2**1100delC variant was absent in that population. Based on the finding of this genetic difference on the population level it must be stated, that the two populations differ with respect to their geographic origin, with the GENICA study originating from the North-West and the KORA survey from the South-East of Germany. Moreover, both studies differ with respect to purpose and eligibility criteria. The GENICA study population was recruited for the purpose of the identification of breast cancer risks and addressed Caucasians only. The KORA survey population was recruited for the identification of cardiovascular disease risks and addressed individuals of German nationality. Although the cause of the discrepancy in prevalence of the *CHEK2**1100delC variant is unknown, our results from the genetic investigation of GENICA and KORA subjects are reminiscent of other known variations in genotype distributions among Caucasian populations that are attributed to possible founder effects and migration. A prominent example is the *C282Y* mutation of the hemochromatosis gene *HFE*, which is the commonest mutation in Caucasians predisposing to hereditary hemochromatosis and known for its decreasing prevalence from North to South in

Europe [29]. Although on a global basis the GENICA and KORA populations may not represent distant populations our data are in agreement with those obtained within the collaborative study of the CHEK2 Breast Cancer Case-Control Consortium [13] in that the *CHEK2**1100delC genotype distribution varied within Europe with frequencies being highest in Finland and The Netherlands. With respect to the latter population it is, however, noteworthy, that in Southwest Netherlands, an area in close neighbourhood to the GENICA study region (greater Bonn area), the *CHEK2**1100delC frequency was 1.8-fold higher compared to that of the GENICA subjects. It may therefore be important to pay attention to fluctuating allele frequencies within short geographic distances, which may have to be considered within the context of population-based disease screening.

Our observation of the overall low prevalence of *CHEK2**1100delC in two German populations and observed higher frequencies elsewhere, call for a careful interpretation of data obtained from studies that do not share uniform designs. Particularly, data from the collaborative analyses of 10 international non-uniform case-control studies reported by the CHEK2 Breast Cancer Case-Control Consortium may be prone to selection bias and constrain breast cancer risk estimates [13]. Although this effort was based on the evaluation of large numbers of more than 10,000 breast cancer cases and more than 9000 controls, it is important to draw attention to the design of the individual studies. In 7 studies cases were selected on the basis of age at diagnosis and in 3 studies on the basis of early diagnosis. In 4 studies controls were hospital-based and/or consisted of blood donors, and in 5 studies controls were not matched by age. Moreover, in half of the studies the frequency matching of controls was 0.5 or less. Thus, the reported approximately twofold increased breast cancer risk for *CHEK2**1100delC carriers may be attributable at least to some extent to the selection of high-risk patients. Our study, although considerably smaller, enrolled incident breast cancer patients from a defined geographic area. Controls were age-matched in order to control for confounding by age and restricted to women of Caucasian origin. These differences in study design may at least in part explain our different findings with respect to a *CHEK2**1100delC associated breast cancer risk that was not reproduced by us.

Inspired by the identification of a significant association of *CHEK2**1100delC with familial, non-*BRCA1/2*-associated breast cancer, we tested for a similar risk association in 86 *BRCA1/2* negative women with early-onset disease [20]. Within this patient cohort the risk to develop breast cancer was increased by 2.56-fold for *CHEK2**1100delC carriers when compared to the GENICA controls. This is the first study in Caucasians reporting this relationship, however, due to the low patient numbers involved in this analysis it will now be important to make an effort towards a more comprehensive analysis in order to test for the significance of this association.

Other results with respect to a *CHEK2**1100delC associated breast cancer risk were similar to those from reported studies. We identified an association of the *CHEK2**1100delC variant with breast cancer for women who were members of *BRCA1/2* negative breast cancer families. The breast cancer risk of *CHEK2**1100delC carriers was at least increased by 1.5-fold, a result that again did not reach statistical significance likely due to small sample size of patients. Similar to findings by Dufault *et al.* [6] the *CHEK2**1100delC variant was not fully penetrant in our study. Interestingly, our risk association is weaker than that described by Dufault *et al.* who reported a 3.3 times increased breast cancer risk for *BRCA1/2* negative *CHEK2**1100delC carriers who originated from across Germany based on the multi-center nature of that study. The higher increase in risk in that study, however, may be explained by the low *CHEK2**1100delC prevalence of 0.5% obtained from randomized non-age-matched female subjects of unspecified regional origin. Alerted by our findings of variations in *CHEK2**1100delC frequencies in two different German populations we suggest, that the true power of risk associations may require a unanimously established *CHEK2**1100delC prevalence across all regions. Thus, although both studies are in agreement with an overall low prevalence of *CHEK2**1100delC in Germany, this issue may point to the need for local, regional and global distribution maps for genetic variants for correct and reliable interpretations of disease risk modulations. This view is supported by the findings of no and infrequent *CHEK2**1100delC prevalence in Spain [11] and Italy [12] as well as the US [10], and beyond that, calls for the fine tuning of *CHEK2**1100delC prevalence among populations of Northern European descent where disease associations of more than twofold were reported for The Netherlands and Finland [7,8,30]. Similar considerations may apply with respect to our findings of the absence of the *CHEK2**1100delC variant in familial breast cancer cases from families with *BRCA1/2* mutations, in cases from *BRCA1/2* positive and negative families with bilateral breast cancer cases as well as patients from families with male breast cancer cases, which matched those of Dufault *et al.* [6] but differed from those of Vahteristo *et al.* [8] in a Finnish study.

Our results underscore the low overall importance of *CHEK2**1100delC in breast cancer susceptibility in German populations. The variant may confer an insignificantly increased risk of breast cancer in women selected for young age and family history, this risk, however, was not apparent in patients with sporadic breast cancer. Due to the low prevalence and low penetrance,

the variant does not represent a relevant disease marker to be recommended for genetic screening programs in Germany.

Conflict of interest

None declared.

Acknowledgements

We gratefully acknowledge all women who participated in this study. We are indebted to the associated members of the *GENICA Network Scientific Advisory Board*: Hermann M Bolt, MD, Ph.D., Institut für Arbeitsphysiologie an der Universität Dortmund, Michel Eichelbaum, MD, Dr. Margarete Fischer-Bosch-Institut für Klinische Pharmakologie, Stuttgart; Hans Vetter, MD, Medizinische Universitäts-Poliklinik Bonn. *Clinicians and hospitals*: Walther Kuhn, MD, Universitätsklinikum Bonn; Uwe-Jochen Goehring, MD, and Horst Wilms, MD, Johanniter-Krankenhaus Bonn; Heinrich Mause, MD, Gemeinschaftskrankenhaus St. Elisabeth-St. Petrus Bonn; Volker Pelzer, MD, Marien-Hospital Bonn; Michael Kaiser, MD, Malteser-Krankenhaus Bonn-Hardtberg; Rolf-Eberhard Herzog, MD, Evangelisches Waldkrankenhaus Bad-Godesberg; Sigrid Milz, MD, and Wolfgang Nohl, MD, St. Johannes Krankenhaus Bad-Honnef; Karl-Heinz Schlensker, MD, Klinikum Rhein-Sieg; Robert Kampmann, MD, St. Josef-Hospital Troisdorf; Bernd Werling, MD, Krankenhaus Troisdorf-Sieglar; Norbert Golz, MD, Marien-Hospital Euskirchen; Matthias Winkler, MD, and Peter-Karl Abramowski, MD, Kreiskrankenhaus Mechernich. *Pathologists*: Reinhard Büttner, MD, Hans-Peter Fischer, MD, Universitätsklinikum Bonn; Magdolna Bollmann MD, Reinhard Bollmann, MD, Dietmar Kindermann, MD, and Rainer Nikorowitsch, MD, Bonn; Jürgen Vogel, MD, Mathias Feldmann, MD, and Jürgen Gerlach, MD, Siegburg. *Epidemiologists*: Ulrich Ranft, Institut für Umweltmedizinische Forschung (IUF), Düsseldorf; Rolf Holle, Ph.D., and Hannelore Nagl, GSF-National Research Center for Environment and Health, Institute of Epidemiology, Neuherberg. *Interviewer*: Mette Besuden, Martin Black, Sandra Gschwendtner, Miriam Knie, Annette Kramer, Robert Primke, Astrid Prömse, Miriam Rossa, Heiko Schneider, Johanna Scholl, Katja Tamme, Matthias Wenghöfer, Bernd Wiesenhütter, Melanie Wollenschein. *Laboratory technical assistants*: Antje Seidel-Renkert and Silke Schöneborn.

This work was in part supported by the Federal Ministry of Education and Research (BMBF) Germany grants 01KW9975/5, 01KW9976/8, 01KW9977/0 and 01KW0114, the Deutsches Krebsforschungszentrum, Heidelberg, Robert Bosch Foundation of Medical Research, Stuttgart, Berufsgenossenschaftliches Forschungsinstitut für Arbeitsmedizin Bochum, and Johanniter Krankenhaus and University of Bonn, Germany.

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