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# Do the Mitochondrial DNA Polymorphisms Correlate With the Occurrence of Breast Cancer in Tunisia?

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## Abstract

**Objectives:** This study seeks to determine whether there is any association between the polymorphism of mitochondrial HV2 region and the risk to develop breast cancer in Tunisia population.
**Methods:** We analyzed the polymorphism of HV2 mitochondrial region in 29 patients with breast cancer and 28 healthy blood donors by PCR-Sequencing.
Results: We revealed a negative significant association between the A>G mitochondrial polymorphism at position 263 and the incidence of breast cancer [13/29 (44.82%), in patients, versus 20/28 (71.42%), in control group] (p= 0.041; OR=0.32).
**Conclusion:** The A>G mitochondrial polymorphism at position 263, present at germ line level, may be a protector factor for breast cancer.

## Introduction

Mitochondria are cellular organelles that perform essential functions in cellular metabolism and are critically important for cell survival [1,2]. In addition to their important role in the fulfillment of cellular energy needs, they are essential in the processing of important metabolic intermediates, for various pathways involved in the metabolism of carbohydrates, amino acids, and fatty acids. These organelles also play an important role in apoptosis which plays a critical role in carcinogenesis process. Recently, mutations in the non-coding (D-loop) and coding region of the mitochondrial DNA have been identified in various types of human cancers [3,4,5,6,7,8,9,10,11,12,13,14,15,16].
Tan and al. have identified somatic mitochondrial polymorphisms in 74% of patients with breast cancer. The majority of these variants (81.5%) were limited to the D-loop region [17]. These results were also reported by another studies [18,19]. Among these D-loop mutations, a mononucleotide repeat situated between nucleotide 303 and 315 was revealed as a frequent hot spot of deletions or insertions in breast tumors. This homopolymeric C-strech is situated in the second hypervariable region (HV2) of the non coding region and involved in the formation of a persistent RNA-DNA hybrid that leads to the initiation of mitochondrial DNA heavy-strand replication [20,21]. All these reports have carried out these analyses, in order to investigate the potential use of mitochondrial DNA mutations as markers for breast cancer detection. However, taking into account, the presence of such polymorphisms, at germ line level, in already studied population we tried to evaluate these markers as genetic risk factors for breast cancer. So we have searched, in a case-control study, for the occurrence of HV2 mitochondrial polymorphisms in Tunisian patients with breast cancer and ethnically matched controls.

## Materials and methods

### Subjects

Blood samples were collected from 29 unrelated patients with breast cancer, admitted in Salah Azeiz Oncology Institute since 2003. Informed consent, using a standardized written form, was obtained from each patient.
Twenty four patients have a family history of breast and/or ovarian cancer (23 females and one male) with at least one first degree relative affected with breast and/or ovarian cancer. The remaining five cases were patients with sporadic breast cancer.
The age of patients is ranged between 31 and 72 years. None of them have received any prior radiation therapy or chemotherapy. The corresponding clinicopathological data were collected for each patient (Table 1).
Twenty eight healthy blood donors, aged from 30 to 60 years were also considered as a control group. All the subjects (patients and controls) were unrelated volunteers who were Tunisian born. Five milliliters of venous blood with 1 ml of EDTA 0,1 M as anticoagulant, were collected from each subject.

### DNA isolation, amplification and sequencing

Genomic DNA was extracted by proteinase K digestion, from peripheral blood mononuclear cells isolated from each sample and then column purified (QIAGEN Inc, Chatsworth, CA, USA). The mitochondrial HV2 region (situate between positions 57 and 372) from each sample was PCR amplified in a total reaction volume of 50 l containing 10mM Tris-HCl, pH 8.3, 50 mM KCl, 1,5-4,5 mM MgCl2, 50 mM dNTPs, 10 M of the primer L16340 (5'AGCCATTTACCGTACATAGCACA3') and 10 M of the primer H408 (5' TGTTAAAAGTGCATACCGCCA3'), 10 ng of genomic DNA and 2 U of Taq polymerase (Go Taq® Flexi DNA polymerase). The PCR cycling program comprised an initial denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 10 sec, annealing of primers at 52°C for 30 sec and extension at 72°C for 30 sec. The amplified products were purified with the Invitrogen PCR purification kit (K3 100-01). The sequencing reaction was

carried out using the Big Dye Terminator (version 3.0) Cycle sequencing kit, with ampliTaq® DNA polymerase (AB Applied Biosystems) and was performed separately on each strand with the primers L16340 and H408. Sequences were run in an automatic sequencer ABIPRISM 377 (AB Applied Biosystems).

**Statistical analysis**

Each sequence from the blood of healthy individuals and of cases was compared to the revised Cambridge Reference Sequence (rCRS) [22] between the positions 64 and 340. Sequences were introduced in an impute Arlequin 3.0 file for Arlequin 3.0 [23] in order to calculate the gene diversity and mean pair wise differences. The mitochondrial DNA polymorphism frequencies were directly estimated. The mitochondrial DNA polymorphism frequencies in cancer patients and controls were compared using Pearson's 2 test or Fisher's exact test (when the number of subjects in a cell is <5). Odds ratios were given with 95% confidence limits. The EPI INFO 6 package program was used for these statistics' analysis (http://www.ensp.fr/services/logiciels/epiinfo_604d_fr.htm).

## Results

The comparison of each sequence of healthy individuals (28) and cases (29) to the rCRS shows 117 polymorphisms for controls with 34 segregating sites and 90 variants for patients with 29 segregating sites (Table 2). Several measures of sequence diversity (Table 3) show that the healthy controls present a higher diversity (Gene diversity = 0.9894+/- 0.0123; MPD = 3.921+/-2.023) than the patients group (Gene diversity= 0.9064+/- 0.0419; MPD = 3.894+/-2.013).

The most frequent polymorphisms in the two groups are a C insertion (at 311-315np), a C insertion (at 303-309np), two A>G transitions at 263np and 73np, respectively. A relatively high instability of D310 mitochondrial microsatellite, within the mononucleotide repeat, between the nucleotide positions 303 and 315, was detected in these samples since we detect these polymorphisms in 25/28 (33,3%) of controls and 28/29 of cases (46,6%) (Table 2).

We carried out a case-control study and compared the occurrence of HV2 polymorphisms in Tunisian patients with breast cancer and ethnically matched controls. The results revealed a negative significant association between the A>G mitochondrial polymorphism at position 263 and the incidence of breast cancer [13/29 (44.82%) in patients, versus 20/28 (71.42%) in control group] (p= 0.041; OR=0.32) (Table 2).

## Discussion

Mitochondrial defects have long been suspected to play an important role in the carcinogenic process [24,3], but their involvement in carcinogenesis has been relatively less studied [25]. These defects include altered expression and activity of respiratory oxidation of NADH-linked substrates, as well as mitochondrial DNA mutations. Some studies suggest the potential use of somatic mitochondrial DNA mutations, as markers for breast cancer detection. Mitochondrial microsatellite instability has been frequently described in breast tumours [15], in non malignant and malignant gastric tumours [26,9]. In cervical cancer, the presence of mitochondrial polymorphisms at somatic level correlated with human Papilloma Virus infection [27].

On the other hand, comprehensive studies examining the mitochondrial DNA polymorphism in breast cancer, published recently, have revealed a high incidence of somatic mitochondrial polymorphisms in the tumour examined tissues [9,11,12,17,28]. However, large investigations are required to evaluate the correlation between these genetic markers and the occurrence and/or the evolution of cancer. For this aim, we have sequenced the second hypervariable region of the mitochondrial genome in 29 patients with breast cancer and 28 healthy blood donors. The 24 patients with family history of breast and/or ovarian cancer have been screened for BRCA1 mutations (because of their BRCA1 suggestivity according to their pedigrees), only two patients present BRCA1 deleterious mutations (Exon 20, 5385inC ; Exon 11, 2789delG) [29]. These two family's cases are negative for the A>G mitochondrial polymorphism at position 263. But each of them present a C insertion polymorphism at nucleotide position 315.

We showed that the mononucleotide repeat located between 303 and 315 nucleotides is very polymorphic, at germ line level and, both in healthy (33,3%) and patient 46,6%) groups. It has also reported by parella and colleagues [15,18] that the D310 mitochondrial microsatellite is a hot spot for mutations in breast cancer.

Moreover, a case-control study was carried out in order to evaluate the mitochondrial polymorphism frequencies, in the second hypervariable region and to search the eventual association between these polymorphisms and the occurrence of breast cancer in Tunisia. A negative association between the 263 mitochondrial DNA polymorphism and the occurrence of breast cancer was found in this study.

Our results would indicate that some mitochondrial DNA polymorphisms, present at germ line level may constitute risk or protector factor for breast cancer. Nevertheless, more extensive studies with larger samples, including different types of cancer and different origins of patients, are required in order to improve the understanding of mitochondrial DNA polymorphism implication in the carcinogenesis process.


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## Tables

Table 1. Patient characteristics

| Clinicopathological factors | Number of patients |
|---|---|
| No. of subjects | 29 |
| Sex | |
| Male | 1 |
| Female | 28 |
| Median (range) age at diagnosis 46,48 (31-72) | |
| 30-40 years | 8 |
| 40-50 years | 14 |
| 50-60 years | 4 |
| 60-70 years | 2 |
| > 70 years | 1 |
| Histological subtype | |
| IDC | 22 |
| IDC multifocal | 1 |
| Bifocal mixte (IDC + LDC) | 1 |
| LDC | 1 |
| microcalcification | 2 |
| Not available | 2 |
| Histological grade | |
| 1 | 3 |
| 2 | 12 |
| 3 | 9 |
| Not available | 5 |
| Lymph node status | |
| Negative | 4 |
| Positive | 18 |
| Not available | 7 |
| Estrogen receptor status | |
| Negative | 4 |
| Positive | 6 |
| Not available | 19 |
| Progesteron receptor status | |
| Negative | 4 |
| Positive | 6 |
| Not available | 19 |

IDC: Infiltrative Ductal Carcinoma; LDC : Lobular Ductal Carcinoma

Table 2. Germinal HV2 mitochondrial polymorphisms in the examined groups

| Patients (n= 29) | | | Controls (n= 28) | | | |
|---|---|---|---|---|---|---|
| Base pair | Polymorphisms | No. of sample | Base pair | Polymorphisms | No. of sample | P value |
| 303-309 | C insertion | 12 | 303-309 | C insertion | 11 | 0.872 |
| 303-309 | CC insertion | 1 | 303-309 | CC insertion | 6 | 0.096 |
| 305 | A insertion | 1 | 305 | A insertion | | 0.321 |
| 311-315 | C insertion | 26 | 311-315 | C insertion | 21 | 0.268 |
| 311-315 | A insertion | 2 | 311-315 | A insertion | 1 | 0.574 |
| 297 | A>C | | 297 | A>C | 2 | 0.142 |
| 295 | C>A | 1 | 295 | C>A | 2 | 0.532 |
| 294 | C>T | 1 | 294 | C>T | | 0.985 |
| 293 | T>C | 1 | 293 | T>C | | 0.985 |
| 285 | C>T | | 285 | C>T | 1 | 0.985 |
| 284 | A>G | 1 | 284 | A>G | | 0.985 |
| 276 | A>G | | 276 | A>G | 1 | 0.985 |
| 272 | A>C | | 272 | A>C | 1 | 0.985 |
| 263 | A>G | 13 | 263 | A>G | 20 | P=0,041 OR= 0,32 |
| 257 | A>C | | 257 | A>C | 1 | 0.985 |
| 253 | C>T | 1 | 253 | C>T | 3 | 0.578 |
| 252 | T>C | 1 | 252 | T>C | | 0.985 |
| 250 | T>C | | 250 | T>C | 1 | 0.985 |
| 245 | T>C | 1 | 245 | T>C | | 0.985 |
| 241 | A>C | 1 | 242 | A>C | | 0.985 |
| 236 | T>A | 1 | 236 | T>C | | 0.985 |
| 220 | T>G | 1 | 220 | T>G | 1 | 0.487 |
| 204 | T>C | | 204 | T>C | 2 | 0.456 |
| 199 | T>C | 2 | 199 | T>C | 2 | 0.629 |
| 198 | C>A | 3 | 198 | C>A | 3 | 0.699 |
| 195 | T>C | 1 | 195 | T>C | 3 | 0.578 |
| 188 | A>G | | 188 | A>G | 1 | 0.985 |
| 185 | G>A | 1 | 185 | G>A | 1 | 0.487 |
| 175 | delT | 1 | 175 | delT | | 0.985 |
| 161 | T>G | 1 | 161 | T>G | | 0.985 |
| 152 | T>C | 2 | 152 | T>C | 5 | 0.391 |
| 151 | C>T | | 151 | C>T | 1 | 0.985 |
| 150 | C>T | 2 | 150 | T>C | 2 | 0.629 |
| 146 | T>C | | 146 | T>C | 1 | 0.985 |
| 141 | C>T | | 141 | C>T | 2 | 0.456 |
| 136 | G>A | | 136 | G>A | 1 | 0.985 |
| 132 | C>T | | 132 | C>T | 1 | 0.985 |
| | | | 132 | C<TC heteroplasmic | 1 | 0.985 |
| 125 | T>C | | 125 | T>C | 1 | 0.985 |
| 114 | C>T | 1 | 114 | C>T | 2 | 0.975 |
| 111 | A>C | | 111 | A>C | 1 | 0.985 |
| 90 | G>A | | 90 | G>A | 1 | 0.985 |
| 83 | T>G | 1 | 83 | T>G | | 0.985 |
| 81 | G>A | 1 | 81 | G>A | | 0.985 |
| 73 | A>G | 8 | 73 | A>G | 13 | 0.140 |
| 72 | T>C | | 72 | T>C | 1 | 0.985 |
| 65 | C>T | 1 | 65 | C>T | | 0.985 |
| | | Total=90 | | | Total=117 | |

Nominal value for comparison, P= 0.05; degree of freedom= 1; OR: Odds Ratio

Table 3. HV2 (from np 64 to np 340) diversity measures in patients and controls

| Samples | Sample size | Haplotypes | Segregating sites | Gene diversity | Mean Pairwise Differences (MPD) |
|---|---|---|---|---|---|
| Patients | 29 | 18 | 29 | 0.9064+/-0.0419 | 3.894+/-2.013 |
| Controls | 28 | 25 | 34 | 0.9894+/-0.0123 | 3.921+/-2.023 |