*Full Length Research Paper*

# Breast cancer risk associated mitochondrial NADH-dehydrogenase subunit-3 (ND3) polymorphisms (G10398A and T10400C) in Bangladeshi women

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**Somatic mitochondrial DNA (mtDNA) mutations have been reported in many types of cancer cells, but very few reports document the prevalence of inherited mtDNA polymorphisms including NADH-dehydrogenase (ND3) subunit polymorphisms in cancer patients compared to healthy control populations. Although, few mitochondrial ND3 subunit polymorphisms were reported in different cancers e.g. breast, esophageal cancer but the effect of polymorphisms on cellular metabolism and growth remain obscure. ND3 subunit with other ND subunits of Complex I and Cytochrome b of complex III of mtDNA Electron Transport Chain (ETC) are the major source of reactive oxygen species (ROS) as a toxic by-product of mitochondrial Oxidative Phosphorylation (OXPHOS), thus polymorphisms in these subunits has been proposed to cause an increased rate of ROS production, therefore, may contribute in developing cancer. In an attempt to investigate mtDNA polymorphisms associated with breast cancer risk, mtDNA from twenty four breast cancer patients and twenty healthy female individuals were studied. We report that two polymorphisms G10398A and T10400C are prevalent in 75% of breast cancer patients, compared to only 35% in healthy female individuals and the difference is statistically significant (P = 0.0138). It is therefore reasonable to assume that G10398A and T10400C single nucleotide polymorphisms may constitute an inherited risk factor for the development of breast cancer in Bangladesh.**

**Key words:** Somatic mitochondrial DNA (MtDNA), NADH, polymorphism, breast cancer, Bangladesh.

## INTRODUCTION

The mitochondrial electron transport chain (ETC) is a major source of ROS (reactive oxygen species), which includes superoxide, hydrogen peroxide and the hydroxyl free radical (Chance et al., 1979). Mitochondrial DNA (mtDNA) complex I (ND1-6, ND4L) and complex III (cytb) involved in ETC system oxidizes molecular oxygen to produce water, ATP and reactive oxygen species (ROS) as a by-product (Fukuda et al., 2007). These oxygen-derived free radicals (ROS) have long been thought to damage both nuclear and mtDNA and play a key role in carcinogenesis (Ames and Shigenaga, 1992). Mitochondrial DNA (mtDNA) polymorphisms, resulting in an amino acid change within the NADH dehydrogenase (ND3) subunit of complex I may increase the production of ROS; therefore, may cause damage to different cellular components including mtDNA (Harman, 1988). It is known that oxidative stress may occur due to imbalance between ROS production and the antioxidant capacity due to mitochondrial dysfunction (Ishikawa et al., 2008). It is also reported that mtDNA is much more vulnerable than nuclear DNA due to its lack of histone proteins and introns; therefore, most of the damage occurs in the coding region, and are thus likely to be of

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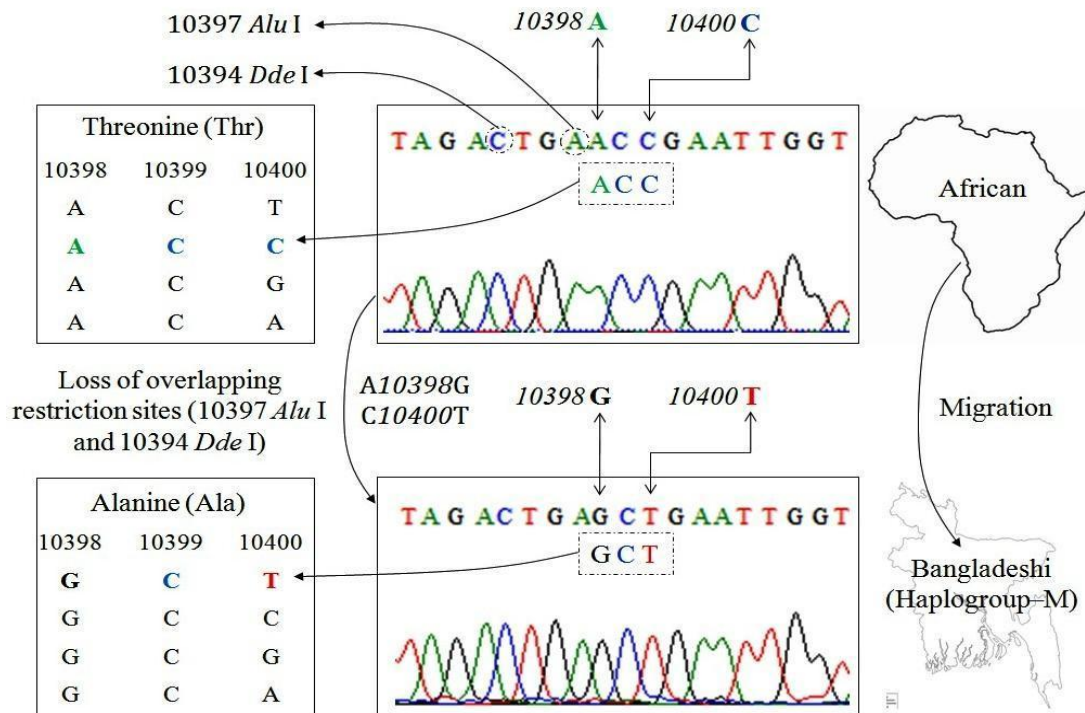
**Figure 1.** Loss of two overlapping restriction sites 10394D*del* and 10397A*lul* in Bangladeshi haplogroup-M mtDNA.

molecular consequence of diseases like cancer (Brandon et al., 2006).

The 10398-nucleotide position (np) in the human mitochondrial genome has been reported highly polymorphic. 10398A, 10400C alleles in Mitochondrial NADH–dehydrogenase subunit 3 (ND3) are prevalent in African populations whereas 10398G, 10400T alleles are frequent in Asians populations including Bangladeshi population (haplogroup M), because of loss of two overlapping restriction sites 10394*Dde*I and 10397*Alu*I in human mitochondrial genome (Figure 1). These variations define a phylogenetic distinguishing feature between African mtDNA and Asian mtDNA (Bandelt et al., 1999). In addition, 10398A allele has been reported as wild type allele in Polish populations and African-American populations whereas 10398G in Northern Indian populations (Czarnecka et al., 2010; Canter et al., 2005; Darvishi et al., 2007).

The mechanism of the adverse effect of the A or G allele at 10398 polymorphic site is still unknown; however, it is presumed to be associated with increased ROS generation due to wild type allele alteration leading to somatic mitochondrial mutations; therefore, it may contribute in developing cancer (Mims et al., 2006). Recently, it has been reported that 10398A allele provides a background risk for breast, esophageal cancer in Northern Indian population and G10398A polymerphism is specific to haplogroup N, thus haplogroup N is associated with increased rate of cancer in that

population. Thus, in Northern Indian population, oxidative stress due to G10398A polymorphism in NADH–dehydrogenase subunit 3 (ND3) may play a role in developing cancers (Darvishi et al., 2007). To verify the adverse effect of the 10398A allele in Bangladeshi populations, the present study has investigated the frequency of 10398A allele in breast cancer patients, and control female individuals.

## MATERIALS AND METHODS

### Sample collection

All approval by the local ethical committee of Bangladesh Medical Research Council (BMRC) and University of Dhaka has been taken. Twenty-four breast cancer patients from Anam Medical College and Hospital, Savar and National Institute of Cancer Research and Hospital, Dhaka, Bangladesh were included for the study in 2010. A healthy cohort of 20 female individuals from mainstream population was also included. All two populations share the same ethnicity and nationality and reside in Bangladesh. Subjects with breast cancer were interviewed. Approximately 3 to 5 mL of blood samples were collected in EDTA coated tubes from breast cancer patients who visited the hospital for treatment. The samples were transported to the laboratory and kept at -20°C until analyzed.

### DNA isolation, PCR and sequencing

Cancer and normal blood DNA was isolated by standard proteinase K treatment followed by phenol/ chloroform/ isoamyl alcohol extraction. DNA was precipitated with 0.3 M sodium acetate (pH

5.2) in 70% ethanol at -20°C overnight and resuspended in Tris-EDTA (TE) buffer (pH 8.0). DNA quantification was performed by taking absorbance at 260 nm and visualized by 0.8% agarose gel electrophoresis (Sambrook et al., 1989). Mitochondrial ND3 region was amplified using one set primer and the resultant amplicons were checked in 20% agarose gel electrophoresis (Rieder et al., 1998). Distinct PCR bands were observed along with diluted 1 Kb$^+$ DNA ladder. 20 µl PCR reaction contained 10 to 20 ng DNA and 0.5 µM primers, 0.2 mM each of deoxynucleotide triphosphate (dNTP), 1U of TaqMan™ DNA Polymerase (Applied Biosystem, USA) and 2.5 mM MgCl$_2$. The PCR amplification of specific regions of mtDNA was performed on the basis of following cycling conditions: initial denaturing at 95°C for 5 min followed by 94°C for 30 s, 58°C for 30 s, and 72°C for 2 min for 35 cycles and final extension step at 72°C for 7 min. In sequencing PCR, the ABI-prism Big Dye Terminator V1.1 containing ampliTaq polymerase, dye terminators (fluorescent label), deoxynucleotide triphosphate, magnesium chloride, was used for direct sequencing of PCR product for specific primers (forward/ reverse primer). The sequencing PCR was performed on the basis of following cycling conditions: initial denaturing at 95°C for 1 min followed by 94°C for 10 s, 55°C for 30 s, and 60°C for 4 min for 35 cycles.

**MtDNA sequence analysis**

The purified sequencing PCR products were analyzed by electrophoresis in the ABI-Prism 3130 Genetic Analyzer (Applied Biosystems, USA). The sequence patterns were observed and edited by using Mac-based software (Auto Assembler V 3.0) and BioEdit Sequence Alignment Editor V 7.0.9.0 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The sequences were aligned by using bl2seq tool of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared with the revised Cambridge Reference Sequence, rCRS (NCBI Reference Sequence: NC_012920.1) (Tanaka et al., 2007; Krystek et al., 2001). MtDNA polymorphisms were compared with the mitochondrial genome database of world population by using Mitomap (www.mitomap.org).

**Statistical analysis**

Two tailed non-directional Fisher-Irwin (Fisher's exact test) has been used to verify the mutation probability difference in cancer and healthy (control) populations (Sheskin, 2007). To confirm the result of this common test in the light of low expected number in the healthy population Yates's chi and un-corrected chi squared test ('N-1' chi squared test) have been used as expected to give relatively low Type I error. The analysis was performed as previously described (Campbell, 2007).

To further understand the significance of G10398A polymorphism related risk factor for unfavorable outcomes (odds ratio, relative risk, difference in proportions, absolute and relative reduction in risk, number needed to treat) and of the effectiveness of a diagnostic criteria (sensitivity, specificity, positive and negative predictive values, positive and negative likelihood ratios, diagnostic and error odds ratios) has been performed. The parameters, as well as the confidence intervals for the estimated parameters are computed by standard methods (Fleiss et al., 2003).

## RESULTS

The present study has sequenced NADH dehydrogenase subunit 3, ND3 (10059→10404 nucleotide position, np) in a total of 24 breast cancer patients and 20 healthy female

individuals. At the 10398, 10400 polymorphic sites, the G10398A with second polymorphism T10400C were present in 18 out of 24 total breast cancer patients (75%), but only 35% in control individuals (Figure 2). This difference is statistically significant P = 0.0138 (Fisher's exact test), which has confirmed both by Yates corrected, ▊ = 5.577 and P = 0.0182 and without correction, 'N-1▊ = 7.114 and P = 0.0076. For the breast cancer population, these 10398A and 10400C SNPs have an odd ratio (OR) = 5.5714 (1.5137 to 20.506 at 95% CI) and relative risk (RR) = 2.14280 (1.1259 to 4.617 at 95% CI). Moreover, these 10398A and 10400C SNPs have high sensitivity = 0.75 (0.617 to 0.854 at 95% CI) and specificity = 0.65421 (0.490 to 0.774 at 95% CI) with subsequent diagnostic odd ratio = 5.571 (1.549 to 20.042 at 95% CI) and number needed to diagnose (NND) = 2.500 (1.592 to 9.333 at 95% CI). The 10398A and 10400C SNPs have a relative risk reduction (RRR) = -1.280 (-3.436 to -0.224 at 95%CI).

## DISCUSSION

The present study has investigated the prevalence of inherited mtDNA polymorphisms associated with breast cancer risk in Bangladesh. Family history being a risk factor suggests possible inheritable genetic susceptibility for breast cancer development. The uniparental (maternal) inheritance of mtDNA polymorphism means wild type allele alterations accumulate slowly over successive generations. Therefore, the altered allele may be an inherited predisposition factor for the development of mitochondrial disorder associated diseases e.g. cardio-vascular disease, cancer, arthritis, cataract, osteoporosis, type 2 diabetes, Alzheimer's disease. We, for the first time, are reportisng the abundance of the G10398A polymorphisms with second T10400C polymorphisms in Bangladeshi breast cancer patients and its frequency in healthy female individuals (Table 1) 10398G, 10400T alleles are common in Bangladeshi population due to mtDNA migration. The mtDNA G10398A polymorphism, resulting in an amino acid change, Alanine (nonpolar, neutral, with hydropathy index of +1.8) to Threonine (polar, neutral, with hydropathy index of -0.7), within the NADH dehydrogenase (ND3) subunit of complex I of mtDNA Electron Transport Chain (ETC), has been proposed to cause an increased rate of electron leakage and ROS production (Czarnecka et al., 2010).
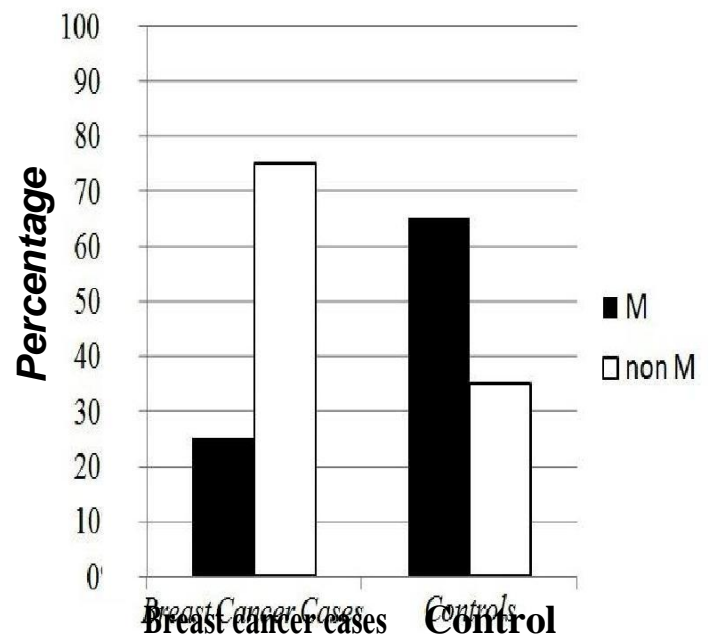
Although T10400C polymorphism causes synonymous amino acid change; however, it occurs as a consequence of G10398A polymorphism because of the presence of two overlapping restriction sites 10394Ddel and 10397AluI in human mtDNA ND3 region (Bandelt et al., 1999). On the basis of mtDNA positions (10398 and 10400), a comparison between breast cancer cases and controls with M and non-M haplogroup background showed that 25% breast cancer cases and 65% controls possessed M mtDNA haplogroups (Figure 3). It has been

**Table 1.** G19398A and T10400C polymorphisms and breast cancer research.

| Population studied | African-American | Northern Indian | Present study |
| --- | --- | --- | --- |
| No of samples | 654 | 124 | 24 |
| Sporadic/familial | Sporadic | Sporadic | Sporadic |
| Control population | 605 | 273 ethnic matched healthy woman | 20 healthy female |
| Result/conclusion | G10398A polymorphism is associated with invasive breast cancer and 10398A is independent risk factor, and is involved in ROS production | 10398A allele provided a background risk for breast cancer, G10398A polymorphism is specific to haplogroup N, haplogroup N is associated with increased rate of breast cancer | G10398A and T10400C polymorphisms increase breast cancer risk in population |
| Allele Freq; Odd Ratio, OR; $P$ value | 10398A = 87% cases (n = 654) 81% controls (n = 605) OR (for A) = 1.6 (1.1-2.31) $P$ = 0.013 | 10398 A = 57.3% cases (n =124) 43.6 % controls (n = 273) OR = (for A) = 1.73 (1.13-2.66) $P$ = 0.01 | 10398A = 75% case (n = 24) 35% controls ($n$ = 20) OR (for A) = 5.5 (1.53-20.5) $P$ = 0.0182 |
| Reference | Canter et al. (2005) | Darvishi et al. (2007) | Present study |
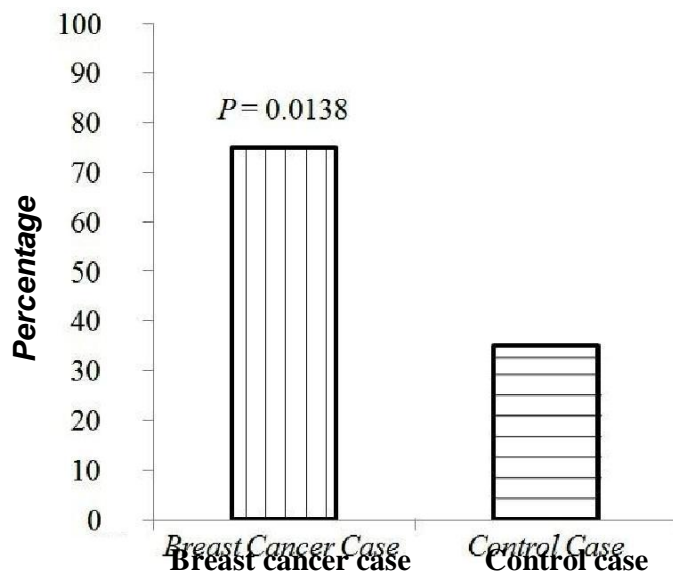


**Figure 2.** Percentage of G10398A polymorphism with second T10400C polymorphism in breast cancer patients compared to healthy females.



**Figure 3.** Frequency of the mtDNA M and non M haplogroups based on mtDNA 10398 and 10400 positions.

reported that 10396G allele is significantly associated with increased breast cancer risk in Polish and African-American populations whereas 10396A is a risk factor of developing breast and esophageal cancer in Northern Indian population with mitochondrial haplogroup N (Czarnecka et al., 2010; Canter et al., 2005; Darvishi et al., 2007). It can be hypothesized from this present study that Bangladeshi population with mtDNA haplogroup-M variants containing 10398G and 10400T alleles may have a decrease in the risk of developing breast cancer, whereas population non M haplogroup variants containing

10398A and 10400C alleles may have an increased risk of breast cancer. Although a large scale population study is required to provide a statement; however, we propose that these identified wild type alterations can be used as a mitochondrial genomic marker to track inherited breast cancer susceptibility in Bangladeshi women, therefore, it may constitute a useful tool to stratify breast cancer risk in Bangladeshi populations.

## ACKNOWLEDGEMENTS

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