

Detection of mitochondrial DNA mutations by high-throughput sequencing in the blood of breast cancer patients

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Abstract. Mitochondrial DNA mutations have been identified in several types of cancer. In breast cancer, germline and somatic mitochondrial DNA (mtDNA) mutations have been identified. A number of mtDNA mutations in breast cancer have been identified in protein-coding regions (in protein-coding genes, such as ND2, COX3, ND4, ND5 and CytB). Mutations in these structure proteins cause impaired electron transport function and lead to electron leakage and increased reactive oxygen species (ROS) production, which in turn increases oxidative stress and oxidative damage to the mitochondria, as well as to cells. These data establish an association between mtDNA mutations and breast cancer; however, there is no reliable prediction of breast cancer predisposition or progression based on mtDNA mutation patterns thus far. In this study, we used high-throughput sequencing to detect mtDNA mutations in the blood of breast cancer patients. Some of these mutations may be used as potential markers for breast cancer diagnosis.

Introduction

Human mitochondrial DNA (mtDNA) is a 16.6 kb circular double-stranded DNA molecule, which is present at a high copy number per cell. Thirteen of the 85 subunits comprising the oxidative phosphorylation system are encoded by mtDNA, including subunits within complex I (ND1, ND2, ND3, ND4, ND4L, ND5, ND6), complex III (CytB), complex IV (COI, COII, COIII) and complex V (ATPase6, ATPase8). Human mtDNA also contains 24 genes encoding 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs) which are essential

for protein synthesis within the mitochondria. Another important region in mtDNA is termed the D-loop, which is the initial site of heavy chain replication and the promoters for heavy and light chain transcription (1,2).

The mitochondrion functions as a powerhouse to generate adenosine triphosphate (ATP) through oxidative phosphorylation (OXPHOS). The mitochondrial OXPHOS also produces most of the cellular reactive oxygen species (ROS) at complexes I and III. It is believed that mtDNA mutations in the subunits of complexes I or III cause aberrant ROS production which may damage mtDNA, as well as nuclear DNA (3). In particular, the mitochondrion has relatively less sophisticated DNA protection and repair systems, and thus it is vulnerable to high mutation rates (4). The mitochondrion also plays central role in apoptosis (5,6), cell proliferation (7) and calcium signaling (8).

Germline and somatic mtDNA mutations have been found in primary breast cancer. One mtDNA population polymorphism has been found to be associated with an increased risk of breast cancer, suggesting that germline mtDNA mutations may be important in the etiology of breast cancer. This mutation occurs in the ND3 gene at nt 10398 in which the 10398A allele was linked to an increased risk of invasive breast cancer in African-American women, compared with African-American women with the 10398G allele. The 10398 allele was an independent risk factor for breast cancer in African-American women but no association was detectable in Caucasian women. It was thought that this mutation results in increased oxidative stress (9). However, Setiawan *et al* argued that there was no association between the 10398 allele and breast cancer in African-American women; however, their study did not provide detail information on the materials and methods that were used (10). Parrella *et al* reported that mtDNA mutations were detected in 61% of patients using direct sequencing. The affected genes included ND4, ND5 and CytB (11). Tan *et al* reported that 14 of the 19 breast tumors (74%) displayed at least one somatic mtDNA mutation. Twenty-seven somatic mutations were found and 22 of them occurred in the D-loop region. The affected genes included ND2, 16srRNA and ATPase6 (12). Gallardo *et al* reported mtDNA mutations of the COI gene in primary breast cancer. The mutation was expected to impair the interaction between

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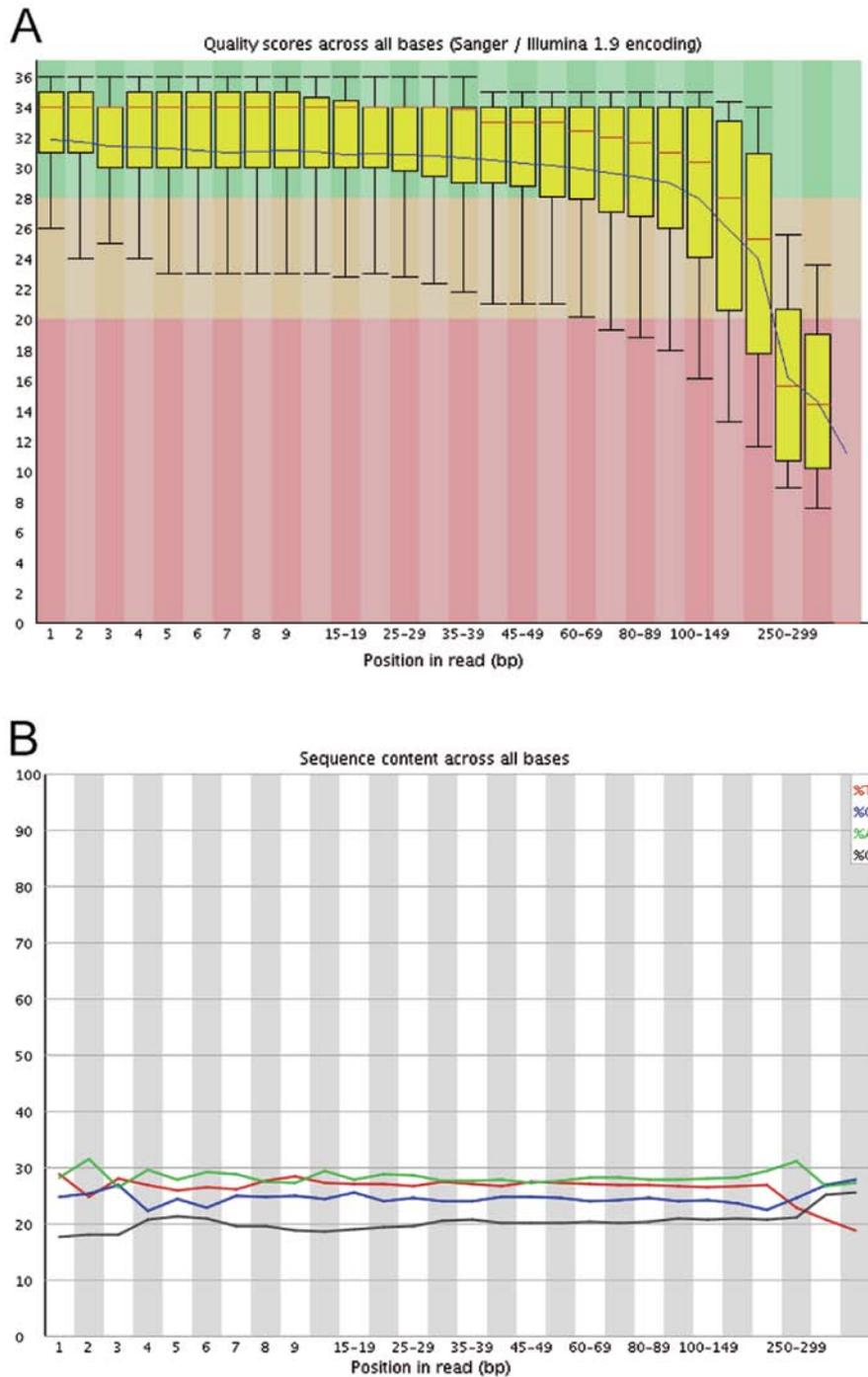


Figure 1. Quality control of sequencing data. (A) Quality scores across all bases. (B) Sequence content across all bases.

subunit I and II of cytochrome *c* oxidase and the mutant had a reduced complex IV activity by 50% (13). Other studies have reported mtDNA mutations in the NDI, CoIII, tRNA-I and tRNA-T genes (11,14). It is believed that mtDNA mutations in structure proteins of the mitochondria cause impaired electron transport function and lead to electron leakage and increased ROS production, which in turn increases oxidative stress and oxidative damage to the mitochondria in the process of transformation and cancer progression. There is no direct evidence on breast cancer to support the ROS theory thus far; however, there is experimental evidence on prostate cancer to support this theory. Petros *et al* reported that cybrids with pathogenic

mtDNA ATP6 T8993G generated tumors 7-fold larger than the wild-type (T8993T) cybrids. The mutant tumors generated significantly more ROS (15).

Studies in this field have established an association between mtDNA mutations and breast cancer. However, evidence for a direct association between these mtDNA mutations and breast cancer is still lacking in terms of the function of the mutant and the development of breast cancer. There has even been some debate between different research groups (9,10,16). Moreover, there is no reliable prediction of breast cancer predisposition or progression based on mtDNA mutation patterns identified thus far.

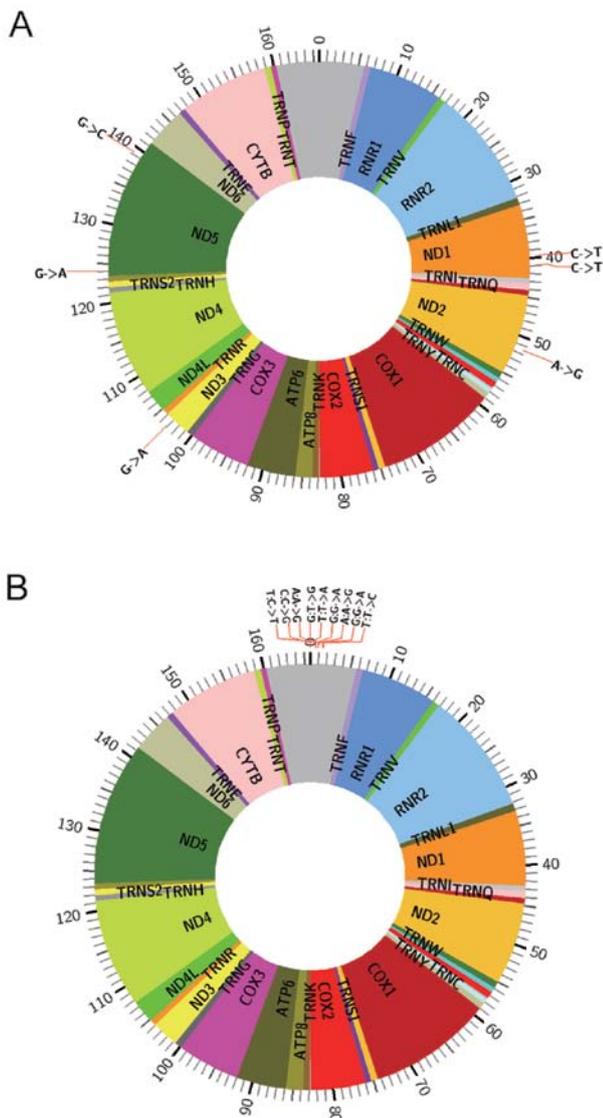


Figure 2. Single-nucleotide polymorphisms (SNPs) and heteroplasmy in pair 1 samples. (A) SNPs, (B) heteroplasmy.

Materials and methods

All blood samples were collected according to procedures approved by the Institutional Review Board of the Guangzhou General Hospital of Guangzhou Military Command. There were 58 blood samples from breast cancer patients and 58 samples from age-matched healthy individuals used in this study. mtDNA from total cellular DNA was enriched by PCR-based strategies.

In PCR-based enrichment, two sets of primers were designed to amplify amplicons that cover the mtDNA genome. The primers used in this study were as follows: Mito-8kb-A-Fwd, GACGGGCTCACATCACCCATAA/Mito-8kb-A-Rev, GCG TACGGCCAGGGCTATTGGT and Mito-8kb-B-Fwd, GGT GGCTGGCAGCAAATTGACC/Mito-8kb-B-Rev, GCC ACACTAACCTCCTCGGACTCCT.

Purified, blunt-ended PCR products were subsequently fragmented by sonication. Fragmented DNA was then end-repaired, A-tailed and ligated with an adaptor oligonucleotide from the Ion Torrent genomic DNA library preparation kit

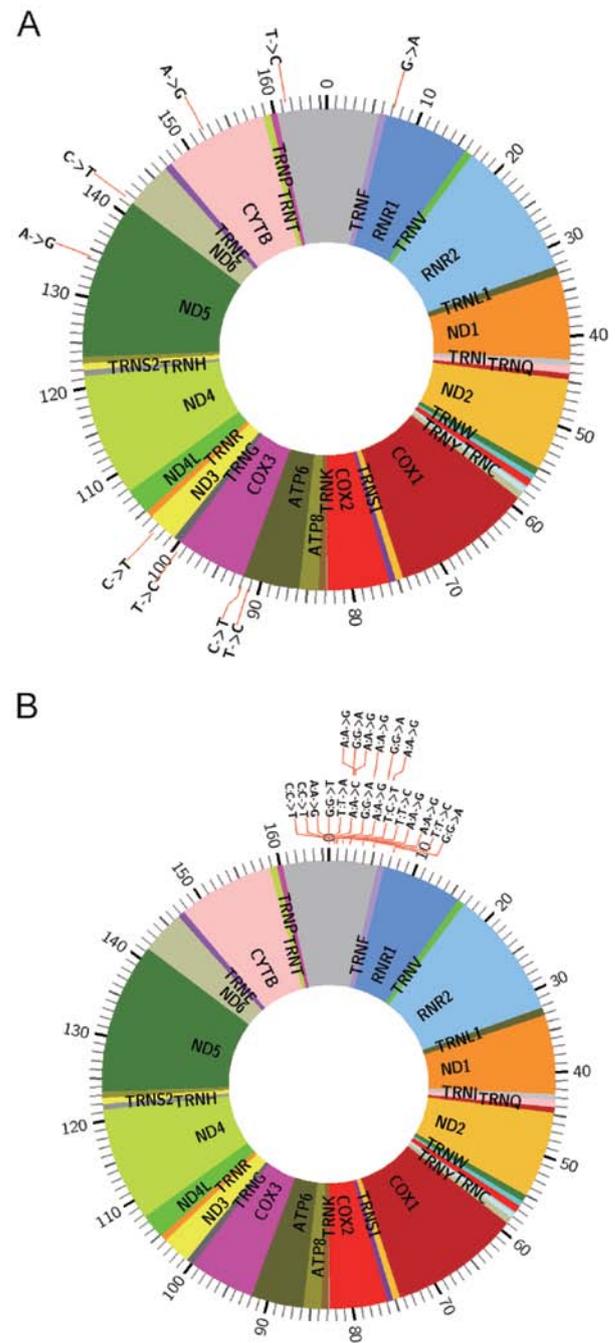


Figure 3. Single-nucleotide polymorphisms (SNPs) and heteroplasmy in pair 2 samples. (A) SNPs, (B) heteroplasmy.

(Life Technologies, South San Francisco, USA) in accordance with the manufacturer's instructions. Adaptor-ligated products were then size-selected by gel purification and sequenced by Ion Torrent PGM. The Mitomap (<http://www.mitomap.org>) and mtDB (<http://www.genpat.uu.se/mtDB/>) databases were used to identify sequence variants.

Results

High-throughput sequencing and quality control were performed. Quality scores were all above 200 and the sequence content was steady (Fig. 1). Thus, the sequence data were ready for use in further analysis.

mutation patterns thus far. In this study, we compared mtDNA sequence data from the blood of breast cancer patients and controls. We confirmed the reported variants and found a new mtDNA variant (13564 A>G). As some studies have suggested, there may be two classes of cancer mtDNA mutations: tumorigenic mutations and adaptive mutations. Tumorigenic mutations would be advantageous in the initial phases of tumor growth, while the adaptive mutations would be advantageous in the late phases of tumor growth when the tumor becomes vascularized (17). Furthermore, since the two classes of mtDNA mutations have different functions in cancer cells, they may be expected to arise and be lost from the tumor cells at different phases during tumor growth. This could be the reason that the heteroplasmid 294 nt ND1 deletion mtDNA has been shown to be present in 50% of the mtDNAs in primary renal cell carcinoma but absent in the subsequent metastatic tumors from the same patient (17,18). The same may occur in breast cancer. Moreover, Rajasimha *et al* reported that the selection against pathogenic mtDNA mutations occurs in a stem cell population. They found that the percentage of pathogenic mtDNA mutations in the blood decreases exponentially over time compared with that in hematopoietic stem cells and leukocyte precursors (19). Therefore, it can be argued that primary breast cancer cells may not be the right cell population to detect tumorigenic mtDNA mutations. In other words, studies carried out on primary breast cancer cells to date have been unable to detect tumorigenic mtDNA mutations. Thus, perhaps it would be more effective to detect tumorigenic mtDNA mutations in breast cancer stem cells.

Solid evidence supports the hypothesis that breast cancer follows a cancer stem cell model. In 2003, Al-Hajj *et al* reported that they were able to identify and isolate a minority group of breast cancer cells using the surface marker, CD44⁺/CD24⁻/low/Lin⁻. Only a few of these cells (only 100 cells) were able to form tumors in mice, whereas of the rest cell population (tens of thousands cells) failed to form tumors. Furthermore, this tumorigenic subpopulation could be serially passaged and the subsequent tumors had a heterogeneity similar to the parental tumor: CD44⁺/CD24⁻/low/Lin⁻ tumorigenic cells, as well as the phenotypically diverse mixed populations of non-tumorigenic cells (20). There may be some concern in terms of species incompatibilities since they used a model in which human breast cancer cells were grown in immunocompromised mice. However, breast cancer stem cells were confirmed in following studies using mouse models of breast cancer. In 2008, Cho *et al* isolated and characterized cancer stem cells in MMTV-Wnt-1 murine breast tumors. They demonstrated that Thy1⁺CD24⁺ cancer cells were highly enriched cells capable of regenerating new tumors compared with the cells of the tumor that did not have this marker profile (21). Similarly, Zhang *et al* identified breast cancer stem cells in a p53-null mouse model of breast cancer using the Lin⁻/CD29^{high}/CD24^{high} marker (22). Vaillant *et al* identified breast cancer stem cells in MMTV-Wnt-1, as well as in p53^{+/-} mouse models using the luminal epithelial progenitor marker, CD61/β3 integrin (23). It seems that several sets of markers can be used to isolate breast stem cells, although the functions of these markers in stem cells remain unclear. In 2007, Ginestier *et al* isolated normal and malignant human mammary stem cells using aldehyde dehydrogenase (ALDH)1 as a marker. They demonstrated that normal and cancer human mammary epithelial cells with increased ALDH activity

had stem cell/progenitor properties. In breast tumors, a high ALDH activity identified the tumorigenic cell fraction which was capable of self-renewal and generating tumors. The subsequent tumors recapitulated the heterogeneity of the parental tumor. Furthermore, the expression of ALDH1 detected by immunostaining correlated with a poor prognosis in a series of 577 breast carcinomas (24). In 2009, Charafe-Jauffret *et al* isolated breast cancer stem cells in 23 breast cancer cell lines using ALDH assay followed by FACS. They confirmed the stem cell properties of ALDH-positive populations *in vitro* and in NOD/SCID xenografts (25). Taken together, using high-throughput sequencing, the findings from our study provide new evidence to support the association between mtDNA mutations and breast cancer. As a next step, it would be helpful to detect tumorigenic mtDNA mutations in breast cancer stem cells to expand these findings.

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