Mitochondrial genotype and breast cancer predisposition

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Abstract. Breast cancer is the most commonly diagnosed cancer in women. Despite recent advances in breast cancer research, a comprehensive set of genetic markers of increased breast cancer risk remain elusive. Recently mitochondrial DNA (mtDNA) mutations have been found in many types of cancer, including breast cancer. To investigate the possible role of mitochondrial genetics in breast cancer predisposition and biology we analyzed the D-loop sequence of cancer patients and assigned mitochondrial haplogroup using RFLP analysis. We detected a significantly greater incidence of mtDNA polymorphisms T239C, A263G and C16207T and a significant lower incidence of A73G, C150T, T16183C, T16189C, C16223T, T16362C in patients with breast cancer compared to database controls. The mitochondrial haplogroup distribution in patients with breast cancer differs from a group of cancer-free controls and the general Polish population in that haplogroup I is over-represented in individuals with cancer. These findings suggest that mitochondrial haplogroup I as well as other polymorphic variants defined by SNPs in the D-loop may be associated with an increased risk of developing breast cancer.

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Introduction

An estimated 1.15 million new breast cancer cases are diagnosed worldwide per year (1). In the United States breast cancer is the most common cancer among women (over 200,000 cases annually) and the second most common cause of cancer mortality (over 40,000 deaths per year) accounting for 15% of cancer deaths among American women and 26% of all new cancer cases among women (2,3). Also in the European Union (EU) breast cancer is the the second most commonly diagnosed cancer and accounts for 17.9 deaths per 100,000 population (4). Multiple diagnostic and prognostic factors have been defined for breast cancer. Prognostic factors generally are related to surgical and pathological findings and include tumor grade, stage and lymph node metastasis status. Few predictive biomarkers are currently available. High throughput molecular technologies are reshaping our understanding of breast cancer and a molecular taxonomy that has stronger predictive power is slowly emerging. In some cases a molecular grading system has been proposed as in the case of ductal cancer *in situ* of the breast (5), and novel prognostic/predictive gene signatures have been identified (6).

There are likely many low-penetrance genes involved in breast cancer carcinogenesis, and their cumulative attributable risk for breast cancer development is substantial. Moreover, 5% of breast cancers are associated with a proven genetic predisposition, transmitted as an autosomal dominant trait. It is known that mutations in the BRCA1 or BRCA2 genes are associated with a high risk of breast or/and ovarian cancer. Women with BRCA1 and BRCA2 mutations have a 65-85% cumulative lifetime risk of developing invasive breast cancer and a 15-65% cumulative lifetime risk of developing

invasive ovarian cancer (7-10). Other genes besides BRCA1 and BRCA2 related to breast cancer susceptibility include the 'guardian of the genome' TP53, and genes that encode proteins in p53-DNA repair-pathways, the PTEN (phosphatase and tensin homolog-mutated in multiple advanced cancers 1) gene, and the CHEK2 (protein kinase CHK2 isoform c) gene (10-12).

Other genes mutated in breast cancer patients include the ATM (ataxia telangiectasia mutated) gene, XPD (ERCC2, excision repair cross-complementing rodent repair deficiency, complementation group 2 protein) and HER-2 (human epidermal growth factor receptor 2) gene (13,14). Susceptibility alleles have been located in the genes *PALB2* (15), *STK11/LKB1*, and *MSH2/MLH1* (1). Recently, genome-wide single nucleotide polymorphism (SNP) association studies revealed genetic risk variants independent of family history in loci of: *FGFR2*, *TNRC9*, *MAP3K1*, *LSP1*, *2q35*, *5p12*, *8q24*, followed by *CASP8*, *FGFR2*, *TNRC9*, *MAP3K1*, *LSP1*, and *rs13387042* on *2q35*, *rs313281615* on *8q24*, and *rs10941679* on *5p12* for individuals of European ancestry (1,16,17). It is likely that additional genetic loci contribute to breast cancer susceptibility. In this regard, mitochondrial genetics may be important in carcinogenesis (18,19) and is an important area in oncology research (20-22). Breast nipple aspirate fluid (NAF) with mtDNA mutations at positions 204, 207 and 16293 has been associated with breast cancer (23) and mtDNA D-loop mutations have been proposed as an independent prognostic marker in breast cancer (24).

At least 50% of women will receive a false-positive result after having annual screening mammograms for a decade, and almost 20% of women will undergo a biopsy for that reason. Also, nearly 25% of women will have a false-positive test at some point during 10 years of regular clinical breast examinations (25). Data from The National Cancer Institute (NCI) puts the false negative rate of mammography as high as 40% for women aged 40-49 and 10% in women over 50. Breast tissue is more dense among younger women, making it more difficult to detect tumors. For this reason, false negatives are twice as likely to occur in premenopausal mammograms (26). Ultrasound is not used for routine breast cancer screening because it does not consistently detect certain early signs of cancer such as microcalcifications, but mammography alone has a cancer detection rate of 7.6 women per 1,000 women screened and adding an ultrasound increases the rate to 11.8 women per 1,000 women screened, an increase of 28% (27). Several risk assessment tools, such as BRCAPRO, the Claus model, and the Tyrer-Cuzick model, are available to help health professionals estimate a woman's breast cancer risk. These tools give approximate, rather than precise, estimates of breast cancer risk based on different combinations of risk factors (28). Because studies of the etiology of breast cancer have failed to identify feasible primary prevention strategies suitable for use in the general population, reducing mortality from breast cancer through early detection has become a high priority. The potential for reducing death rates from breast cancer is contingent upon increasing mammography screening rates and subsequently detecting the disease at an early stage, when more treatment options are available and survival rates are higher. Therefore, additional accurate genetic markers of increased risk would be valuable (29).

There is an association between somatic mtDNA mutations and cancer development, progression and metastasis (18,21,30-37). In addition, recent studies have shown that inherited polymorphisms or mutations of the mitochondrial genome may modulate the risk of developing cancer, including prostate, oral and colorectal cancers (18,38-41). Somatic mtDNA mutations have been described in various types of human cancers including bladder, brain, breast, colon, head and neck, lung, ovaries, prostate, thyroid, and breast cancers (20,21,31,33). At the same time inherited polymorphisms have been identified as contributing factors in cancer development (35,39,40).

Our current research builds upon other studies indicating that mtDNA mutations may be more powerful in detecting tumor cells in bodily fluids and cytological specimens than mutations found in nuclear DNA (34,41). Fliss and colleagues (42) have reported facile detection of mtDNA mutations in diagnostic samples, reliably detecting known mutations of mtDNA against the background of normal mtDNAs present in diagnostic specimens.

In the present study, we examined the genetic alterations in the D-loop region of mtDNA in primary human breast cancer tissues and paired control tissues. We have also investigated the distribution of haplogroups in the patient cohort, in an effort to address the significance of mtDNA alterations and inherited polymorphisms in breast tumorigenesis. In order to test for association with cancer susceptibility and frequency of mtDNA variants in BC patients we analysed the frequency of mtDNA polymorphisms and compared it with the frequency of those polymorphisms in healthy controls, centenarians and the general Polish (43) and European (44,45) populations.

Materials and methods

Patients. We studied 44 breast cancer (BC) patients who had undergone surgery (total mastectomy) at the Monument Institute of Polish Mothers Health Center, Lodz, Poland. Adjacent benign tissue was available in all cases. The patients received no chemotherapy, radiotherapy, or hormonal therapy before surgery. Histological analysis was based on the standard AJCC classification (46). For each specimen we determined histological type and grade, depth of infiltration, estrogen receptor (ER), progesterone receptor (PR) expression and the presence or absence of metastases in the lymph nodes.

The cancers were predominantly stage T1 including 5 pT1b patients, and 19 pT1c patients. Eighteen women were diagnosed with stage pT2 disease. Twenty-three patients were lymph node negative while 20 were node positive (all pN1) and one patient was Nx. Estrogen receptor (ER) and progesterone receptor (PR) staining were as follows: ER(0), 16; ER(+), 17; ER(++), 2; ER(+++), 9; PR(0),15, PR(+), 16; PR(++), 3; PR(+++), 10. The mean age of the breast cancer population was 57, median age was 58.

The healthy cohort (control group 1) of 100 individuals was recruited at the Department of Genetics and Pathology, International Hereditary Cancer Center, Pomeranian Medical University. The healthy cohort consisted of healthy females with a negative family history of cancer. The females were part of a population-based study of 1.5 million Polish residents designed to identify familial aggregations of cancer by the

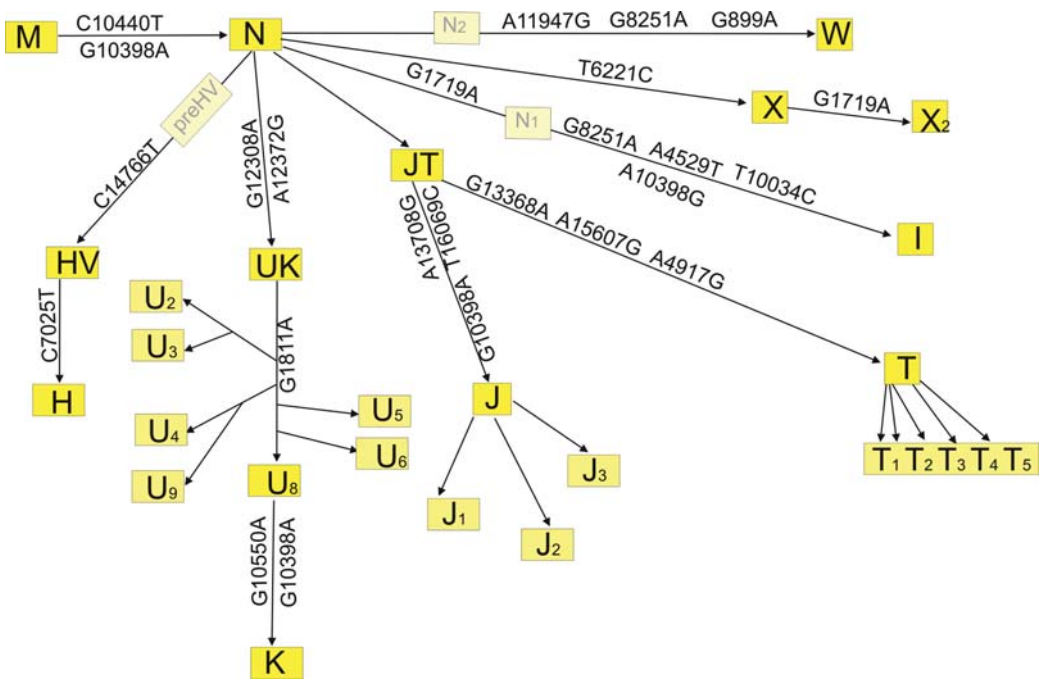

Figure 1. Haplogroup assignement pattern.

International Hereditary Cancer Center. These controls were interviewed in 2007. Women affected with any malignancy, or with any cancers diagnosed in a first-degree relative were excluded from this control group. The mean and median age of this healthy, breast cancer-free population was 55 years. Thus, the healthy control group is both free of breast cancer and unlikely to have inherited a genetic predisposition to the development of breast cancer.

One hundred and twenty-six centenarians (control group 2) with negative cancer history and negative family history of cancer were recruited from Polish Centenarians project of the International Institute of Molecular and Cell Biology in Warsaw (47). The control and general Polish populations have been described previously (43). All three populations share the same ethnicity, nationality, parentage, ancestry and currently reside in Poland. This study did not include any patients of Asian, African or Jewish origin.

The project was approved by the local Ethics Committee at the Medical University of Warsaw, Warsaw, Poland (KB-0/6/2007 to AMC). The Centenarians database was registered at the Bureau of the Inspector General for the Protection of Personal Data in May 1999. An agreement from the Bioethical Committee at the Central Clinical Hospital of the Military Medical Academy in Warsaw was granted for the Polish Centenarians Program. The centenarians or their relatives signed an informed consent for participation in the project (47). The Hereditary Cancer Registry at the Pomeranian Medical University in Szczecin, Poland contains clinical and epidemiological data collected since 1997, and was approved by the Ethics Committee of Pomeranian Medical University in Szczecin, Poland and all participants gave informed consent prior to enrolling in the study. All women received genetic counseling prior to and at the provision of their test results (48).

Tissue collection and DNA isolation. Tissue obtained at surgery was sub-divided into two parts. One portion was fixed in buffered formalin (pH 7.4) for routine histopathological assessment, while the rest was immediately frozen in liquid nitrogen and stored at -80˚C. Non-neoplastic material was also collected at surgery and stored at -80˚C until assayed.

DNA isolation. Cancer and matched normal tissue DNA was isolated using a PureGene DNA Purification Kit (Gentra Systems) according to the manufacturer's specifications. Control group DNA was isolated using phenol/chloroform extraction and ethanol precipitation from 10 ml blood samples. Centenarians' DNA was isolated from lymphoblast cell culture with NucleoSpin Blood L (Machery-Nagel) according to the manufacturer's specifications. We have previously demonstrated that haplotype-defining SNPs may be determined on either blood or tissue DNA without changing haplotype assignment (49).

Polymorphism analysis. The mtDNA in normal tissue of individual patients was used to assign that individual's mitochondrial haplotype when different from the reference sequence and that difference was defined as a germline (inherited) polymorphism. The revised Cambridge sequence (CRS) (50) of the relevant position, in each case, is shown for reference in Table I.

Haplogroup analysis by RLFP. Common polymorphisms in mtDNA determine classes of related genotypes, referred to as haplogroups. These were detected by restriction fragment length polymorphism (RFLP) analysis. Haplogroup RLFP analysis, restriction enzymes and primers used are summarized in Fig. 1 and Table I.

Table I. RFLP pattern used to establish haplogroups.

	Haplogroup									
Restriction site	H	I	J	K	T	U	V	W	X	M
-1715 Dde I		+							+	
-1806 Psi I	+					+	+			
+4220 Nla III	+		+		+					
-4529 Hae II		+								
-4577 Nla III							+			
-4915 Bfa I		+			+					
+6230 Mnl I					+				+	
-7015 Alu I	+									
+8249 Ava II		+						+		
-8994 Hae III								+		
-9052 Hae II				+						
+10028 Alu I		+								
+10394 Dde I		+	+	+						+
+10397 Alu I										+
+10497 Nla III				+						
+11949 BsF I		+						+		
+12308 Hinf I				+		+				
+13366 Bam HI					+					
-13704 Bst NI			+							
-14766 Mse I	+						+			
+15606 Alu I					+					
-15606 Alu I										
+15904 Mse I							+			
-15925 Msp I					+					
-16065 Hinf I			+							
+16389 Bam HI		+								

Haplogroup analysis by multiplex-PCR/sequencing. To verify haplogroups established by RFLP (as in Table I) a multiplex-PCR/primer extension analysis as described previously was performed (51). Moreover, haplogroups were also assigned based on specific D-loop polymorphisms according to concensus data (52-54). If any non-specific RFLP or multiplex-PCR/primer extension variants were found, sequencing with appropriate primers listed in Table I was performed in order to unambiguously determine the polymorphism.

PCR amplification of a D-loop segment of mtDNA. MtDNA fragments (15587-964) spanning the D-loop region (spanning nucleotide 16024 to 576) were amplified using eight pairs of primers. The primer pairs used and the sizes of the amplified products are shown in Table II.

Fifty-microlitre reactions contained 10 ng DNA and 0.5 μM primers, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 1 U of FIREPol® DNA Polymerase (Solis BioDyne, Estonia) or *Pfu* DNA Polymerase (Fermentas AB, Lithuania) and 2.5 mM $MgCl_2$. DNA was subjected to the following cycling conditions: initial denaturing at 95˚C for 3 min followed by 94˚C for 1 min, 55˚C for 30 sec, and 72˚C for 1 min for 40 cycles and final extension step at 72˚C for 7 min. Two microlitres of PCR products were analysed on an ethidium bromide-stained, 3% agarose gel (40 min at 70 V) to demonstrate the presence of the amplification product and for its quantification.

mtDNA sequence analysis. Sequence analysis was performed by: FinchTV Version 1.4.0 (Geospiza Inc., USA) and BioEdit version 7.0.5.3 (Copyright Tom Hall 1999-2007), contig assembly was performed with Sequencher 4.1.4 (Gene Codes Corp., Ann Arbor, MI USA) and multiple sequence alignment was performed with Clustal W (55). Normal and cancer tissue mtDNA sequences were compared with the revised Cambridge Reference Sequence (CRS) and sequence variants were recorded (50,56).

Statistical analysis. Two tailed non-directional Fisher-Irwin (Fisher's exact test) was used for statistical analysis (57). Statistical analysis was performed with PAST-PAlaeontological STatistics, ver. 1.34 and Analyse-it for Microsoft Excel General and Clinical Laboratory modules Version 1.73 [Analyse-it Software, Ltd., Copyright© 1997-2005].

Table II. Sequences of primers used for mtDNA D-loop sequencing (listed according to start position in mtDNA).

Primer	Primer sequence (5'-3')	Amplified mtDNA region (CRS)
108F	AGCACCCTATGTCGCAGTATC	108-638
276R	TCTGTGTGGAAAGTGGCTGTG	16344-276
315F	CGCTTCTGGCCACAGCAC	315-803
548F	CCAACCAAACCCCAAAGAC	548-964
559R	GGGTTTGGTTGGTCCGGG	16495-559
638R	GGTGATGTGAGCCCGTCTAAAC	108-638
803R	GGTGTGGCTAGGCTAAGC	315-803
964R	GGGAGGGGGTGATCTAAAAC	548-964
15587F	CTCCGATCCGTCCCTAACAAAC	15587-16185
15879F	AATGGGCCTGTCCTTGTAG	15879-16545
16098F	ACATTACTGCCAGCCACCATG	16098-16456
16185R	GGTTTTGATGTGGATTGGGT	15587-16185
16344F	CAGTCAAATCCCTTCTCGTCCC	16344-276
16456R	CCGGAGCGAGGAGAGTAGC	16098-16456
16495F	CGACATCTGGTTCCTACTTC	16495-559
16545R	AACGTGTGGGCTATTTAGGC	15879-16545

The difference was considered statistically significant at $p<0.05$. In selected cases, to confirm the result of Fisher's test, Yates's χ and uncorrected χ^2 test ('*N*-1' χ^2 test) were used as expected to give relatively low type I error in case of a small research cohort. The statistics were performed according to published statistical standards (57). To further understand the significance of specific polymorphisms as factors for favorable outcomes (odds ratio, relative risk, difference in proportions, absolute and relative reduction in risk) and of the effectiveness of a diagnostic criterium (number needed to diagnose, specificity, positive and negative predictive values, positive and negative likelihood ratios, diagnostic and error odds ratios) additional analysis was performed. The parameters, as well as the confidence intervals for the estimated parameters are computed by using standard definitions and methods (59,60).

Results

In order to test the hypothesis that the inherited mitochondrial genotype of women with breast cancer is different from controls, several comparisons were made. The standard 9-haplogroup RFLP analysis of the patients with breast cancer was compared to a carefully selected breast cancer-free control group of 100 (control group 1), 101 centenarians (control group 2) and the general Polish population (described previously). To further determine whether the inherited SNP profile was different than population controls, we performed D-loop sequencing and compared the results to the frequencies found in the on line searchable database mtDB.

Haplogroup distributions differs breast cancer patients. Altogether, our analysis of 44 breast cancer (BC) patients has shown that 16 (36%) belong to haplogroup H, 6 (14%) to haplogroup I, 5 (11%) to haplogroups K and V, 3 (7%) to haplogroup T, 2 (5%) to haplogroups J, U and W, 1 (2%) to X. In 2 (4%) cases, no specific haplogroup was assessed due to non-specific polymorphisms in haplogroup-defining positions (Fig. 2). Control group 1 (healthy cancer-free woman) were 50% H, 6% I, 13% J, 6% K, 8% T, 0% U, 15% V, 1% W and 1% X. Statistical analysis revealed also that haplogroup distributions of the BC cohort and general Polish population is different (Table III; Fig. 1). Most striking is the over-representation of haplogroup I individuals among BC patients (14 vs. 3%; p=0.017) in comparison to the general Polish population. The significance of the cancer specific haplogroup distribution is even more pronounced if the cancer cohort is compared to a large Polish cohort (n=436) haplogroup distribution, 14 vs. 2%; (p=0.001) for haplogroup I (61). Nevertheless, this haplogroup is characterized by many RFLP-markers and no single mtDNA position may serve as a cancer marker. Therefore, it is possible that the interactions of multiple mitochondrial DNA variant modify breast cancer risk in the case of haplogroup I (62), possibly with special role of A10398G polymorphism (35,37). Other haplogroups seem to be represented in the cancer population at a frequency similar to the general Polish population.

At the same time BC cohort individuals if compared to centenarians carry haplogroup H less frequently (38 vs. 58%; p=0.019). Since polymorphism T7028C defines haplogroup H (7028C), therefore, it seems that 7028C variant may be classified as protective against cancer development. It is for the reason that centenarians carry the 7028C 58% of the time while in the breast cancer group patients inherited the 7028C SNP 36% of the time (43). This difference is statistically significant p=0.019 with Fisher's exact test, which was confirmed both by Yates corrected χ^2, 5.118 and p=0.024 and 'N-1' χ^2, 'N-1' χ^2, 5.93, and p=0.01. These tests have been used as expected to give relatively low type I error.

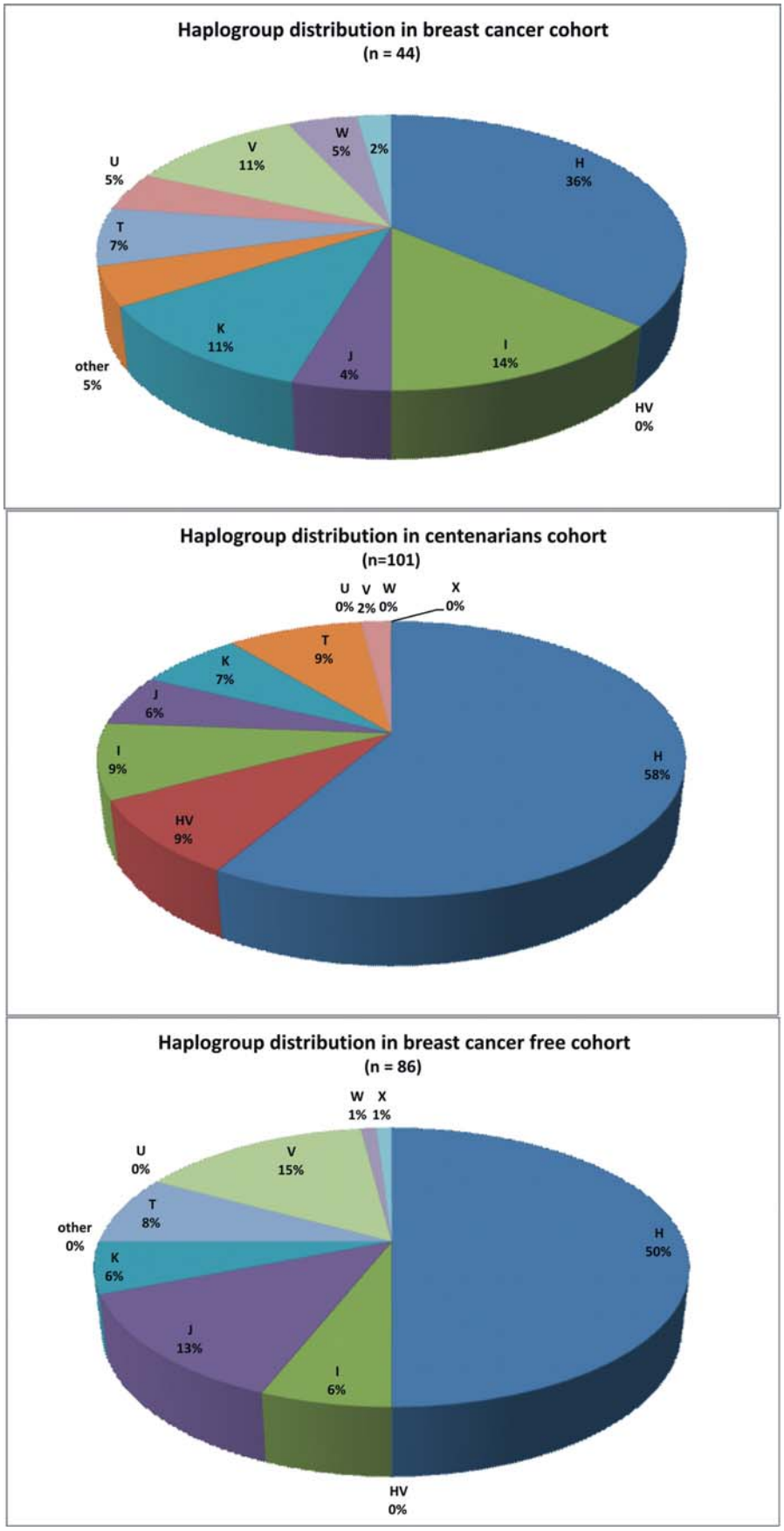

Figure 2. Haplogroup distribution in breast cancer, centenarians and healthy woman cohorts.

The statistics was performed as previously suggested (57). Moreover, in centenarians 7028C is found at relative risk RR=1.31 (95% CI: 1.0473<R.R.<1.6414), which is 31% more often than in the cancer cohort. This gives odds ratio

Table III. Germ-line polymorphisms in the D-loop region of mtDNA of the breast cancer patients.

mtDNA position (CRS)	Polymorphism	No	A/G/C/T/del frequency (mtDB)	Tissues where sequence was found (MITOMAP)	P-value	Region/population where sequence variant predominantly was found
64	T→C	1	0/0/**1838**/27	Aging brains	0.482	Africa, Japan, Autralia
73	A→G	16	309/**1555**/1/0	Aging brains, POLG/PEO and control muscle, thyroid tumor, oral cancer	**0.001 U**	Very common
146	T→C	2	1/0/**190**/1674	Elderly fibroblasts, aging/AD brains, POLG/PEO and control muscle, prostate tumor, ovarian cancer	0.310	Africa, Japan, Taiwan, Finland Italy, Spain, Algerian Jew, India, Polynesia, Caucasian
150	C→T	2	0/2/**1616**/247	Elderly fibroblasts/leukocytes, lung tumor, thyroid tumor	**0.001 U**	China, Japan, Berbers, Italy
152	T→C	4	0/0/**396**/1469	Aging brains, elderly fibroblasts, ovarian cancer, oral cancer	0.059	Africa, China, Japan, American, Finland, Italy
153	A→G	1	1840/**25**/0/0 Top of Form Bottom of From	Polymorphism	0.457	Japan, Finland, Italy
189	A→G	2	1782/**75**/8/0	Elderly muscle, POLG/PEO muscle and fibroblasts, aging brains, prostate tumor	0.697	Japan, Finland, India
195	T→C	4	11/0/**280**/1574	Elderly fibroblasts, lung-cancer cells, aging/AD brains, thyroid tumor, oral cancer	0.301	Africa, Japan, American, Finland, Italy, Caucasian
199	T→C	1	0/0/**121**/1744	Ovarian cancer, POLG/MNGIE muscle	0.361	China, Japan, Finland, India
204	T→C	3	0/0/**123**/1741	Oral cancer, prostate tumor	0.765	Japan, Finland, India
207	G→A	3	**123**/1741/0/0	Oral cancer, prostate tumor, thyroid tumor	0.765	Japan, Finland, India
225	G→A	1	**10**/1855/0/0	Polymorphism	0.227	
226	T→C	1	0/0/**21**/1863	Oral cancer	0.400	
228	G→A	1	**57**/1805/0/3	Polymorphism	1.00	American, Finland, India, Caucasian
239	T→C	3	0/0/**4**/1861	POLG/PEO muscle, ovarian tumor, oral cancer	**0.001 O**	Extremely rare, Italy
242	C→T	1	0/0/1854/**11**	POLG/PEO muscle	0.245	Extremely rare, American, Finland
250	T→C	1	0/0/**17**/1848	Oral cancer	0.344	Africa, Japan, Taiwan, Finland, Italy, Spain, Algerian Jew, India, Polynesia, Caucasian
263	A→G	32	6/**1861**/0/0	POLG/MNGIE muscle, oral cancer	**0.001 O**	Africa, Japan, China, Australia, American, Finland, India
295	C→T	2	4/0/1788/**75**	POLG/MNGIE muscle	**0.697**	American, Finland, India, Caucasian
303	C7→C8 (ins)	15	Nn	Multiple tumor types	-	Africa, Japan, Taiwan, Finland, Italy, Spain, India, Polynesia, Caucasian, Ashkenazi Jew, American, Australia
310 (ins)	C5→C6	32	Nn	Multiple tumor types	-	Africa, Japan, Taiwan, Finland, Italy, Spain, India, Polynesia, Caucasian, Ashkenazi Jew, American, Australia
462	C→T	1	0/0/2073/**71**	Thyroid tumor	1.00	American, Finland, India, Caucasian

Table III. Continued.

mtDNA position (CRS)	Polymorphism	No	A/G/C/T/del frequency (mtDB)	Tissues where sequence was found (MITOMAP)	P-value	Region/population where sequence variant predominantly was found
477	T→C	1	0/1/**19**/2124	AD brains, ovarian tumor	0.335	Africa, China, Japan, American, Finland, Italy, India
513	A→AA (ins)	4	Nn	-	-	Nn
514	delC	5	Nn	Head/neck tumor, ovarian cancer and control tissue, thyroid, prostate and breast tumors		Nn
515	delA	3	Nn	-	-	Nn
16111	C→T	1	1/0/1830/**36**	Colonic crypts somatic mutations, oral cancer	0.581	Japan
16126	T→C	3	0/0/**166**/1701	Oral cancer	1.00	China, Japan, American, Finland, Italy, India
16136	T→C	1	0/0/**24**/1843	Polymorphism	0.443	Very rare Japan, Melanesia
16172	T→C	1	0/0/**150**/1717	MNGIE tissues, head/neck tumor back-mutation, oral cancer	0.253	Japan, Morocco, Finland, Italy
16183	T→C	1	1541/12/**237**/ 0/77	Lung tumor back-mutation, prostate tumor	**0.036 U**	China, Japan, Finland, Italy, India
16189	T→C	4	0/0/**522**/1345	Prostate tumor	**0.004 U**	Africa, Japan, India, Italy, Finland
16192	C→T	3	0/0/1808/**59**	Oral cancer	0.169	Rare, Japan, Finland, Italy
16207	C→T	1	1863/4/0/**0**	Polymorphism	**0.023 O**	Nn
16222	C→T	1	0/0/1852/**15**	Polymorphism	0.312	Maroco, India, America
16223	C→T	2	0/0/992/**875**	Oral cancer	**0.001 U**	Africa, Japan, China, Australia, India, Finland, Ashkenazi Jews
16224	T→C	2	0/0/**107**/1760	Oral cancer	1.00	Rare, Japan, American, Finland, Ashkenazi Jews
16230	A→G	1	1854/**13**/0/0	Polymorphism	0.279	Extremely rare, South Asia, Africa
16235	A→G	2	1846/**21**/0/0	Oral cancer	0.097	Extremely rare, Africa, Italy
16249	T→C	1	0/0/**87**/1780	Prostate tumor	0.720	Rare, Africa, Japan, Italy
16256	C→T	2	0/0/1838/**29**	Oral cancer	0.159	Very rare, Asia, Finland, India
16261	C→T	2	0/0/1756/**111**	Oral cancer	1.00	Japan, Taiwan, Finland, India
16269	A→G	1	1855/**12**/0/0	Polymorphism	0.262	Extremely rare, Japan, Italy
16270	C→T	3	0/0/1802/**65**	Oral cancer	0.204	Very rare, Finland, Italy, India
16278	C→T	1	0/0/1724/**143**	Oral cancer	0.251	Rare, Africa, Japan, Italy, India
16291	C→T	1	0/3/1816/**48**	Polymorphism	1.00	Very rare, Asia, Italy, Australia
16292	C→T	2	0/2/1801/**64**	Breast, ovarian, head/neck tumor, oral tumor	0.663	Very rare, Japan, Finland, Italy
16293	A→G	1	1848/**17**/2/0	Glioblastoma	0.344	Extremely rare, Africa, Italy, India
16294	C→T	2	0/0/1760/**107**	Oral cancer	1.00	Rare, Japan, India, Finland, American
16296	C→T	1	0/0/1823/**44**	Polymorphism	1.00	Very rare, Japan, American, Finland
16304	T→C	1	0/0/**140**/1727	Esophageal, breast and prostate tumors, oral cancer	0.252	Asia, America, Italy
16311	T→C	6	0/0/**340**/1526	Oral cancer	0.554	Africa, Japan, China, American, Finland, Italy, India

Table III. Continued.

mtDNA position (CRS)	Polymorphism	No	A/G/C/T/del frequency (mtDB)	Tissues where sequence was found (MITOMAP)	P-value	Region/population where sequence variant predominantly was found
16325	T→C	2	0/0/**47**/1820	Polymorphism	0.312	Very rare, Asia, Native American, South America
16327	C→T	1	0/0/1843/**24**	Oral cancer	0.443	Extremely rare, Africa, Asia
16343	A→G	2	1852/**14**/1/0	Polymorphism	0.051	Extremely rare, Africa, Melanesia, Polynesia
16354	C→T	1	0/0/1862/**5**	Polymorphism	0.131	Extremely rare, Melanesia, China, Finland
16362	T→C	1	1/0/**444**/1422	Oral cancer	**0.001 U**	Africa, China, Japan, Italy
16390	G→A	1	**62**/1805/0/0	Breast, ovarian tumor, oral cancer	1.00	Very rare, Africa, Asia, Ashkenazi Jew
16391	G→A	1	**17**/1850/0/0	Oral cancer, ovarian tumor	0.344	Extremely rare, Finland
16482	A→G	1	1864/**3**/0/0	Polymorphism	0.089	Extremely rare, Italy
16519	T→C	16	0/0/**1115**/752	Oral cancer, gastric, lung, ovarian tumor	**0.003 U**	Africa, Japan, Caucasian, China, American, Finland, Italy, India
16526	G→A	2	**19**/1848/0/0	Polymorphism	0.083	Extremely rare, Asia, Finland

U, polymorphism under-represented in breast cancer population; O, polymorphism over-represented in breast cancer population.

OR=2.4583, which means that a 7028C carrier had a 2.45 higher chance not to develop cancer with specificity 0.636 (0.513-0.747). The cancer-free status positive predictive value (precision rate PPV) of 7025C test is 0.787 (0.714-0.851 at 95% CI), posterior probability (odds): 79%, with relative risk reduction RRR=-0.311 and number needed to diagnose NND=4.53.

Inherited haplogroup-related polymorphisms analyzed shown no correlation with TNM or clinical stage. At this time it is probably the effect of lack of effective population-vide screening annual programs. TNM/stage at detection is more dependent on public awareness of early detection's, access to detection and diagnostic centers and organization of screening programs in place which seem not to be satisfactory (63).

D-loop polymorphisms: C-tract insertions in breast cancer patients. There were two common insertion polymorphisms seen in breast cancer patients. The first is the insertion of a single cytosine (C) in the poly-C tract spanning nucleotide positions 310-315. The reference sequence contains a run of 5 consecutive Cs, while the expansion makes this a run of 6 Cs. We found this 5C-6C insertion event in 32 breast cancer patients. The second similar polymorphism is also a single C insertion, but in the 7C run between nucleotide positions 303-310. We found this 7C-8C variant in 15 patients. Of these 15 patients, 14 also had the single C insertion in the 310-315 tract, while a single patient had the C insertion at the 303-310 position without the corresponding insertion in the 310-315 position. In other words, the insertion of one C in the 303-315 tract is almost always accompanied by a second insertion in the 303-310 tract. Of the 32 patients with the 310-315 insertion, 14 also had the insertion in the 303-310 tract. Thus, an insertion is the 310-315 tract is accompanied by a second insertion in the 303-310 tract 44% of the time.

D-loop SNPs specific found for breast cancer. As outlined in the methods section, 1120 bases of the D-loop were sequenced in all breast cancer patients (spanning nucleotide positions 16024 to 576) and differences from the reference Cambridge sequence were identified and their frequency compared to the frequency in the online searchable database mtDB. A complete list of all changes and their statistical analysis is listed in Table III. There were only a small number of SNPs that reached statistical significance. These included 7 SNPs that were seen at significantly lower frequency in breast cancer cases compared to the diverse population represented in mtDB (A73G, C150T, T16183C, T16189C, C16223T, T16362C, T16519C) and 3 that were over-represented in the breast cancer patient group (T239C, A263G, and C16207T).

Discussion

The fact that family history is a risk factor for breast cancer suggests possible inherited genetic susceptibility. There is broad agreement that there are many genes involved in the process of breast carcinogenesis and that risk for breast cancer increases with each relevant genetic alteration present. In particular, breast cancer development is associated with

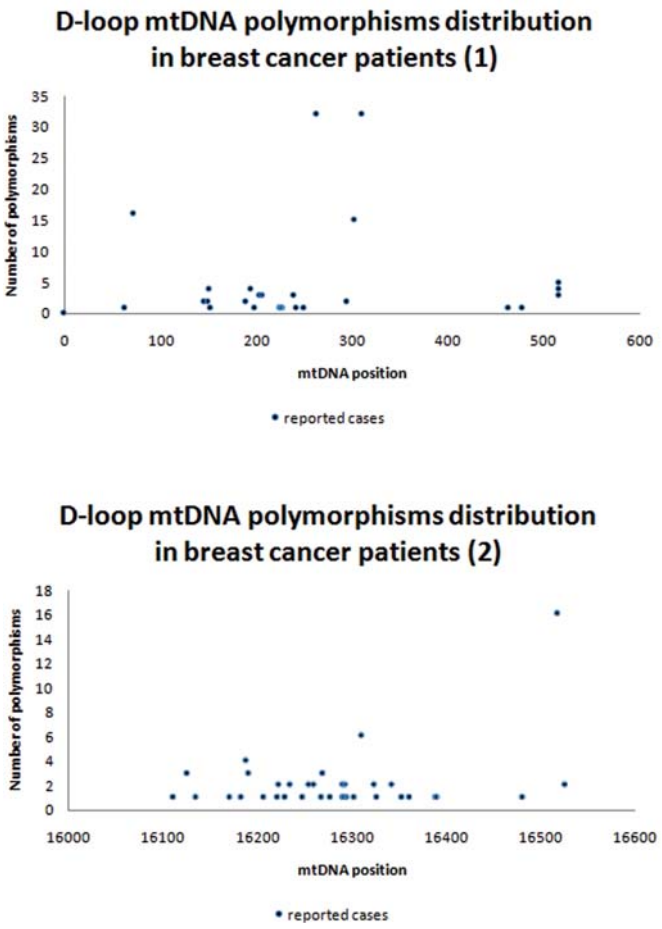

Figure 3. mtDNA D-loop polymorphisms distribution pattern in breast cancer cohort.

autosomal dominant inheritance of mutated BRCA1 or BRCA2 genes. Despite the established role of several genes important in breast cancer, the list of genetic factors involved in this disease is still incomplete and more effort is needed to identify biomarkers and genetic alterations that would allow the selection of individuals with increased risk for intensive screening, prevention and treatment programs (1,10,17).

The use of mitochondrial DNA polymorphisms as a biomarker is rapidly expanding in metabolic diseases, aging, cancer, the analysis of human migration patterns, and human identification in forensic sciences (20-22). In breast cancer mtDNA analysis seems to be an attractive clinical target, as mtDNA mutations and/or polymorphisms can be detected in the material obtained from serum and WBC (64) and also in nipple aspirate fluid (23,65) and ductal lavage (66). So far, the prevalence of mtDNA mutations in human BCs has been analyzed mostly in patient cohorts recruited from China and Taiwan (24,67,68), India (69,70), or African-Americans (71,72) but no large-scale research included Europeans or Caucasians (21,33,37).

Tumor-associated mitochondrial genomic instability is seen in breast cancer patients and the presence of one or more somatic point mutations is found in 29% (73) to 93% of patients (74). The mononucleotide repeat (D310) appeared as a mutation 'hot-spot' in primary tumors (21,31,33). In sporadic BC the inherited 10398G polymorphism was associated with susceptibility to BC (35,75). In familial breast cancer inheritance of G9055A, A10398G, T16519C polymorphisms were shown to increase breast cancer risk, while T3197C and G13708A decreased breast cancer development risk (76). Other patient populations were shown to carry different patterns of mtDNA mutations including changes not only in the D-loop, but also ND5, ND1, ND4, CYTB (77) or ATPase8, ATPase6, COII, ND5 (24) genes. To date, no correlation between the pattern of mitochondrial abnormalities and clinical or histopathological features has been found (73).

Our analysis of the D-loop revealed no known pathogenic mtDNA mutations but multiple polymorphisms, including microsatellite instability in the 'C-tract' (between nucleotides 303 and 315) in 32 (73%) of BC patients. We did not detect any somatic D-loop mtDNA mutations which is different from what some others have reported (23,73). We believe that this is a result of the stringent quality control we used (34,78), similar to the mitochondrial breast cancer project by Wang *et al*, who also reported no somatic mutations in the patient cohort (79). As previously pointed out by Salas *et al* (80) mtDNA sequencing and analysis techniques contain inherent problems, particularly with regard to the generation of authentic and useful data. It is important to employ standardized procedures based on scientific grounds, in order to have mtDNA-based evidence that can be accepted in clinical practice. Therefore, we have put much effort into the refinement of our amplification and sequencing strategies, as well as quality control of mtDNA sequencing (34,35,81). We believe that careful analysis of possible technical errors at any stage of the analysis, DNA extraction, amplification, mutation screening, mtDNA sequencing, and patient documentation may prevent misinterpretation and we have put much effort in quality control (82). To further increase specificity of our mutation screen, we took advantage of detailed multiple-database analysis, as it has proven to be an effective and successful method of data evaluation for mtDNA (78,80,83). Our summary polymorphism table should be treated and interpreted judiciously, as previously reported cancer mutations may have arisen from technical errors in previous projects (83,84).

Multiple interesting mtDNA polymorphisms and mutations exist in prostate (35) thyroid (92) and oral cancers (39). Thus, others have hypothesized that patients bearing some of the common mitochondrial polymorphisms are at higher risk of cancer development. Also some of the mtDNA variants reported as possible tumor-specific somatic mtDNA mutations are actually common in the general population and proposed therefore a paradigm has been proposed for evaluation of sequence quality and for detection of potential problems with inferring a pathogenic status of a particular mutation and to avoid false positive results (20,22,87). At the same time, some mtDNA mutations and polymorphisms may develop before cell transformation and may be found in precancerous tissues (88) including tumor margins, as it is in the case of TP53 mutations in lung cancer (89). The possibility of pre-cancerous mtDNA variation should also be considered in experiment planning and data interpretation to avoid false negative results (34).

Because the complement of germline polymorphisms in cancer patients and controls are different, it is possible that the inheritance of specific mitochondrial genotypes predisposes individuals to cancer (90,91). Moreover, the co-occurrence of multiple inherited mtDNA SNPs may influence the disease phenotype as in the case of 12308G and 10398G in BC (62) or G10680A and T14484C in Leber hereditary optic neuropathy (LHON) (92). One polymorphism may have a potential modifier role in increasing the penetrance and expressivity of other mtDNA genome alternations. In particular, the mitochondrial haplotypes may play a synergistic role in the development of cancer as is the case of vision loss in the families carrying the LHON-associated primary mtDNA mutations. In LHON patients the mitochondrial haplotype has been shown to influence the clinical expression (93). Therefore, our project covers haplogroup related polymorphisms as BC biomarkers. Our strictly European population is exclusively distributed among the nine haplogroups designated: H, I, J, K, T, U, V, W, and X, and no analysis of haplogroups characteristic for Asian or African populations was included (54). Our research was also informed by the fact that a significant increased risk in breast cancer development was reported for haplogroup K (76). We have also chosen to focus on haplogroup-specific polymorphisms as we and others have previously correlated A10398G polymorphism characteristic for haplogroup I, J, K with BC development (35,70,71,75,94). The BC cohort haplogroup distribution was different from the healthy Polish population and cancer-free centenarians with haplogroup H underrepresented in BC patients. We believe that this might suggest a protective role of haplogroup H in cancer development. Our hypothesis seems plausible as mtDNA haplogroup H is very common in Caucasoids, reaching frequencies of ~50%. Recent studies have suggested haplogroup H3 is highly protective against AIDS progression (95). Moreover, mtDNA haplogroup H is a strong independent predictor of outcome during severe sepsis (96).

Our polymorphism analysis also focused on the D-loop region of mtDNA, as it contains two hypervariable regions: HV1 (16024-16383) and HV2 (57-333) which were reported as somatic mutation 'hot spots' in many types of cancer including sequence length poly-C polymorphisms (21,97). Breast cancer patients with multiple mtDNA D-loop (CA) (n) polymorphisms have significantly poorer disease-free survival than those with one copy of the mtDNA D-loop (CA) (n) polymorphism (98). We also believe that mtDNA nucleotide variations, particularly those in the D310 segment, might be involved in the breast carcinogenesis and could be included in a panel of molecular biomarkers for cancer susceptibility or early-detection strategies. In particular, C16189T could be of interest as this mtDNA variant has been associated with susceptibility to dilated cardiomyopathy (DCM) (99), insulin resistance and development of diabetes mellitus (DM) in adult life and vascular pathologies involved in stroke (lacunar cerebral infarction) and cardiovascular diseases (100).

Precise understanding of the molecular mechanisms whereby specific mtDNA polymorphisms predispose to cancer is currently lacking. Although the mechanisms of generation and functional impact of mtDNA polymorphisms are still not clear, there is a high incidence and broad distribution in human cancers making them a potential marker for cancer detection, but the role of mtDNA polymorphisms in the maintenance of tumor cell phenotype or in tumorigenesis remains to be elucidated (21,22,31,33,101,102). Although it is generally believed that polymorphisms may have pathogenic potential, it remains to be established whether these polymorphisms contribute to neoplastic transformation by changing cellular energy capacities, increasing mitochondrial oxidative stress, or modulating apoptosis. The significance of each individual polymorphism for mitochondrial function and tumorigenesis is unknown and only functional analysis in relation to cell behavior, proliferation, and apoptosis can determine the significance of these polymorphisms and their association with tumorigenesis. We believe that polymorphisms in mtDNA, both in the D-loop and in the coding region (including haplogroup specific positions) may cause subtle differences in the encoded protein structure and function (40). Particular mtDNA genotypes may predispose an individual to an earlier onset of degenerative cellular processes, such as the accumulation of somatic mtDNA variation, decline in OXPHOS capacity or faster cancer progression, as shown in transmitochondrial cybrids (103). The mtDNA haplotype may also influence its further mutagenesis, as has been shown for haplogroup J that predisposes the mitochondrial genome to mutate at locus 14484, possibly through near-neighbor effects as previously described to occur during mtDNA evolution (104).

Finally, from a broader perspective we believe that cancer control efforts in the postgenome era should be focused at both population and individual levels to develop novel risk assessment and treatment strategies that will further reduce the morbidity and mortality associated with the disease. The discovery that mutations in particular genes increase the risk of breast cancer has radically transformed our understanding of the pathology of breast cancer. Moreover, a better understanding of tumor biology may lead to improvements in the multidisciplinary management of breast cancer, and its pathologic evaluation if routine clinical examination was enhanced with molecular analysis. A number of genetic biomarkers should be developed for routine use. The immediate challenge now is to learn how to use the molecular characteristics of an individual to improve early detection and ultimately to decrease breast cancer morbidity (1,18,34,37).

The inherited mitochondrial genotype of women with breast cancer differs from that of multiple comparison groups including a rigorously defined no-cancer control group, the general Polish population, and a group of Polish centenarians. This is documented by differences in the standard mitochondrial haplotype analysis (haplogroups) as well as SNPs in the highly variable D-loop. This finding suggests that inherited mtDNA variation may predispose to or protect from the development of breast cancer in women. We found no somatically acquired or tumor-specific mutations in the D-loop area of the mitochondrial genome, a finding that is consistent with at least one other report, but is at odds with other reports, perhaps due to our more stringent and redundant analysis of DNA sequence. The identification of specific SNPs that are over- and underrepresented in cancer patients suggests the possibility of their application

in identifying high risk groups for the clinical care of women, particularly in the areas of screening and detection, though the utility of these genetic markers has not been tested in this circumstance. Our results are similar to findings in other tumor types, especially prostate and renal cancers, where inherited mitochondrial genotype has been shown to vary significantly between individuals with cancer and no-cancer controls.

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