Interaction of Werner and Bloom syndrome genes with p53 in familial breast cancer

Michael Wirtenberger1, Bernd Frank1, Kari Hemminki1-2, Rüdiger Klaes3, Rita K.Schmutzler4, Barbara Wappenschmidt4, Alfons Meindl2, Marion Kiechle3, Norbert Arnold6, Bernhard H.F.Weber7, Dieter Niederacher8, Claus R.Bartram9 and Barbara Burwinkel1

1Division of Molecular Genetic Epidemiology, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 580, 69120, Heidelberg, Germany, 2Department of Biosciences at Novum, Karolinska Institute, Huddinge, Sweden, 3Institute of Human Genetics, University of Heidelberg, Heidelberg, Germany, 4Division of Moleculargynaecology-Oncology, Department of Gynaecology and Obstetrics, Center of Molecular Medicine Cologne (CMMC), University Hospital of Cologne, 5Department of Gynaecology and obstetrics, Klinikum rechts der Isar at the Technical University, Munich, Germany, 6Division of Oncology, Department of Gynaecology and Obstetrics, University Hospital Schleswig-Holstein, Kiel, 7Institute of Human Genetics, University of Regensburg, Regensburg, 8Division of Molecular Genetics, Department of Gynaecology and Obstetrics, Clinical Center University of Düsseldorf, Düsseldorf, Germany and 9Helmholtz-university Group Molecular Epidemiology, German Cancer Research Center (DKFZ), Heidelberg, Germany

To whom correspondence should be addressed. Tel: +49 6221 421811; Email: m.wirtenberger@dkfz.de

Mutations of the human RecQ helicase genes WRN and BLM lead to rare autosomal recessive disorders, Werner and Bloom syndromes, which are associated with premature ageing and cancer predisposition. We tested the hypothesis whether three polymorphic, non-conservative amino acid exchanges in WRN and BLM act as low-penetrance familial breast cancer risk factors. Moreover, we examined the putative impact of p53 MspI 1798G>A, which is completely linked to p53PIN3, a 16 bp insertion/duplication that has been associated with reduced p53 expression, on familial breast cancer risk. Genotyping analyses, performed on 816 BRCA1/2 mutation-negative German familial breast cancer patients and 1012 German controls, revealed a significant association of the WRN Cys1367Arg polymorphism with familial breast cancer (OR = 1.28, 95% CI 1.06–1.54) and high-risk familial breast cancer (OR = 1.32, 95% CI 1.06–1.65). The analysis of p53 MspI 1798G>A, which is completely linked to p53PIN3, showed a significantly increased familial breast cancer risk for carriers of the 16 bp insertion/duplication, following a recessive mode (OR = 2.15, 95% CI = 1.12–4.11). WRN Cys1367Arg, located in the C-terminus, the binding site of p53, is predicted to be damaging. The joint effect of WRN Cys1367Arg and p53 MspI resulted in an increased breast cancer risk compared to the single polymorphisms (OR = 3.39, 95% CI 1.19–9.71). In conclusion, our study indicates the importance of inherited variants in the WRN and p53 genes for familial breast cancer susceptibility.

Introduction

Breast cancer is the most common cause of cancer-related deaths in women worldwide and after lung cancer, the second most frequent cancer in the world (1). About 10% of all breast cancers are associated with a family history of the disease (2). Large twin and family studies have shown that inherited factors account for about one-third of the total variability in breast cancer incidence (3,4). Familial aggregation of breast cancer risk is mainly due to heritable causes (5). The two major high-penetrance susceptibility genes, BRCA1 and BRCA2, are responsible for about 25% of the excess familial breast cancer risk in the investigated German study population (6). Other high-risk susceptibility genes, such as ATM, p53 and PTEN, account only for a minor percentage of familial breast cancers (7–11). According to the polygenic model of inherited breast cancer, unfavourable combinations of polymorphic genetic variants in low-penetrance susceptibility genes jointly contribute to the excess familial breast cancer risk, and most of these genes remain to be discovered (12,13).

DNA helicases play an essential role in the process of genetic recombination, transcription, DNA replication and DNA repair (14–16), in order to maintain genome stability (17). Mutations of the human RecQ helicase genes, WRN (18,19), BLM (20) and RECQ4 (21), lead to rare autosomal recessive genomic instability disorders, referred to as Werner syndrome (WS) (22), Bloom syndrome (BS) (23,24) and Rothmund–Thomson syndrome (RTS) (25), associated with premature ageing and cancer predisposition. Polymorphisms in RecQ helicase genes may be risk factors for cancer (17). Thus, we tested the hypothesis whether WRN and BLM act as low-penetrance familial breast cancer susceptibility genes. RECQ4 was not investigated, since a single nucleotide polymorphism (SNP) search in this gene did not reveal any coding polymorphism.

The tumour suppressor gene p53 encodes a key cellular component in maintaining genomic stability by either arresting cell cycle to allow DNA repair or by inducing apoptosis (26–28). Germline mutations in p53 lead to an autosomal dominant disorder, the Li-Fraumeni syndrome, associated with an 18-fold higher risk for developing breast cancer before the age of 45. While mutations in constitutional DNA of p53 account for less than 1% of all breast cancers, somatic mutations in p53 are reported for 19–57% of all breast cancers (8,29).

A recent study indicates that p53PIN3, a 16 bp insertion/duplication in intron 3 of p53, is associated with an increased colorectal cancer risk and reduced p53 mRNA levels (30). Here, we examined whether the p53 MspI 1798G>A variant, which is completely linked to p53PIN3, is also responsible for familial breast cancer susceptibility. p53 binds to WRN and BLM and inhibits their helicase activities (31–34). Moreover p53-dependent apoptosis is attenuated in Werner and Bloom syndrome cells (35–37), and the reduced levels of
p53-mediated apoptosis can be restored by complementation with microinjected WRN and BLM CDNA (38). We analysed whether the interaction of WRN and BLM with p53 may influence familial breast cancer risk.

This is the first association study examining the putative effects of polymorphic non-conservative amino acid exchanges in WRN (Leu1074Phe, Cys367Arg) and in BLM (Pro868Leu), as well as investigating the possible influence of a 16 bp insertion/duplication in intron 3 of p53 (p53PIN3) on familial breast cancer risk using a large German study cohort.

Materials and methods

Study population

Genotyping analyses were performed on genomic DNA of BRCA1/2 mutation-negative index patients from 816 German breast cancer families, among a subset of 458 high-risk breast cancer cases (risk category A1 and B, see below) and 73 bilateral breast cancer cases, and 1012 unrelated German controls. All breast cancer cases were classified into six categories (6): (A1) families with two or more cases of breast cancer including at least two cases with onset under the age of 50 years; (A2) families with at least one male breast cancer case; (B) families with one or more cases of breast and at least one ovarian cancer; (C) families with two or more cases of breast cancer including one case diagnosed before the age of 50 years; (D) families with two or more cases of breast cancer diagnosed after the age of 50 years; (E) a single case of breast cancer with diagnosis before the age of 35 years. Categories A1 and B were chosen as high-risk categories, since they have shown the highest BRCA1/2 mutation frequencies in a German study population, 35% for A1 and 52% for B (6). The breast cancer cases comprised unrelated women that had been tested BRCA1/2 mutation-negative by applying the denaturing high performance liquid chromatography (DHPLC) method on all exons, followed by direct sequencing of conspicuous exons (6). The samples were collected during the years 1997–2005 by six centres of the German Consortium for Hereditary Breast and Ovarian Cancer (centres of Heidelberg, Wu¨rzburg, Cologne, Kiel, Düsseldorf and Munich, see authors affiliations). Index patients were first diagnosed with breast cancer and then referred to a family registry. All breast cancer patients gave informed consent.

The control population included healthy and unrelated female blood donors collected by the Institute of Transfusion Medicine and Immunology (Mannheim), sharing the ethnic background and sex with the breast cancer patients. The age distribution in the controls and cases was nearly identical (controls: median age 45.6 years, median age 45 years; cases: median age 45.1 years, median age 45 years). According to the German guidelines for blood donation, all blood donors were examined by a standard questionnaire and gave informed consent. They were randomly selected during the years 2004–2005 for this study and no further inclusion criteria were applied during recruitment. The study was approved by the Ethics Committee of the University of Heidelberg (Heidelberg, Germany).

Results

The present case–control study focussed on the potential impact of frequent, non-conservative amino acid exchanges in WRN and BLM on familial breast cancer risk (WRN Leu1074Phe and Cys367Arg, BLM Pro868Leu, retrieved from the NCBI dbSNP database). A SNP search in RECO4 did not reveal any coding polymorphism. The potential effect of the p53PIN3 variant on breast cancer risk was investigated for technical reasons by genotyping the p53 MspI 1798G>A variant, since it has been reported that p53PIN3 and MspI 1798G>A are completely linked (41). This complete linkage was confirmed in the present study by sequencing 57 randomly chosen German control samples.

We performed a case–control study using genomic DNA of BRCA1/2 mutation-negative female index patients from 816 unrelated families, among a subset of 458 high-risk and 73 bilateral breast cancer cases, and 1012 female unrelated

Table 1. TaqMan primers and probes for allelic discrimination assays used for genotyping breast cancer cases and controls

<table>
<thead>
<tr>
<th>db SNP #</th>
<th>Variation</th>
<th>Amino acid exchange</th>
<th>TaqMan primer sequence</th>
<th>TaqMan probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs11852361</td>
<td>35476C&gt;T†</td>
<td>Pro868Leu</td>
<td>F: AGGTTCAGCTATGTCCTTAACAGACAT R: TGCCTTCTGATCCATCTTAGCAAT</td>
<td>VIC-TTTTFAAGCCTTTTCGCTGTAATA FAM-TTTTTAGCCTTTTTCCAGTAAATA</td>
</tr>
<tr>
<td>WRN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs2725362</td>
<td>83316G&gt;T†</td>
<td>Leu1074Phe</td>
<td>F: AGAGCTTCCATCCTCCAGCTAATG R: GCACATTAAAAACTACAGAGTTACAAGAAAGAAAA</td>
<td>VIC-CCAAACAGTTCTTTCTCGC FAM-AAAAAGATGTTCTTTCTCGC</td>
</tr>
<tr>
<td>rs1346044</td>
<td>108690T&gt;C†</td>
<td>Cys367Arg</td>
<td>F: AGATCCCTTCACATGCTGCA</td>
<td>VIC-CATCACTGAAAGGTG FAM-ACATCACTGAAAGGTG</td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1625895</td>
<td>1798G&gt;A†</td>
<td>Intron 6</td>
<td>F: GGTTAAACGGTGGGTCTGAGT R: AGGAAGAAGCCCCCTACT</td>
<td>VIC-CCCTCAGGTTGAGC FAM-CCCTCGGGTGGAGC</td>
</tr>
</tbody>
</table>

†Position relative from ATG according to NT_086832.
‡Position relative from ATG according to NT_086740.
§Position relative from ATG according to NT_010780.

Genotyping

The polymorphic non-conservative amino acid exchanges, Leu1074Phe and Cys367Arg of WRN, Pro868Leu of BLM and the intronic polymorphism 1798G>A of p53, which is completely linked to p53PIN3, were analysed with TaqMan allelic discrimination assays according to earlier descriptions (39). Used primers and probes are described in Table I. The SNP assays were validated by re-genotyping 10% of all samples.

Statistical analysis

The Hardy-Weinberg equilibrium (HWE) test was undertaken using the chi-square ‘goodness-of-fit’ test. Genotype-specific odds ratios (ORs), 95% confidence intervals (95% CIs) and P values were computed by unconditional logistic regression using a tool offered by the Institute of Human Genetics, Technical University Munich, Munich, Germany (http://thg.gsdf.org/cgi-bin/hw/hwa1.pl) and SAS version 9.1 (SAS Institute Inc., Cary, NC). P values were calculated using two-sided chi-square test. Given our sample size, we calculated a power of 80% to detect an OR of 1.52 (BLM, Pro868Leu), 1.36 (WRN, Leu1074Phe), 1.31 (WRN, Cys367Arg) and 2.53 (p53, intron 6, 1798G>A) using the power and sample size calculation software PS version 2.1.31 (http://www.mcp.huber.ETH/vparm/ps/index.htm) (40). Chi-square test for trend (Mantel extension) and the associated P value was calculated using the software Epi Info 2000 version 3.2 (http://www.cdc.gov/epiinfo/). Haplotypes of WRN polymorphisms, Leu1074Phe and Cys367Arg, were determined using SNPHAP 1.3 software by David Clayton (http://archimedes.well.ox.ac.uk/pse/snphap-simple.html). Each individual was assumed to carry the most likely pair of haplotypes. The distribution of each haplotype was compared relative to the most common one between cases and controls.
The results of the chi-square test for trend indicated a dose-by-re-genotyping 10% of all samples and concordance rates of were consistent with the HWE. The SNP assays were validated German controls. Genotype distributions in cases and controls relative to the most common one between cases and controls. The haplotypes GC and TC, containing the risk allele C of WRN Cys Arg, were more frequent among breast cancer cases than among controls, resulting in an association with familial breast cancer and having a similar significance compared to the results of the single SNP (data not shown).

The AA genotype of the p53 variant MspI 1798G>A, which is completely linked to p53PIN3, was significantly more frequent among cases than controls, resulting in an increased familial breast cancer risk following a recessive mode (OR = 2.15, 95% CI 1.12–4.11, P = 0.018, Table II). The OR in bilateral cases was 3.09 but not significant (data not shown). Carriers of the WRN Cys1367Arg TC + CC and p53 MspI AA genotype combination were at a higher breast cancer risk compared to carriers of WRN Cys1367Arg TT and p53 MspI GG+GA (OR = 3.39, 95% CI 1.19–9.71, P value = 0.023). The result of the test for interaction was not significant (Pinteraction = 0.602). ORs were calculated by comparing the distribution of each genotype combination with the most common one between cases and controls (Table III). Stratification of cases and controls according to different age groups (<50 years or ≥50 years) did not influence the risk of the investigated polymorphisms, and age adjustment had no appreciable effect on the ORs.

### Discussion

The tumour suppressor genes p53, WRN and BLM play a crucial role in maintaining genome stability (17). Germline mutations in each of the three genes lead to rare syndromes associated with cancer predisposition (8,17,42). The present case–control association study focussed on the putative impact of the most frequent polymorphic non-conservative amino acid exchanges in WRN (Leu1074Phe, Cys1367Arg) and BLM (Pro868Leu) on familial breast cancer risk. The potential effect of the p53PIN3 variant on familial breast cancer risk was investigated by the genotyping of p53 MspI 1798G>A variant, which is completely linked with p53PIN3 (41) and confirmed in our analyses.

The strength of the present study is the large sample size of familial breast cancer cases, since the power of association studies based on cases with a familial history of the disease is at least twice as high as the power of a study using unselected cases (43,44). Only BRCA1/2 mutation-negative familial breast cancer cases were included in the study to avoid the effects derived from mutations in these high-penetration susceptibility genes (6). Concerning WRN Leu1074Phe and BLM Pro868Leu, genotype frequencies between breast cancer cases and control samples were similar, showing no significant association with familial breast cancer, although the latter SNP is located in close proximity to conserved domains of the gene (45).

A 1.28-fold risk for familial breast cancer was revealed for carriers of the rare C allele of WRN Cys1367Arg. The OR of high-risk familial breast cancers was 1.32, and the OR of bilateral cases was 1.40 but not significant (data not shown). Recent studies have shown an increase in genotype effects when high-risk or bilateral breast cancer cases have been examined (46–49). However, especially, the results of bilateral cases must be interpreted with caution, since this subgroup consisted only of 73 cases. Due to the large confidence intervals, the OR of bilateral cases might be inflated.

In the case of WRN Cys1367Arg (108690T>C), homozygous and heterozygous variant allele carriers showed an elevated breast cancer risk (TC–OR = 1.26, CC–OR = 1.39); thus we used the dominant, allele dose-dependent model to examine this association.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotypes</th>
<th>Cases</th>
<th>Controls</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro868Leu</td>
<td>CC</td>
<td>706/4</td>
<td>897/897</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>92/11</td>
<td>97/9.7</td>
<td>1.21</td>
<td>0.89–1.63</td>
<td>0.225</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>1/0.1</td>
<td>6/0.6</td>
<td>0.21</td>
<td>0.03–1.76</td>
<td>0.113</td>
</tr>
<tr>
<td>WRN</td>
<td>GG</td>
<td>263/3</td>
<td>304/30.5</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GT</td>
<td>402/49.5</td>
<td>492/49.5</td>
<td>0.94</td>
<td>0.77–1.17</td>
<td>0.596</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>148/18.2</td>
<td>202/20.2</td>
<td>0.85</td>
<td>0.65–1.11</td>
<td>0.225</td>
</tr>
<tr>
<td>Cys1367Arg</td>
<td>TT</td>
<td>407/50.2</td>
<td>565/56.2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TC</td>
<td>339/41.8</td>
<td>375/37.5</td>
<td>1.26</td>
<td>1.03–1.52</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>65/8.0</td>
<td>65/6.5</td>
<td>1.39</td>
<td>0.96–2.00</td>
<td>0.079</td>
</tr>
<tr>
<td>BLM</td>
<td>CC</td>
<td>404/49.8</td>
<td>440/43.8</td>
<td>1.28</td>
<td>1.06–1.54</td>
<td>0.010</td>
</tr>
</tbody>
</table>

*p<0.05*

**Table III.** Joint effect of WRN Cys1367Arg (108690T>C) and p53 MspI (1798G>A)

<table>
<thead>
<tr>
<th>WRN</th>
<th>N (controls/cases)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT</td>
<td>550/389</td>
<td>1/10</td>
</tr>
<tr>
<td>TC + CC</td>
<td>426/389</td>
<td>1/5</td>
</tr>
</tbody>
</table>

**Pinteraction = 0.602**

German controls. Genotype distributions in cases and controls were consistent with the HWE. The SNP assays were validated by re-genotyping 10% of all samples and concordance rates of more than 99.5% were attained for all investigated SNPs.

Genotype frequencies of WRN Leu1074Phe and BLM Pro868Leu were similar between breast cancer cases and control samples, showing no significant association with familial breast cancer (Table II). The analysis of the WRN Cys1367Arg polymorphism revealed a significant association with familial breast cancer (OR = 1.28, 95% CI 1.06–1.54, P = 0.010) and high-risk familial breast cancers (OR = 1.32, 95% CI 1.06–1.65, P = 0.015, data not shown). The OR in bilateral cases was 1.40 but not significant (data not shown). The results of the chi-square test for trend indicated a dose-dependent association between the rare C allele of Cys1367Arg and an increased familial breast cancer risk (P<0.010, Table II). The distribution of WRN Leu1074Phe (83316G>T) and Cys1367Arg (108690T>C) haplotypes was compared relative to the most common one between cases and controls. The haplotypes GC and TC, containing the risk allele C of Cys1367Arg, were more frequent among breast cancer cases than among controls, resulting in an association with familial breast cancer and having a similar significance compared to the results of the single SNP (data not shown).
The Cys1367Arg polymorphism leads to a non-conservative amino acid exchange from a sulphydryl group, necessary for disulfide bonds, to a positively charged residue with a guanidino group. According to PolyPhen (http://www.bork.emb-lheidelberg.de/PolyPhen/), Cys1367Arg is predicted to be damaging. Moreover, Cys1367 is conserved in the orthologous genes of mouse and orangutan. It is located in the C-terminus of WRN, the binding site of p53 (31), in close proximity to the nuclear localization signal (50). Functional studies of Cys1367Arg showed little changes (less than 2-fold) in the helicase activity relative to the wild type WRN protein (51). However, the functional relevance of Cys1367Arg for the WRN and p53 interaction and for the p53-mediated apoptosis has not been investigated. The minor allele frequency of WRN Cys1367Arg in the present study was 0.29 among the controls. Previous case–control studies have reported similar estimates for the study was 0.13 among the controls. Previous breast cancer large German breast cancer study cohort, we showed here a ¼ the single SNPs (OR breast cancer risk, which showed higher ORs compared to breast cancer risk, we used the recessive model to examine this association. In addition, earlier reports have shown a significant association of p53 Arg72Pro with increased breast cancer risk (66,76,77). Likewise, it has been reported that Arg72 plays a functional role in the ability of the protein to induce apoptosis (78), and it may affect the function of p53 mutations in breast carcinomas (79). Meta-analyses by Dunning et al. (80) (412 cases) have revealed a moderately increased significant breast cancer risk associated with the variant allele of p53 Arg72Pro. In contrast, meta-analyses by deJong et al. (29) (552 cases) and other studies (74,81,82) have shown a decreased breast cancer risk for homozygous variant carriers. However, since p53 Arg72Pro is in strong linkage disequilibrium to MspI (1798G>A), our results suggest that the variant allele of p53 Arg72Pro might be a breast cancer risk factor rather than possessing a protective effect. Our data are supported by another study on a large German breast cancer cohort, which has shown a borderline significant association of p53 Pro72 with an increased breast cancer risk (64).
In the present study, we investigated the possible impact of putative functional candidate SNPs in genes with a strong a priori biological relevance and probability to be involved in carcinogenesis. Thus, adjustment for multiple comparisons was not taken into account even though four different SNPs were analysed. The consistency of the effects of WRN Cys1367Arg in the genotype and haplotype analyses, the dose-dependency of the association, the consistency of the results of p53PIN3 with the data of previous colorectal and breast cancer studies, the large German breast cancer study cohort, and finally, the further increased risk of WRN Cys1367Arg and p53PIN3 genotype combination carriers argue against a chance finding.

In conclusion, our results suggest that WRN Cys1367Arg might interfere with the binding of p53 to WRN, leading to an attenuated apoptotic function of p53, which is in line with the data on WS cells (35–38). Carriers of p53PIN3 alleles with reduced mRNA levels (30) would be at risk for developing breast cancer, which is further enhanced in carriers of WRN Cys1367Arg.

Acknowledgements

The authors are grateful to Bowang Chen and Justo L. Bermejo for statistical analyses as well as Dagmar Beisse and Julia Schmutzhard for performing TaqMan assays. The German breast cancer samples were collected within a project funded by the Deutsche Krebshilfe, supported by the Center of Molecular Medicine, Cologne (CMMC) and coordinated by Rita K.Schmutzler. This study was supported by the EU, LSHC-CT-2004-503465.

Conflict of Interest Statement: None declared.

References

M.Wirtzberger et al.


Received December 2, 2005; revised January 25, 2006; accepted February 14, 2006.