

HRM SCREENING OF THE *UBC9* GENE ENCODING THE SUMO-E2-CONJUGATING ENZYME — CASE-CONTROL STUDY IN BREAST CANCER

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Aim: *UBC9* (E2) small ubiquitin-like modifier conjugating enzyme plays a key role in the post-translational modification of proteins named sumoylation. Defects in small ubiquitin-like modifier modification may contribute to breast carcinogenesis. In the present work, we examined *UBC9* genetic variation. **Materials and Methods:** *UBC9* genetic variation was analyzed by using the high resolution melting (HRM) method. HRM study was conducted on 173–182 healthy women and 188–190 women with breast cancer. **Results:** During HRM screening, we analysed three known single-nucleotide polymorphisms in introns: rs4984806, rs909916 and rs909917, and one known single nucleotide polymorphism rs8063 in exon 7, in a non-coding region. The genotype frequencies for all polymorphisms were in accordance with Hardy — Weinberg equilibrium among the control subjects and breast cancer patients. The linkage disequilibrium analysis displayed that there was one polymorphism block, which consisted of three single nucleotide polymorphisms: rs909916, rs909917 and rs4984806. We identified two common haplotypes CCG and TTC, but we did not find significant differences in the distribution of these haplotypes between cases and controls. **Conclusion:** Our study showed no differences in the occurrence of indicated polymorphisms in the *UBC9* gene in a group of healthy women compared to women with breast cancer. These results suggest that the polymorphisms of the *UBC9* gene — rs4984806, rs909916, rs909917 and rs8063 can be not associated with breast cancer risk.

Key Words: *UBC9* gene, gene polymorphism, sumoylation, breast cancer, high resolution melting method.

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Sumoylation is a type of post-translational modification of proteins characterized by covalent and reversible binding of small ubiquitin-like modifier (SUMO) to a target protein. SUMO (SUMO-1, 2, 3 and 4) proteins are small molecules having a molecular weight about 11 kDa, and have a similar three-dimensional structure compared to ubiquitin. In contrast to ubiquitin, SUMO proteins have a 10–25 amino-acid tails at N-terminal domains. SUMO proteins show about 20% identity to ubiquitin. SUMO-2/3 proteins are identical to each other by 95% and they are identical to SUMO-1 protein by 50% [1]. Sumoylation cycle is preceded by activation of SUMO proteins, which involves the removal of the C-terminus and the exposure of two molecules of glycine. This activation step is carried out by SENP proteins (Sentrin/SUMO-specific protease). Then, SUMO protein is bound by heterodimeric enzyme E1 — SAE (SUMO-activating enzyme), consisting of two subunits, SAE1 and SAE2. SUMO-E1 complex is formed, wherein the cysteine at position 173 (Cys173) of SAE2 subunit binds by a thioester bond with glycine at position 97 (Gly97) at the C-terminus of SUMO protein. The next stage is a transfer of SUMO protein to conjugat-

ing enzyme E2, called UBC9 protein (SUMO-E2-conjugating enzyme). SUMO-UBC9 complex is created by a thioester binding between Gly97 of SUMO protein and Cys93 residue in the N-terminus of UBC9 protein. UBC9 enzyme catalyses the isopeptide bond formation between a carboxyl group of glycine at the C-terminus of SUMO protein and ϵ -amino group of lysine in a target protein. E3 enzymes called SUMO ligases can also occur in this step of SUMO cycle. Sumoylation modifies function, subcellular localization and stability of proteins involved in various cellular processes including DNA damage response, transcriptional regulation, nuclear transport and cell cycle control [2, 3].

Previously, we examined the *UBC9* gene polymorphism in women with breast cancer in the Polish population [4, 5]. Our first work was focused on association between the c.73G>A (p.Val25Met) (rs11553473) polymorphism of the *UBC9* gene and an efficiency of DNA double-strand breaks repair in breast cancer patients [4]. We indicated that efficacy of DNA double-strand breaks repair in breast cancer is decreased in carriers of the variant allele of the c.73G>A polymorphism (rs11553473), and it can be considered as functional implication of this polymorphism. Moreover, we noticed that the variant allele is strongly inversely related to HER2 (human epidermal growth factor receptor 2) negative breast cancer patients (OR 0.03, 0.00–0.23 95% CI) [4]. In the work of Wozniak et al. [5] we investigated an association between three polymorphisms of the *UBC9* gene:

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Abbreviations used: HRM — high resolution melting; HWE — Hardy — Weinberg equilibrium; LD — linkage disequilibrium; OR — odds ratios; SNP — single nucleotide polymorphism; SUMO — small ubiquitin-like modifier.

c.73G>A (rs11553473), c.430T>G (rs75020906) and g.1289209T>C (rs7187167) and a risk of ductal breast cancer occurrence. The obtained results suggested that genetic variants of the *UBC9* gene, c.73G>A (rs11553473) and g.1289209T>C (rs7187167) may play a role in the development of ductal breast cancer. The results of our previous studies, as well as reports in the literature on the potential role of *UBC9* protein in breast cancer encouraged us to explore the *UBC9* gene in order to search for genetic differences between healthy women and women with breast cancer.

MATERIALS AND METHODS

DNA samples. Human genomic DNA samples were derived from anonymous Polish unrelated women who declared to be healthy (controls). Samples were randomly selected from the “normal Polish population” genetic collection at the Biobank LAB, Department of Molecular Biophysics, University of Lodz. Genetic material for this collection was sampled in 2011–2012 within the EU-funded TESTOPLEK project. This collection was involved in creation of a retrospective POPULOUS collection (POPULATION — LODz UNIVERsITY Biobank) and registered since 2013 in the Biobanking and BioMolecular resources Research Infrastructure catalog of the populations collection [6]. All subjects gave their written informed consent to participate in the study. This study was approved by the relevant regional ethical committee (Research Bioethics Commission, University of Lodz — Decision no. 8/KBBN-UL/II/2014 and Statement of the Research Bioethics Commission, University of Lodz from 17th of June 2010) and all procedures were performed in accordance with the Declaration of Helsinki.

Saliva was collected into Oragene OandG-500 DNA collection/storage receptacles (DNA Genotek, Kanata, Canada) and genomic DNA was subsequently isolated by the MagNA Pure LC DNA Isolation Kit — Large Volume (Roche, Basel, Switzerland) with final concentration normalized to 200 pg/μl [7]. A total of 173 (rs4984806) and 182 (rs909916, rs909917 and rs8063) samples were enrolled in the scanning study.

In the case of cancer patients blood samples were collected from 188 (rs909916 and rs909917), 189 (rs8063) and 190 (rs4984806) women (mean age 60.2 ± 11.8 years) diagnosed with breast cancer and treated at the Department of Surgical Oncology, N. Copernicus Hospital (Lodz, Poland). The clinical characteristic of breast cancer patients is presented in Table 1. Blood was collected before surgical treatment and chemotherapy. The Bioethics Committee of Regional Medical Chamber in Lodz (nr K.B.-4/07) approved the study and each patient gave a written consent. Genomic DNA was prepared from peripheral blood of breast cancer patients by using of commercial Blood Genomic DNA Miniprep Kit (Axygen Biosciences, CA, USA), as recommended by the manufacturer. Each sample was normalized to final concentration equal 200 pg/μl.

Screening of *UBC9* gene by high resolution melting (HRM) method. Investigation of *UBC9* ge-

Table 1. The clinical characteristics of breast cancer patients

Characteristic	Number of patients
Age	
Range: 32–86	190
Mean age $\pm$ SD	60.18 ± 11.79
Carcinoma ductale	127
Carcinoma ductale infiltrans	5
Carcinoma intraductale	5
Carcinoma lobulare	16
Carcinoma lobulare infiltrans	2
Carcinoma mammae	1
Carcinoma metaplasticum	1
Carcinoma mucinosum	4
Carcinoma papillare	1
Other	28
Tumor grade by Bloom-Richardson grading system	
1	12
2	55
3	60
Not determined	63
Metastases in lymph nodes	
Positive	36
Negative	128
Not determined	26
Estrogen receptor status	
Positive	130
Negative	42
Not determined	18
Progesterone receptor status	
Positive	113
Negative	48
Not determined	29

netic variation was conducted by using the HRM method, as was previously described in the work of Słomka et al. [8].

Polymorphism detection. The *UBC9* genomic DNA sequence (NM_194260.2) was obtained from GenBank (www.ncbi.nlm.nih.gov) and used as a reference sequence during analysis of sequencing results by CodonCode Aligner software (Codon-Code Corporation, Centerville, USA).

Availability of supporting data. For each of the polymorphisms detected in this study in *UBC9* gene, the following parameters were assigned: dbSNP IDs (rs numbers) (www.ncbi.nlm.nih.gov/SNP), nucleotide position within or relative to the coding sequence based on the NM_003345.4, and amino acid position in the protein for single nucleotide polymorphisms in the coding sequence based on the reference sequence NP_919236.1. These data were obtained from GenBank (www.ncbi.nlm.nih.gov) and were used in this paper as the nomenclature of variants.

Linkage disequilibrium (LD) and haplotype blocks analysis. The observed genotype distribution was determined for all the detected polymorphisms by performing the Hardy — Weinberg Equilibrium (HWE) exact test assuming consistency for *P*-value higher than 0.001. LD and haplotype block analysis was performed by Haploview 4.2 software (www.broad.mit.edu/mpg/haploview/). The LD analysis was made using *D'* and *r*² parameters for each variation pair. *D'* coefficient was preferentially used to model recombination rates in examined population, *r*² parameter to model association power [9, 10].

RESULTS

The aim of this study was an analysis of variation of the *UBC9* gene. We screened coding regions and exon/intron boundaries with intronic flanking sequenc-

es. For maximal optimisation of detection level, tested polymerase chain reaction product was never longer than 250 base pairs. HRM scanning covered 6 exons, and exon 3 was divided into two separate scanned regions. It was necessary to divide the exon 3, because we could not scan this exon probably due to the presence of GC-rich region. We chose for analysis three known single nucleotide polymorphisms (SNPs) in introns and one known SNP in exon 7, in a non-coding region (Table 2). The selection of studied polymorphisms was mainly determined by their potential biological significance — the localization of the SNPs was in areas near exons or regulatory regions of genes. The polymorphic variant c.67-13T>C (rs4984806) is located 12 base pairs from exon 3, the c.333+19T>C (rs909916) is located 18 base pairs from exon 5 and the c.333+25C>G (rs909917) is located 24 base pairs from exon 5. The SNP c.*24A>G (rs8063) is located in exon 7, in non-coding region. The minor allele frequency for each SNP was determined for control subjects and breast cancer patients (see Table 2). The genotype frequencies for all polymorphisms were in accordance with HWE among the control subjects and breast cancer patients (see Table 2). The genotypes and allele distribution and odds ratios (OR) of polymorphisms detected by HRM method are shown in Table 3. There was no difference in the frequency of the genotypes and alleles of the polymorphic variants of the *UBC9* gene between patients and controls.

In our study, we also examined links between the *UBC9* gene polymorphisms predicted by Haploview program. LD analysis displayed that there is one SNPs

block in the *UBC9* gene. Among four selected SNPs, three SNPs: c.333+19T>C (rs909916), c.333+25C>G (rs909917) and c.67-13T>C (rs4984806) were included in this block. Allelic variants of these polymorphisms form two the most common haplotypes in the block (Figure). Haplotype analysis results are

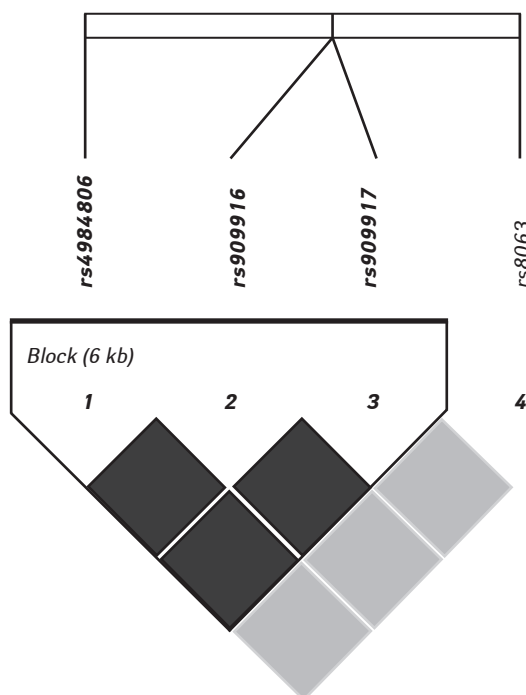


Figure. The haplotype structure of three *UBC9* SNPs detected in this study. The Haploview program generated this plot. One haplotype block was determined

Table 2. Summary of *UBC9* gene variants detected by HRM method

dbSNP ID	Variant residue NM_003345.4	Intron/exon	HWE P-value ^a		MAF ^b		
			HWE (case)	HWE (control)	MAF (case)	MAF (control)	MAF (total)
rs4984806	c.67-13T> C	Intron 2	0.47	0.24	0.100	0.124	0.126
rs909916	c.333+19T> C	Intron 5	0.99	0.54	0.104	0.126	0.130
rs909917	c.333+25C> G	Intron 5	0.99	0.54	0.104	0.126	0.130
rs8063	c.*24A> G	Exon 7	-	0.97	-	< 0.001	< 0.001

Note: ^aP-value is consistent with HWE if $p > 0.05$; ^bMAF – minor allele frequency. Minor allele shown in bold font in column 2.

Table 3. The genotype and allele distribution and OR of rs4984806, rs909916, rs909917 and rs8063 polymorphisms of the *UBC9* gene in breast cancer patients and controls

Genotype or allele	Breast cancer patients (n=190)	Controls (n=182)	OR	OR (95% CI)	P-value
rs4984806					
R/R	1	1	0.96	0.24–3.83	0.73
R/V	36	41	0.75	0.45–1.25	0.96
V/V	153	131	1.33	0.80–2.19	0.33
R	38	43	0.78	0.49–1.24	0.36
V	342	303	1.28	0.80–2.03	0.36
rs909916					
R/R	2	2	0.96	0.13–6.91	0.67
R/V	35	42	0.76	0.46–1.26	0.35
V/V	151	138	0.77	0.47–1.26	0.36
R	39	46	0.80	0.51–1.26	0.40
V	337	318	1.25	0.79–1.97	0.40
rs909917					
R/R	2	2	0.97	0.13–6.94	0.68
R/V	35	42	0.76	0.46–1.26	0.35
V/V	151	138	1.30	0.79–2.13	0.36
R	39	46	0.80	0.51–1.26	0.4
V	337	318	1.25	0.79–1.97	0.4
rs8063					
R/R	0	0	-	-	-
R/V	0	1	-	-	-
V/V	189	181	-	-	-
R	0	1	-	-	-
V	378	362	-	-	-

Note: R – reference allele; V – variant allele.

summarized in Table 4. No significant differences in the distribution of haplotypes between breast cancer patients and control subjects were detected.

DISCUSSION

In this study, we analysed the *UBC9* gene in order to search for genetic differences between healthy women and women with breast cancer. Genetic variability may affect expression and activity of the *UBC9* gene or/and protein and may have an impact on breast cancer occurrence and progression. Abnormalities in SUMO modification may contribute to carcinogenesis by affecting post-translational modification of key proteins, e.g. hormone receptors including estrogen receptor alpha [11] and progesterone receptor [12]. It is hypothesised that SUMO modification contributes to the aggressive nature of breast cancer, particularly those associated with features similar to breast carcinoma arising in patients with *BRCA1* germline mutations [2]. The SUMO modification was shown to be involved in *BRCA1* regulation of gene expression and maintenance of genome stability. *BRCA1* is modified by SUMO in response to genotoxic stress and co-localizes at site of DNA damage with SUMO proteins and *UBC9* [13, 14]. Analysis of *UBC9* and *BRCA1* proteins interactions demonstrated that *UBC9* protein was required for *BRCA1* ubiquitin ligase activity [15]. It was also demonstrated that *UBC9* participated in *BRCA1* ubiquitin ligase mediated degradation of estrogen receptor alpha. Interestingly, the *UBC9* protein interacted with *BRCA1* and affected its activity not only in the context of sumoylation, but also in SUMO independent manner.

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death in women worldwide, accounting for 23% of total cancer cases and 14% of all cancer related mortalities [16]. The incidence of breast cancer in Poland in 2013 was 17 142 (21.9%), and the number of deaths was 5816 (13.87%) [17]. It is well known that chemotherapy is an effective way to treat cancer, but is associated with significant side effects, which are often the cause of death of patients. Literature data indicate that genes and proteins of the SUMO cycle may be good targets of tumor therapy. Therefore, we decided on the exact genetic analysis of the *UBC9* gene in women with breast cancer compared to healthy women.

Our research was focused on searching genetic variation in the *UBC9* gene using HRM method. The base of the method is dsDNA denaturation which temperature melting depends on length and nucleotide composition. Even a single base change generates different melting curve and several variants could be detected by this way.

Table 4. Haplotype block analysis of the *UBC9* gene according to Gabriel et al. [20]

Haplotype block	Haplotype ^a	Freq (total)	Freq (case)	Freq (control)	χ^2	P ^b
Block 1	CCG	0.886	0.897	0.874	1.031	0.3099
	TTC	0.110	0.095	0.126	1.886	0.1696

Note: ^aHaplotypes with total frequency less than 5% were omitted; ^bP-value for the χ^2 test.

We did not find any differences between healthy women and patients with breast cancer in terms of the *UBC9* gene variation (see Table 3). Our results may indicate that the coding regions of the *UBC9* gene and areas flanking exons are highly conserved in both healthy women and patients.

In other studies an association between the polymorphic variants of the *UBC9* gene and the risk of breast cancer has been shown [18, 19]. An association between four *UBC9* SNPs: rs7187167, rs11248866, rs8052688 and rs8063, and the risk of grade 1 breast tumors was found [19]. The strongest association was observed for rs7187167, and the effect appeared to be stronger in familial cases. The last of these SNPs rs8063 was also detected in our present study. Although, we did not demonstrate its relationship with breast cancer.

We found one SNP block including three polymorphic sites analysed during this study: c.333+19T>C (rs909916), c.333+25C>G (rs909917) and c.67-13T>C (rs4984806) (see Figure). To determine haplotype block in the *UBC9* gene we used the method described previously by Gabriel et al. [20] based on confidence bounds on D'. Based on this method we identified two common haplotypes CCG and TTC. We did not find any significant differences in the distribution of haplotypes between cases and controls (see Table 4).

CONCLUSION

Although our study showed no difference in the polymorphic variants of the *UBC9* gene detected by HRM method between healthy women and women with breast cancer, we believe that such research should be continued. We suggest that these studies should be carried out in more specific groups of breast cancer patients, for example with mutations in the *BRCA1/2* genes or in triple-negative breast cancer. On the other hand we also suggest expanding genetic research of the *UBC9* gene on the analysis of introns and untranslated regions as sequences rich in polymorphic variants, which may have clinical relevance as well.

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COMPETING INTERESTS

We have no competing interests.

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