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Mitochondrial NADH-dehydrogenase polymorphisms as sporadic breast cancer risk factor

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Abstract. Breast cancer is the most frequently diagnosed female cancer all over the world. Although the molecular genetics of this disease has been the focus of many projects for over 20 years, the number of prognostic markers used in clinics is still unsatisfactory. Mitochondrial DNA mutations have been reported in many breast cancer studies. To investigate the possible role of mitochondrial inherited polymorphisms in breast cancer development we analyzed the sequence of NADH-dehydrogenase genes in cancer samples and their corresponding normal tissues. We detected increased incidence of mtDNA polymorphisms, in particular very rare polymorphisms such as A4727G, G9947A, A10044G, A10283G, T11233C, and C11503T. Our report supports the notion that mtDNA polymorphisms establish a specific genetic background for breast cancer development and that mtDNA analysis may help in selection of cohorts that should undergo intensive screening and early detection programs.

Introduction

An estimated 1,152,161 new breast cancer (BC) cases are diagnosed worldwide per year (1). Although much effort has been made, molecular studies of breast cancer pathology and etiology have so far failed to identify successful primary prevention strategies. The reduction of mortality from BC is now only possible thanks to early detection that must become the highest priority. The reduction of death rate from BC may result from increased mammography/sonography screening programs and subsequent detection of the disease at an early stage. Biomarkers and tests that indicate additional risk could help in the selection of populations at higher risk and should be used in BC (2). Today it seems possible that a molecular mtDNA-analysis-based approach may fulfill at least some of the BC biomarker requirements.

This interest in mitochondrial function in carcinogenesis was reported as early as in the 1920s, when Otto Warburg discovered that cancer cells have a high glycolytic rate and produce increased levels of lactate in the presence of oxygen. This discovery has opened a new field of research currently referred to as 'mitochondrial medicine' and 'mitochondrial oncology'. Multiple reports have found an association between somatic mtDNA mutations and cancer development, progression or metastasis and inherited mtDNA polymorphisms have been indicated as contributing factors in cancer development (3-5).

For the last few years in parallel to nuclear genome research, mtDNA has also been screened for mutations specific for breast cancer. Breast nipple aspirate fluid with mtDNA mutations at positions 204, 207 and 16293 has been suggested as an indicative for breast cancer (6) and mtDNA D-loop mutations have been proposed as independent prognostic marker in breast cancer (7). We have shown that individuals who inherited the A10298G polymorphism are at higher risk for developing BC (8). As A10398G is located in the ND3 (NADHd-dehydrogenase subunit 3) we have been interested if any other polymorphisms are abundant in ND genes in the BC cohort. Our interest was further increased as human mammary carcinoma cells were shown to have depressed expression levels of complex I (CI, NADH-ubiquinone oxidoreductase) genes (9). Also in prostate cancer ND6 transcript levels are significantly decreased in tumor samples when compared to their paired normal tissue (10). CI gene expression is also reduced in glioblastoma (11) and in photodynamic therapy-resistant colon carcinoma cells (12). At the same time benign renal oncocytomas were shown to be deficient in electron transport chain complex I proteins and to harbor point mutations in CI genes (13). Moreover,

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Table I. Primers used for sequencing mitochondrial NADH-dehydrogenase genes.

CRS no.	5'-primer-3'
3160F	AGCGCCTTCCCCCGTAAATGA
4630R	TCTGTGGAACGAGGGTTTAT
4021F	ACTACAATCTTCCTAGGAAC
5880R	TGAGTGAAGCATTGGACTGT
9641F	CTGAGCTCACCATAGTCTAA
10760R	TTGGAGTAGGTTTAGGTTAT
10221F	TTCTTCTTAGTAGCTATTAC
11190R	TTCAGGCGTTCTGGCTGGTT
10511F	CTCACTTCTAGGAATACTAG
12450R	GGATTTTACATAATGGGGGT
12241F	CATGTCTAACAACATGGCTT
14320R	TAATAGTGTAGGAAGCTGAA
8201F	TTCATGCCCATCGTCCTAGA
4980R	GAATGAGTAGGCTGATGGTT
8381F	ACTACCGTATGGCCCACCAT
9360R	GTGTGTTGGTTAGTAGGCCT

the HPLC study of ND4 mutations in transitional cell carcinomas has proven that most mito-chondrial mutations identified in tumors pre-exist in the heteroplasmic state in a minority of mtDNA molecules in the cell. These are too low in quantity to be detected by methods such as DNA sequencing. The authors suggested that mtDNA mutations occur before tumorigenesis and become apparent in cancer cells (14). Gasparre *et al* hypothesised that the clonal amplification of mtDNA with mutated CI genes in tumors demonstrates that these alterations are selected and therefore favor or trigger growth (13). In light of these data we believe that specific inherited mtDNA variants may influence the cancer development susceptibility of an individual in addition to nuclear DNA and environmental factors. In our opinion, such mitochondrial abnormalities as NADH-ubiquinone oxidoreductase polymorphisms may play a role in modifying an individual's risk to breast cancer (15) and should be included in the diagnostic algorithm for identification of individuals with hereditary predisposition to breast cancer along with BRCA pathway mutations and clinical parameters (16). In the present study, we examined the genetic alterations in the NADH-dehydrogenase region of mtDNA in primary human BCs and their paired control samples.

Materials and methods

Patients. The population of patients originated from our previous study (8). The project was approved by the local Ethics Committee at the Medical University of Warsaw, Warsaw, Poland (KB-0/6/2007 to AMC).

PCR amplification of D-loop segment of mtDNA. mtDNA fragment NADH dehydrogenase in particular regions of genes: ND1-NADH dehydrogenase subunit 1 (3307-4262), ND2-NADH dehydrogenase subunit 2 (4470-5511), ND3-NADH dehydrogenase subunit 3 (10059-10404), ND4L-NADH dehydrogenase subunit 4L (10470-10766), ND4-NADH dehydrogenase subunit 4 (10760-12137), ND5-NADH dehydrogenase subunit 5 (12337-14148), ND6-NADH dehydrogenase subunit 6 (14149-14673), were amplified. The primer pairs used are shown in Table I.

Fifty-microlitre reactions contained 10 ng DNA and 0.5 μM primers, 0.2 mM each of deoxynucleotide triphosphate (dNTP), 1 U of AmpliTaq Gold® DNA Polymerase 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 was 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 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 (17). Normal and cancer tissue mtDNA sequences were compared with the revised Cambridge Reference Sequence (CRS) and sequence variants were recorded.

Results

Breast cancer population of patients is characterized by 28 germ-line polymorphisms (Table II) that differentiate BC patients from haplogroup H (18,19), the most common haplogroup in Poland, and the haplogroup we have shown to be at lower risk for BC development. In particular, 68% (19) of discovered polymorphisms were very rare (20), while polymorphism T5082C has not been reported before (21). We have classified polymorphisms including A4727G, A4745G, T5082C, G9947A, A10044G, A10283G, T11233C, C11503T, A13722G as very rare. These have been reported before in <20 cases (20). The majority of polymorphisms described by us are synonymous and only four -G5046A, G5460A, G8557A and G13759A- polymorphisms change the amino acid sequence of the protein. Comparing cancer tissue and normal breast (control) tissue, we did not detect any somatic mtDNA mutations, point mutations, insertions or deletions.

Discussion

A number of studies have proven that mitochondrial DNA mutations are more powerful in detecting tumor cells in bodily fluids and cytological specimens than mutations in nuclear DNA (5) and we believe this can be applied to BC screening. Thus, we suggest that analysis of mtDNA polymorphisms and possibly also mutations may be useful to select a population at increased risk of cancer development.

Germ-line polymorphisms in the NADH-dehydrogenase mtDNA of the breast cancer patients.

mtDNA position (CRS)	Polymorphism	Case no.	No.	A/G/C/T/del frequency (mtDB)[a]	Tissues where sequence was found (MITOMAP)[a]	Amino acid change	Gene
3915	G>A	200	1	**22**/2682/0/0	Polymorphism	Syn	ND1
4727	A>G	200	1	2695/**9**/0/0	Polymorphism	Syn	ND2
4745	A>G	207	1	2689/**15**/0/0	Polymorphism	Syn	ND2
4769	A>G	200, 201, 202, 203, 204, 205, 206, 207, 208, 227, 230, 231	12	30/**2674**/0/0	Polymorphism	Syn	ND2
5004	T>C	206	1	0/0/**29**/2675	Pancreatic cancer	Syn	ND2
5046	G>A	231	1	**79**/2625/0/0	Polymorphism	**V-I**	ND2
5082	T>C	204	1	0/0/**0**/2704	Polymorphism	Syn	ND2
5426	T>C	230	1	0/0/**27**/2677	Polymorphism	Syn	ND2
5460	G>A	231	1	176/**2528**/0/0	Polymorphism	**A-T**	ND2
5656	A>G	207	1	2660/**44**/0/0	Polymorphism	NC	NC4
8269	G>A	206	1	37/**2667**/0/0	Polymorphism	NC	COII
8557	G>A	231	1	21/**2681**/2/0	Colonic crypts	ATP6:**A-T** ATP8:syn	ATP6/8
8860	A>G	200, 201, 203, 204, 205, 206, 207, 208, 227, 228, 230, 231	11	6/**2698**/0/0	rCRS rare pm, abdominal aortic aneurysm	T-A (consensus)	ATP6/8
9947	G>A	227	1	**13**/2691/0/0	Polymorphism	Syn	CO3
10034	T>C	205	1	0/0/**37**/2667	Polymorphism	NC	NC
10044	A>G	206	1	2688/**16**/0/0	Polymorphism	NC	NC
10238	T>C	205	1	0/0/**83**/2621	Polymorphism	Syn	ND3
10283	A>G	207	1	2699/**5**/0/0	Polymorphism	Syn	ND3
10589	G>A	200	1	**44**/2660/0/0	Polymorphism	Syn	ND4L
10876	A>G	229	1	2683/**21**/0/0	Polymorphism	Syn	ND4
11233	T>C	203	1	0/0/**3**/2701	Polymorphism	Syn	ND4
11467	A>G	201, 207,	2	2357/**347**/0/0	Oral tumor	Syn	ND4
11503	C>T	229, 230,	2	0/0/2703/**1**	Polymorphism	Syn	ND4
11719	G>A	201, 205, 207, 231	4	**2100**/604/0/0	Polymorphism	Syn	ND4
12308	A>G	201, 206, 229	3	2357/**347**/0/0	Haplogroup U marker renal and prostate cancer, CPEO, CM, stroke	NC	TL2
12372	G>A	201, 206	2	**390**/2314/0/0	Sporadic parathyroid adenoma, prostate tumor	Syn	ND5
13722	A>G	231	1	2691/**13**/0/0	Polymorphism		ND5
13759	G>A	229	1	**39**/2665/0/0	Polymorphism	A-T	ND5

[a]Unless indicated otherwise, the data are from MITOMAP (32) and mtDB (20) databases and the references therein. Syn, synonymic mutation; NC, non coding region.

Cancer control efforts in the postgenomic era should be focused at both population and individual levels to develop novel risk assessment strategies that will reduce the morbidity and mortality associated with breast cancer. Mitochondrial polymorphisms described by us as specific in BC population have been shown before as good candidates for identification purposes in criminal and forensic medicine. It has been proven that T5004C may be used to resolve identity of people with otherwise identical mitotype and A5656G may be used even in the case of samples with highly degraded DNA (22). Position 11719 in the MTND4 gene was described as a 'hot spot' for base substitutions and as a position suitable for identification purposes in Legal Medicine (23). At the same time some of our BC-associated polymorphisms have been described before as typical for particular haplogroups. This further supports the hypothesis that some haplogroups are preferential candidates for cancer development and that haplogroup specific polymorphisms are sequence variation hot-spots. The abundance of polymorphisms such as A5656G and G11719A in cancer population seem to favour this hypothesis. In fact A5656G was associated exclusively with mtDNA haplogroup U (24) and G11719A is a marker of non-HV haplogroups (25).

Few of the polymorphisms discovered by us have been analyzed before as disease-associated factors. In particular, 11719A is a marker of reduced sperm motility (25) and the allele A at 12308 in tRNA(Leu) was reported to increase the risk of oral cancer (26). The transition A10044G in the tRNA(Gly) gene was associated with sudden unexpected death in a family with severe encephalopathy (27). In addition, the missense mutation G5460A affecting the ND2 was reported in pathologically proven Parkinson and Alzheimer's diseases (28). Therefore, it cannot be excluded that mtDNA polymorphisms may result in disturbed protein synthesis, synthesis rate or finally in changed protein structure and therefore influence mitochondrial and cell physiology with the consequence of cancer development susceptibility (3-5,8,29).

In conclusion, we suggest that mitochondrial research will enable the establishment of biomarkers helping to identify individuals at high risk for developing specific cancer types and to develop screening approaches for early diagnosis of cancer. Molecular assessment of mitochondrial abnormalities of cancer cells could represent a promising tool for early diagnosis of neoplasia (30,31).

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