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Polymorphisms in *BRCA2* resulting in aberrant codon-usage and their analysis on familial breast cancer risk

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Abstract Mutations in *BRCA1* and *BRCA2* are associated with increased breast cancer risk. While numerous non-synonymous SNPs in *BRCA1/2* have been investigated for breast cancer risk, the impact of synonymous SNPs has not been studied so far. Recently, it has been reported that synonymous SNPs leading to an aberration from the preferred codon-usage can have functional effects and consequently be associated with disease. This motivated us to search for SNPs with the tendency to differential codon-usage in *BRCA1/BRCA2*. Based on defined criteria, two codon-usage-changing variants, Ser455Ser (1365A > G) and Ser2414Ser (7242A > G), were detected in *BRCA2*, whereas no such variant could be identified in *BRCA1*. We investigated the impact of these variants on breast cancer risk in a large case–control study. However, both SNPs, *BRCA2* Ser2414Ser (7242A > G) and Ser455Ser (1365A > G), showed no

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association with breast cancer risk. This indicates that these codon-usage-changing SNPs have no major impact on familial breast cancer risk.

Keywords Breast cancer risk · Case–control study · Codon-usage · *BRCA1* · *BRCA2*

Introduction

Breast cancer is the most frequent cancer in women, and after lung cancer the second most frequent cancer in the world [1]. Up to 10% of women who are diagnosed with breast cancer report a family history [2, 3]. A large twin and family study has shown that inherited genetic factors account for about 28% of all breast cancers, while the remaining cases are due to shared or non-shared environmental factors [4, 5]. Furthermore, familial aggregation of breast cancer suggests a major contribution of hereditable genetic factors [6, 7].

Several genes contribute to familial breast cancer risk. Among them, the breast cancer 1 gene (*BRCA1*) and breast cancer 2 gene (*BRCA2*) are the most important ones. Mutations in these genes confer a high-penetrance and account for about 5–30% of familial breast cancer cases [8–10]. Missense mutations, intronic variants and in-frame deletions or insertions in either *BRCA1* or *BRCA2* confer a lifetime risk of breast cancer from 60 to 85% [11–14]. The risk of *BRCA1* and *BRCA2* mutation carriers can be further modified by other genetic or environmental factors [15–18].

It has been suggested that a single nucleotide polymorphism (SNP) can alter the amino acid residue in the primary protein sequence and consequently impact on protein folding, target recognition, transcription regulation and even the long-range enhancer regulation [19–22]. Thus, some SNPs could affect the disease processes and clinic response [23, 24]. However, these studies mainly focused on the non-synonymous SNPs, whereas the effects of synonymous SNPs are largely unknown.

Recently, Chava Kimchi-Sarfaty et al. [25] reported a synonymous SNP in the Multidrug Resistance 1 (MDR1) gene leading to an aberration from the preferred codon-usage. An ATC-codon (20.9% usage) is changed to an ATT–codon (15.8% usage). As a result, the use of the less preferred codon appears to influence the translation rate, which in turn affects protein folding and finally leads to altered drug and inhibitor interaction, although the mRNA and protein levels are similar to the wild-type [25, 26]. Consequently, this SNP has been found to be associated with MDR1 activity in B-cell chronic lymphocytic leukaemia [27]. One of the most recent works reported a synonymous SNP in beta-arrestin 2, which suggested that the rare codon AGT is associated with increased risk of Tardive dyskinesia occurrence [28]. A silent mutation was also found by Knobe et al. [29] in five of total 86 families with haemophilia B in Sweden. This SNP is also an aberration from the preferred codon-usage (GTG 28.2% usage to GTA 7.1% usage) [29]. Anton A. Komar suggested that infrequent codons in mRNA appear to be slowly translated, whereas frequent codons are rapidly translated [30]. Thus, the use of rare codons influences the translation rate of mRNA, which consecutively affects protein folding secondary structure [31–33]. Furthermore, amino acids encoded by GC third bases appear to be more preferred to cell function and survival than those encoded by AT third bases [34, 35], indicating that the codon-usage might be one of the possible explanations for the bias of the alleles in synonymous SNPs. Other reports have suggested that synonymous SNPs can also affect the stability of mRNA or protecting from deleterious mutations [36, 37].

In the present study, we investigated whether there are synonymous polymorphisms in *BRCA1 and BRCA2* with less preferred codon-usage and consequently whether these SNPs could be associated with an increased breast cancer risk.

Materials and methods

Study population

The database of the German consortium for hereditary breast and ovarian cancer (GC-HBOC), which comprises indentified polymorphisms and variants of 3,564 analysed *BRCA1/BRCA2* mutation-negative familial breast cancer index cases, was used to select for silent SNPs. These SNPs were further investigated for an alteration of the preferred codon-usage in Homo sapiens and within the *BRCA1/2* genes by comparison with the codon-usage database (http://www.kazusa.or.jp/codon).

Genotyping was performed on genomic DNA of *BRCA1/2* mutation-negative index patients from 811 German breast cancer (BC) families, among them a subset of 351 high-risk breast cancer cases (A1 group: families with two or more cases of breast cancer including at least two cases with onset of the disease under the age of 50 years; B group: families with one or more male breast cancer cases), and 1,330 unrelated healthy German women. The breast cancer cases are comprised of 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 [8]. The BC samples were collected during the years 1997–2007 by three centres of the German Consortium for Hereditary Breast and Ovarian Cancer

(centres of Heidelberg, Cologne 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 an informed consent for the study.

The control population included healthy and unrelated female blood donors collected by the German Red Cross Blood Service of Baden—Wuerttemberg—Hessia and Institute of Transfusion Medicine and Immunology (Mannheim), sharing the same ethnic background as the breast cancer patients. The age distribution in controls and cases is similar (controls: mean age 43.9 years, median age 42 years, age from 18 to 68 years old; cases: mean age 45.4 years, median age 45 years, age from 19 to 87 years old). According to the German guidelines for blood donation, all blood donors were examined by a standard questionnaire and gave their informed consent. They were randomly selected during the years 2004–2007 for this study and no further inclusion criteria were applied during recruitment. This study was approved by the Ethics Committee of the University of Heidelberg (Heidelberg, Germany).

Genotyping

Two SNPs in *BRCA2*, Ser455Ser (1365A > G) and Ser2414Ser (7242A > G), were selected from the GC-HBOC database following defined criteria described in the discussion section. These SNPs were analysed using *Taq*Man allelic discrimination assays according to earlier descriptions [38]. Sequences of primers and probes are available upon request. The SNP assays were validated by re-genotyping 10% of all samples.

Statistical analysis

Hardy–Weinberg equilibrium test was undertaken using the χ^2 'goodness-of-fit' test by a tool from the Institute of Human Genetics, Technical University Munich, Munich, Germany (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). Genotype-specific odds ratios (OR), 95% confidence intervals (CI) and *P* values were computed by unconditional logistic regression using SAS version 9.1 (SAS Institute Inc, Cary, NC). Age, treated as a continuous variable was included in the regression as covariate. *P* values were calculated using two-sided χ^2 test. The power ($\alpha = 0.05$) was calculated using the power and sample size calculation software PS version 2.1.31 (http://www.mc.vanderbilt.edu/prevmed/ps/index.htm) [39]. SNPs linked with Ser455Ser and Ser2414Ser with $r^2 \geq 0.8$ and block definition were identified using HaploView version 3.32 (http://www.broad.mit.edu/mpg/haploview). The linked SNPs' breast cancer associations were further checked if they have being analysed in the Cancer Genetic Markers of Susceptibility genome wide association study (CGEMS) (https://caintegrator.nci.nih.gov/cgems/browseSetup.do).

Results

We searched for SNPs in *BRCA1* and *BRAC2* leading to a less preferred codon-usage in familial breast cancer patients, by investigating the database of the German consortium for hereditary breast and ovarian cancer (GC-HBOC). This database included genetic variations indentified in 3564 *BRCA1/2* mutation-negative index familial breast cancer cases.

From this database, seven synonymous variations were found in *BRCA1* and nine synonymous variations were found in *BRCA2*. Some of these synonymous SNPs were also detected by Loizidou et al. [40]. We selected the most interesting candidates by applying the following criteria. The SNP induces a reduction of codon-usage of more than 50% investigating the general codon-usage in Homo saklpiens. Furthermore, the allele frequency should be larger than 1% (Table 1). Using these criteria, no such variants in *BRCA1* but two variants in *BRCA2*, Ser455Ser (1365A > G) and Ser2414Ser (7242A > G) (Table 1) could be identified. These two SNPs were reported by Loizidou M et al. but with limited samples' investigation [40]. Thus, we investigated the impact of these two variants on breast cancer risk performing a large case-control study on familial *BRCA1/2* mutation-negative breast cancer cases and controls.

The genotype analysis of *BRCA2* Ser455Ser (1365A > G) and Ser2414Ser (7242A > G) was performed on genomic DNA of *BRCA1/2* mutation-negative index patients of 811 German breast cancer families with a mean age of 45 years, and 1,330 unrelated German control individuals with a mean age of 44 years. Genotype distribution in both controls and cases were consistent with the Hardy–Weinberg equilibrium (HWE). The SNP assays were validated by re-genotyping 10 % of all samples attaining concordance rates of more than 99.5% for both investigated SNPs.

Allele and genotype frequencies of *BRCA2* Ser2414Ser (7242A > G) and Ser455Ser (1365A > G) were similar between familial breast cancer cases and controls. Ser2414Ser (7242A > G) ([G] vs. [A], OR = 1.00, 95% CI 0.86–1.17, $P = 1.00$; [AG] vs. [AA], OR = 1.01, 95% CI 0.83–1.22, $P = 0.96$; [GG] vs. [AA], OR = 1.00, 95% CI 0.83–1.21, $P = 0.98$; $P_{\text{trend}} = 1.00$, Table 2) and Ser455Ser (1365A > G) ([G] vs. [A], OR = 0.94, 95% CI 0.65–1.37, $P = 0.76$; [AG] vs. [AA], OR = 1.02, 95% CI 0.69–1.50, $P = 0.92$; $P_{\text{trend}} = 0.76$, Table 2), showing no association with familial breast cancer. We also analysed if individuals carrying the rare allele in both SNPs versus homozygous wild-type carriers in both SNPs are more frequent in cases than in controls, but did not find any significant association ($P = 0.16$, data not show).

Table 1 Codon usage alternation in *BRCA1/2* synonymous variations

Variant	Consequence	Numbe[a]	Codon change	Homo sapiens codon-usage alteration[b]
BRCA1 A5A (15T > C)	Synonymous	71	GCT > GCC	18.4 > 27.7
BRCA1 C197C (591C > T)	Synonymous	9	TGC > TGT	12.6 > 10.6
BRCA1 P359P (1077A > G)	Synonymous	8	CCA > CCG	16.9 > 6.9
BRCA1 S694S (2082C > T)	Synonymous	1,079	AGC > AGT	19.5 > 12.1
BRCA1 L771L (2313T > C)	Synonymous	1,070	CTT > CTC	13.2 > 19.6
BRCA1 S1436S (4308T > C)	Synonymous	1,073	TCT > TCC	15.2 > 17.7
BRCA1 Q1604Q (4812A > G)	Synonymous	4	CAA > CAG	12.3 > 34.2
BRCA2 S455S (1365A > G)	Synonymous	116	TCA > TCG	12.2 > 4.4
BRCA2 H743H (2229T > C)	Synonymous	115	CAT > CAC	10.9 > 15.1
BRCA2 Q961Q (2883G > A)	Synonymous	0	CAG > CAA	34.2 > 12.3
BRCA2 K1132 K (3396A > G)	Synonymous	898	AAA > AAG	24.4 > 31.9
BRCA2 S1172S (3516G > A)	Synonymous	9	TCG > TCA	4.4 > 12.2
BRCA2 V1269 V (3807T > C)	Synonymous	690	GTT > GTC	11.0 > 14.5
BRCA2 L1356L (4068G > A)	Synonymous	18	TTG > TTA	12.9 > 7.7
BRCA2 S1733S (5199C > T)	Synonymous	45	TCC > TCT	17.7 > 15.2
BRCA2 S2414S (7242A > G)	Synonymous	727	TCA > TCG	12.2 > 4.4

[a] Number of identified synonymous variants in 3,356 investigated familial, *BRCA1/2* negative breast cancer cases

[b] Homo sapiens general codon usage frequencies for the respective amino acid were selected from codon usage database, http://www.kazusa.or.jp/codon

Table 2 Genotype frequencies of codon-usage-changing SNPs in *BRCA2*

SNP	Genotypes	Case (%)	Control (%)	OR	95% CI	*P*
	AA	747 (94.4)	1,229 (94.1)	1		
BRCA2	AG	44 (5.6)	74 (5.7)	1.021	0.694–1.502	0.916
Ser455Ser	GG	0 (0.0)	3 (0.2)	–	–	0.977
1365A > G	[G] vs. [A]			0.942	0.648–1.370	0.755
rs1801439						$P_{trend} = 0.758$[a]
	AA	483 (61.5)	815 (62.2)	1		
BRCA2	AG	262 (33.4)	431 (32.9)	1.005	0.829–1.218	0.960
Ser2414Ser	GG	40 (5.1)	64 (4.9)	1.003	0.834–1.205	0.978
7242A > G	[G] vs. [A]			1.000	0.857–1.166	0.999
rs1799955						$P_{trend} = 0.999$[a]

Adjusted for age; all analyses done with SAS Version 9.1 Proc Logistic

[a] χ^2 test for trend

The two SNPs in *BRCA2*, Ser455Ser (1365A > G) and Ser2414 (7242A > G), were found to be not in linkage disequilibrium to each other ($r^2 = 0.0090$). Furthermore, a haplotype analysis was performed and investigated for a putative association with breast cancer risk. As a result, none of the three haplotypes (rs1801439-rs1799955: A–A, A–G and G–A) of the two polymorphisms revealed any association with breast cancer risk (data not shown). To compare our results with findings from genome wide studies, we analysed 200 kb flanking regions of *BRCA2* Ser455Ser (1365A > G) and Ser2414Ser (7242A > G), which covers the whole *BRCA2* gene. Neither of the SNPs, Ser455Ser (1365A > G) and Ser2414Ser (7242A > G) themselves, nor SNPs in linkage disequilibrium with Ser2414Ser ($r^2 \geq 0.9$) have been analysed in the Cancer Genetic Markers of Susceptibility genome wide association study (CGEMS) (https://caintegrator.nci.nih.gov/cgems/browseSetup.do). However, one SNP in linkage disequilibrium with Ser455Ser (1365A > G), rs11571684 (located at 14.5 kb downstream; $r^2 = 1.0$), has been analysed in CGEMS. This variant did not show any trend for an association with breast cancer ([AG] vs. [AA], OR = 0.79, $P = 0.96$), thus confirming our results.

Discussion

Inherited mutations in the *BRCA1* and *BRCA2* tumour suppressor genes are among the strongest genetic risk factors for breast cancer. According to a world wide study [41], it has

been estimated that 0.7–29 % of familial heritage are accounted for by mutations in *BRCA1*, and 1.5–25 % are accounted for by mutations in *BRCA2*, varying a lot in populations from different geographic regions and ethnicities. Nonsense-, frame shift-, missense-, splice site-, deletion-, insertion-mutations and large deletions/genomic rearrangements have been identified in *BRCA1/2* [41–44]. In addition, several non-synonymous SNPs in *BRCA1/2* have been associated with breast cancer risk [45–47]. It is crucial to understand the contribution of different types of genetic variants within *BRCA1/2* to familial breast cancer risk.

Our work is the first study focussing on the possible impact of synonymous SNPs leading to an alteration from the preferred codon-usage in *BRCA1 or BRCA2* on familial breast cancer risk. Whereas no obvious codon-usage changing polymorphism could be found in *BRCA1*, two codon-usage changing SNPs in *BRCA2*, Ser455Ser (1365A > G) and Ser2414Ser (7242A > G), were identified and investigated in a large case–control study population. As mutations in *BRCA1* and *BRCA2* are account for about 30% of familial breast cancer cases in Germany [8], only *BRCA1/2* mutation-negative familial breast cancer cases were included in our study to avoid the effects attributable to disease-associated mutations. Given our sample size, we had a power of 80% ($\alpha = 0.05$) to detect an OR of 1.64 for Ser455Ser and of 1.30 for Ser2414Ser, not considering the usage of familial cases. The power of an association study based on familial cases is even about two times higher compared to a study of unselected cases [48, 49].

Normally, there is a bias in the use of redundant codons. A comparative study on five mammals for "extreme conservation" showed that there are three times fewer synonymous than non-synonymous mutations [50]. This indicates that synonymous codons are under strong evolutionary selection. Thus, the variation of the codon-usage may result in functional impact. Chava Kimchi-Sarfaty et al. [25] reported a functional synonymous SNP whose rare allele reduces the codon-usage frequency from 20.9 to 15.8% (ATC–ATT). This results in a delayed translation and changes the folding of the protein. In consequence, this SNP was associated with altered ligand binding efficiency.

The two codon-usage-changing SNPs in *BRCA2* introduce an A–G transition in the third base position of a codon for Serine: Ser455Ser (1365A > G) and Ser2414Ser (7242A > G), changing the TCA-codon (12.2% usage in Homo sapiens, Table 1) to the less preferred TCG-codon (4.4% usage in Homo sapiens, Table 1). But here, both variants, Ser455Ser (1365A > G) and Ser2414Ser (7242A > G), showed no association with familial breast cancer risk.

According to the work of Chava Kimchi-Sarfaty et al. the change from ATC-codon-usage to ATT-codon-usage is only 20.9–15.9%, but nevertheless this specific change alters the drug and inhibitor interactions of P-glycoprotein [25, 26]. Therefore, in the present study, although the two investigated SNPs in *BRCA2* showed no association with familial breast cancer risk, it is still unknown whether these two SNPs which induced the greatest reduction of preferred codon-usage in Homo sapiens are associated with breast cancer therapy or drug treatment. We also can not exclude the possibility that other synonymous variants found in *BRCA1* and *BRCA2* showing less codon-usage alternation are associated with breast cancer risk.

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