Original Article

Association of Mitochondrial DNA 10398 Polymorphism in Invasive Breast Cancer in Malay Population of Peninsular Malaysia

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Abstract

Background: **The mitochondrial DNA (mtDNA) 10398 polymorphism is hypothesised to alter a mitochondrial subunit of the electron transfer chain and is associated with several neurodegenerative disorders and cancers.**

Methods: **In this study, an mtDNA polymorphism at nucleotide position 10398 was screened in 101 Malay female patients with invasive breast cancer and 90 age-matched healthy female controls using minisequencing analysis.**

Results: **The Malay women with the 10398G variant showed a significantly increased risk of invasive breast cancer (OR = 2.29, 95% CI 1.25–4.20, *P* = 0.007). Immunohistochemistry analysis was conducted to investigate the effect of this polymorphism on the levels of apoptosis in breast cancer cells. The level of Bax (a pro-apoptotic protein) expression was significantly higher than that of Bcl-2 (an anti-apoptotic protein) in patients carrying the G allele (*P* = 0.016) but not in those carrying the A allele (*P* = 0.48).**

Conclusion: **Based on these findings, we propose that the mtDNA 10398 polymorphism may be a potential risk marker for breast cancer susceptibility in the Malay population.**

Keywords: *breast cancer, DNA sequencing, genetic marker, mitochondrial DNA, oncology, single nucleotide polymorphism*

Introduction

Mitochondria are a major site and target of intracellular reactive oxygen species (ROS), which are a natural by-product of electron transport chain activity (1,2). Mitochondrial DNA (mtDNA) is vulnerable to the effect of these molecules and has a limited ability to repair itself. Therefore, the excessive formation and continuous accumulation of ROS could lead to a cellular stress response and the inhibition of apoptosis (3). Several findings (4–6) show that both defects and reduction in the apoptosis threshold can extend the life span of the cell, contributing to continuous proliferation that may lead to cancer development. However, the exact role of mtDNA mutations in inhibiting apoptosis, either by suppression of pro-apoptotic genes or by activation of anti-apoptotic genes, has not been defined.

Several mutations, including single nucleotide polymorphisms in certain genes in both the nuclear and mitochondrial genomes, are implicated in breast cancer susceptibility (7,8). An A to G polymorphism at nucleotide position 10398 in the mitochondrial genome causes a non-conservative amino acid substitution from threonine to alanine within the NADH dehydrogenase (ND3) subunit of Complex I (9,10). This particular polymorphism has also been reported to alter both mitochondrial pH and intracellular calcium levels (11,12); these alterations have been associated with the modulation of ATP production and apoptosis (13). The structural alteration and impairment of Complex I may lead to increased production of free radicals and has been associated with increased risk of several mitochondrial disorders, such as Parkinson's disease (14,15) and bipolar disorders (16).

The association of the mtDNA 10398 polymorphism in Complex I with breast cancer was first studied by Canter et al. (17), which showed that the risk of invasive breast cancer was significantly higher in African-American women carrying the 10398A allele compared with non-carriers. This polymorphism is also associated

with an increased risk of prostate cancer in African-American men (18) as well as an increased risk of breast cancer and oesophageal cancer in individuals of Indian descent (19). However, the opposite was observed in Polish populations and non-Jewish European American populations as the frequency of the 10398G variant was significantly higher percentage in breast cancer patients of these groups compared with the controls (20). Similar results were obtained in a study of a non-Jewish European American population (21). Finally, the 10398G variant was reported at high frequency in several Asian populations (22,23). As different populations have a variable risk of breast cancer susceptibility associated with this polymorphism, in this work, we determined whether this association held in the Malay population of Peninsular Malaysia. The effect of this polymorphism on the levels of apoptosis in breast cancer tissues was studied through the expression levels of the pro- and anti-apoptotic proteins, Bax and Bcl-2, respectively, using immunohistochemical analysis.

Materials and Methods

Sample collection

Ethical clearance for this study was obtained from the human ethics committee, Universiti Sains Malaysia. A total of 101 paraffin-embedded breast cancer tissue samples of Malay females (from 2003 until 2009) were obtained from the Department of Pathology, School of Medical Sciences, Universiti Sains Malaysia. Buccal swabs were collected from 90 age-matched healthy Malay females with no history of breast cancer in their family as controls.

DNA extraction and PCR amplification

Total DNA was extracted from buccal swab samples using a Puregene DNA Extraction Kit (Gentra System, US), while DNA extraction from paraffin-embedded tissues was performed using a QIAamp DNA FFPE Tissue Kit (Qiagen, USA), both according to the manufacturer's protocol. mtDNA fragments bearing the 10398 polymorphism were amplified by the polymerase chain reaction (PCR) technique using the following primers: 10342-F 5' CAT CAT CCT AGC CCT AAG TC 3' and 10518-R 5' GAA GTG AGA TGG TAA ATG CTA G 3'. The final PCR product was 176 bp in length. The following PCR thermal cycle conditions were performed: 94 °C for 10 min, followed by 30 cycles of 94 °C for 30 s, 49 °C for 30 s, 72 °C for 45 s, and a final extension step at 72 °C for 10 min.

Minisequencing

Minisequencing analysis was performed using the ABI PRISM SNaPshot Multiplex Kit (Applied Biosystems, US). The PCR products were purified using 5 µL (1 unit/µL) of shrimp alkaline phosphatase (SAP) and 0.1 µL (20 unit/µL) of *Exo* I. The minisequencing reactions contained 1.0 µL (10 pM/µL) of HPLC purified minisequencing primer (5' CTA CAA AAA GGA TTA GAC TGA 3'), 3 µL of SNaPshot Multiplex Reddy Mix and 1 µL of purified PCR template. Single base extension of 25 cycles were performed on GeneAmp PCR System 9700 (Applied Biosystems, US) using the following conditions: 96 °C for 10 s, at 48 °C for 5 s, and 60 °C for 30 s. SAP (1 unit) was added to the post-extension product and incubated at 37 °C for 1 h. The product was then incubated at 75 °C for 15 min to deactivate the enzyme. The purified product was subjected to electrophoresis in ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, US). Each well contained 9 µL of Hi-Di formamide, 0.5 µL of minisequencing product, and 0.5 µL of GeneScan-120 LIZ size standard. The results were analysed using GeneMapper ID software.

Immunohistochemistry

Paraffin-embedded breast tissues were cut into 4 µm sections and mounted on poly-L-lysine treated microscope slides. After deparaffinisation and hydration, antigen retrieval step was performed using antigen retrieval solution (citrate buffer, pH 7.6) in a stainless steel pressure cooker. Samples were incubated for 30 min with either monoclonal anti-human Bcl-2 (Dako, US) or polyclonal rabbit anti-human Bax (Dako, US) overnight at 1:100 dilutions. The slides were then incubated with the corresponding biotinylated secondary antibodies for 30 min, followed by incubation with streptavidin–horseradish peroxidase solution for 30 min. The samples were subsequently incubated with the substrate, diaminobenzidine, in the presence of H_2O_2. After washing with Tris-buffered saline, the slides were counterstained and mounted. Negative and positive controls (tonsil tissue for Bcl-2 and Hodgkin's lymphoma for Bax) were included routinely.

Protein expression, visualised as a brown staining pattern, was assessed using light microscopy (Nikon, JP). The areas with the highest levels of antibody staining within the tumour sections were assessed at low magnification (40×) according to Van Diest et al.'s method (24) to obtain a general impression

of the overall distribution of tumour cells. The percentage of immunopositive cells (Figure 1) was quantitated at higher magnification (400×) (25,26) independently by 2 investigators without knowledge of the scoring data. The scoring method used was based on Umemura et al., (27) with some modifications: 1 (< 10%), 2 (< 50%), 3 (< 80%), and 4 (> 80%).

Statistical analyses

Statistical analyses were performed using the SPSS version 12.0 for Windows (SPSS Inc., Chicago, Illinois, US). Chi-square analysis was carried out to establish the association of the 10398 polymorphism with invasive breast cancer. The Wilcoxon signed ranks test was used to compare the expression of both Bax and Bcl-2 proteins in patients with 10398A and 10398G variants. A *P* value of less than 0.05 was considered significant.

Results

Statistical analysis revealed a significant correlation between the 10398 polymorphism and invasive breast cancer in the Malay samples (Table 1). A chi-square test indicates a *P* value of 0.007 ($P < 0.05$) with an odds ratio (OR) of 2.29 (95% CI 1.25–4.20). The frequency of the 10398G allele (73%) is much higher than that of the 10398A allele (27%) in breast cancer patients. The frequency of 10398G is also higher in patients (73%) compared with controls (54%).

Paraffinised tissues from 20 patients carrying the 10398A allele and 50 patients carrying 10398G allele were successfully immunostained for both Bax and Bcl-2 antibodies. The level of Bax (pro-apoptotic protein) expression was significantly higher than Bcl-2 (anti-apoptotic protein) expression in patients carrying the G allele ($P = 0.016$), but not in those carrying the A allele ($P = 0.48$) (Figure 2).

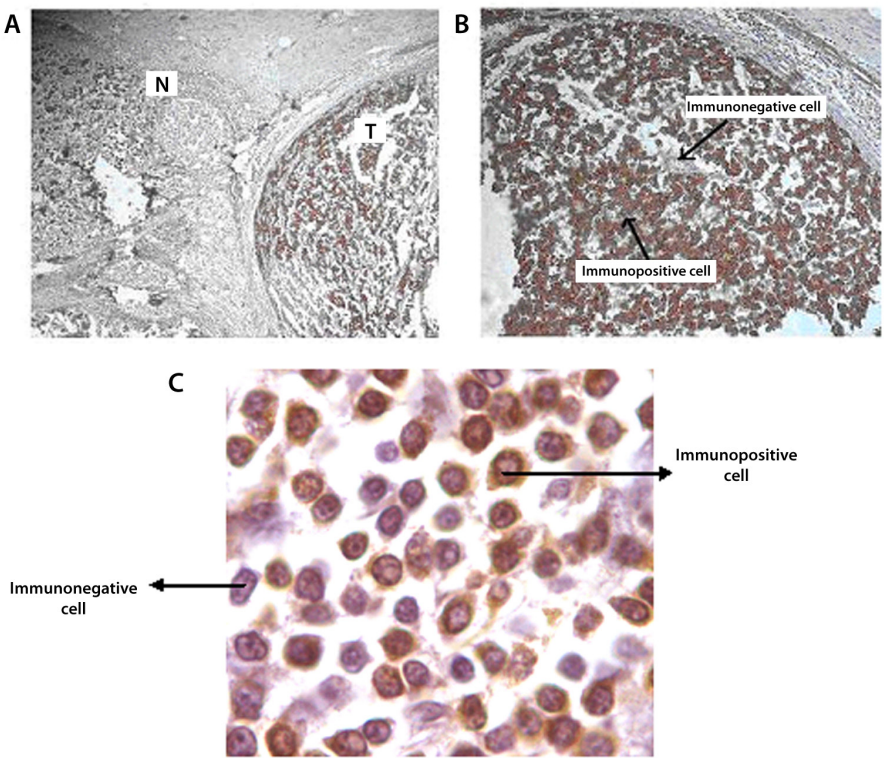

Figure 1: Immunohistochemistry staining of the Bax protein in breast cancer tissue. A: The protein expression levels (stained brown) were assessed in both tumour cells (T) and normal cells (N) (40× magnification). B: Immunopositive and immunonegative cells within the tumour sections (100× magnification). C: Immunopositive and immunonegative cells within the tumour sections (400× magnification).

Table 1: Frequencies of the mtDNA 10398 variant in the Malay population.

Allele	Patients (n = 101)	Controls (n = 90)	OR (95% CI)	P value
A	27 (27%)	41 (46%)	2.29 (1.252–4.200)	0.007
G	74 (73%)	49 (54%)		

Abbreviations: OR = odds ratio, CI = confidence interval.

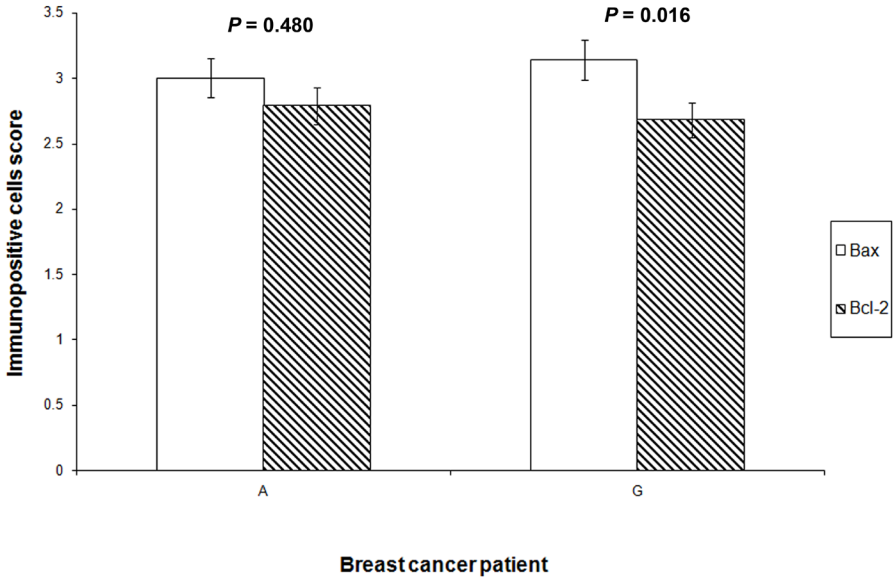

Figure 2: The expression of Bax and Bcl-2 (immunopositive score) in breast cancer cells in relation to the 10398 variant.

Discussion

Alterations or polymorphisms of the mtDNA have emerged as new biomarkers in population studies and for the detection of a variety of diseases and tumours (28,29). Various mutations in both the non-coding and coding regions of the mitochondrial genome are associated with an increased risk of breast cancer (29,30). Despite the established role of both nuclear and mitochondrial genes in breast cancer susceptibility, the list of genetic factors involved is still incomplete. More effort will be required to determine additional genetic alterations that would identify individuals with increased risk, who then would undergo intensive screening, prevention, and treatment programmes (28,29).

The mtDNA 10398 polymorphism has been reported to alter the ND3 subunit of the electron transport chain Complex I and to cause oxidative stress (9–12). Recently, an increasing number of studies have reported the significance of this polymorphism in cancers. However, the literature contains multiple conflicting reports regarding which nucleotide is associated with cancer. Several reports suggested that the 10398A allele is associated with breast cancer susceptibility (17–19), while others suggested the association of the 10398G allele with invasive breast cancer (20,21). The differing results may be due to the variability of risk modifiers that exist in diverse geographical areas (32–34).

In this study, we found that the 10398G allele can be considered a potential risk marker for breast cancer susceptibility in the Malay population. To the best of our knowledge, this study represents the first mtDNA polymorphism screening in breast cancer in Malaysia. Understanding the pathological impact of this polymorphism is important, and indeed, several papers have reported on the mtDNA 10398 polymorphism in cancer. Although no apoptosis data associated with this polymorphism are currently available, we hypothesise that the mtDNA

10398 polymorphism increases the production of ROS as a result of altered Complex 1 function, thus inhibiting the apoptotic mechanism. The mtDNA 10398G variant was found to be associated with breast cancer susceptibility in the Malay population of our study, suggesting that cells carrying the 10398G allele may express a high level of anti-apoptotic protein Bcl-2. It was reported that Bcl-2 expression increased in cells with respiratory defects involving Complex I (35,36). However, in the present study, Bax expression levels were found to be significantly higher than Bcl-2 expression levels in patients carrying the G allele, but the expression levels of these 2 proteins showed no significant difference in patients carrying the A allele.

This result is not in agreement with previous studies (37,38), which reported that levels of the pro-apoptotic protein Bax are low and undetectable compared with the significantly higher levels of Bcl-2 in human breast cancer. Other studies showed similar findings in which Bax was expressed along with Bcl-2 (26,39). We suggested that alterations in Complex I of the electron transport chain may cause dysfunction of proteins involved in apoptosis signaling cascades and sensitise normal cells to undergo apoptosis, allowing cancerous cells, which are protected against the induction of cell death, to become the dominant, surviving cells. The presence of anti-apoptotic proteins other than Bcl-2 in breast tumours may also counteract the suppression of cell death, presumably by forming heterodimers with Bax; however, excessive of anti-apoptotic proteins may also inhibit apoptosis by antagonizing the activated conformation of Bax (5,26,40,41). Based on this finding, we suggest that the 10398 polymorphism demonstrates a relationship between the levels of apoptosis and the incidences of breast cancer. Further understanding of this polymorphism at the molecular and biochemical level is necessary to elucidate its role in breast cancer.

Conclusion

From the present study, we can conclude that mtDNA 10398 polymorphism may be useful as a risk marker for breast cancer susceptibility in the Malay population. Intensive screening program for individuals carrying this polymorphism should perhaps prevent late detection and increase the chance of recovery. Significant increase of Bax expression observed in patients carrying the G allele may indicate its role in the apoptosis signaling cascades in breast cancer.

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Authors' Contributions

Conception and analysis, obtaining of funding, critical revision and final approval of the article, administrative, technical, or logistic support: ZZ
Provision of study materials: JH
Collection and assembly of the data, drafting of the article: TBN
Analysis and interpretation of the data: JH, TBN

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