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Title: The Relevance of Somatic Mutations in Mitochondrial D-loop Region and Their Relationship to TP53 Mutations in Breast Cancer

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Keywords: Mitochondrial DNA; D-loop; Breast cancer; TP53; Prognosis

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Abstract: The objective of this study was to investigate whether somatic mutations in the mitochondrial DNA (mtDNA) D-loop region correlate with known prognostic factors (age, tumor size, lymph node status, metastasis, TNM stage, lymphvascular invasion, and statuses of the progesterone receptor (PR), estrogen receptor (ER), Her2/neu and TP53 determined by immunohistochemistry), and their relationship to TP53 mutations in human breast cancer. Thirty BRCA-unrelated breast tumors as well as the adjacent non-tumorous tissues were genotyped for the mtDNA D-loop region, and the promoter as well as the coding region of the TP53 gene. Clinicopathological parameters were recorded and assessed. A total of 17 somatic mtDNA D-loop mutations was identified in 13 of 30 tumor samples (43%), of these, 2 (544C>T and 16510A>C) were novel. Four TP53 mutations were found in six tumor samples (20%), of these, 2 (c.437G>A and c.706T>C) appear to be novel. Statistic analyses showed only the status of PR correlated with the number of somatic mtDNA D-loop mutations (likehood chi-square test, $p<0.05$). Somatic mutations in the mtDNA D-loop and in TP53 seemed to be independent to each other (Fisher's exact test, $p>0.05$). These results suggested that the number of somatic mtDNA D-loop mutations may be an indicator of poor prognosis through a mechanism independent of TP53.

Cover Letter

Dear editors

Thank you for giving us the chance to revise and improve our ms, and kindly accepting our revised ms.

Sincerely yours,

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Dear Dr Chen,

According to the reviewer of your manuscript, "The Relevance of Somatic Mutations in Mitochondrial D-loop Region and Their Relationship to TP53 Mutations in Breast Cancer", the revised version is now acceptable for publication.
Please submit your last version of your revised manuscript, so we can process it for publication.
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Response to reviewer' comments

Dear editor and reviewer:

The objective of this study was to investigate whether somatic mutations in the mitochondrial DNA (mtDNA) D-loop region correlate with known prognostic factors (*i.e*. age, tumor size, lymph node status, metastasis, TNM stage, lymphvascular invasion, and statuses of the progesterone receptor (PR), estrogen receptor (ER), Her2/neu and TP53 determined by immunohistochemistry), and their relationship to *TP53* mutations as well as to the methylation status of the *TP53* promoter region in human breast cancer. We performed extensive analyses because recent studies suggested that multiple factors including somatic mtDNA D-loop mutations, *TP53* mutations as well as the methylation status in the *TP 53* promoter involve in carcinogenesis of breast cancer, but the relationship among these underlying factors was poorly understood and litter literature has been reported.

We consider the results of this study not only extend the mutation spectrum of mtDNA D-loop and *TP53* gene associated with breast cancer but also provided additional valuable findings, including: (1) the number of somatic mutations in the mtDNA D-loop correlates with a negative PR; (2) the occurrence of somatic mtDNA D-loop and *TP53* mutations (and the *TP53* promoter methylation) seemed to be independent of each other in the breast cancer patients we studied.

In the current revised manuscript (ms), **CGC-D-09-00266R3**, a radical change indeed has been made according the comments from the respected reviewer. We apologize for the previous incidents that may cause uncomfortable to the reviewer and editor you. We believe there is misunderstanding caused by our own fault. We have generated a detailed point-to-point response to you and the reviewer. We hope you and the reviewer can have a look, not only to express we indeed made the changes the reviewer kindly suggested but also try to get a second chance for revision.

We number the comments proposed by the reviewer and answered them in order as below:

Reviewer: 1

The study involves extensive experimental effort, but presentation of the paper has to be critically restructured:

1. What was the reason to undertake all mentioned experiments? What was the expected novelty of the study? I would advise to systematically review all breast cancer studies, which analyzed D-loop mutations, alone or in combination with p53 status. What was the gap in the knowledge, which the authors were going to fill? What was the justification for performing

p53 promoter methylation analysis in the context of the mtD-loop and p53 mutation testing?

> We have described the reasons to explain why we performed extensive analyses in this study, the results of a systematical review, the gap of knowledge in the somatic D-loop mutations alone and/or with *TP53* status, and the novelties of our findings in the "**ABSTRACT**", "**INTRODUCTION**" and "**DISCUSSION**" of our revised ms, **CGC-D-09-00266R3**. Since the ms has been restructured and radical change has been made, we showed the major parts we revised below **(for detail, please see pages 2, 3-5 and 14-15)**.

(1) ***Reasons why we performed the analyses (mtDNA D-loop and TP53 mutation screenings, and TP53 promoter methylation analysis) and the systematical review:***

> **(a) line 46, page 3 – line 21, page 4**

High frequency of somatic mutations was also found in the mtDNA D-loop of breast cancer, which was approximately 60 times more susceptible to mutation than the mtDNA coding region [7]. One recent study from Taiwan found that 30% (18/60) of breast cancer patients have somatic mtDNA D-loop mutations and claimed that it is an independent prognostic factor [8]. However, another recent study of early-stage breast cancer in the Chinese population found that only two of ten patients carried somatic mtDNA mutations, suggesting the prevalence of somatic mutations in breast cancer in the previous literature may have been an overestimate [23].

The fact that mitochondria are vitally important in energy metabolism, generation of reactive oxygen species, aging and apoptosis suggests that mitochondria may serve as a key switch in shifting the cell from death to abnormal cell growth, thus contributing to the neoplastic process [24]. Although mitochondrial genome instability and somatic alterations have been demonstrated in breast cancer, the correlation between the mtDNA mutations and the clinicopathological parameters of breast cancer remains poorly understood.

(b) line 31, page 4 – line 6, page 5

The TP53 has been shown to have close functional interaction with mitochondria in maintaining normal mitochondrial homeostasis including its genomic stability. The concomitant presence of mutations in *TP53* and mtDNA were predominant in breast cancer with poor prognosis. However, among the tumors with mutations in both the mtDNA and in the *TP53* gene, the non-D-loop mutations contributed more significantly to the poor prognosis than the D-loop mutations [27]. Because the mutation occurs much more frequently in the mtDNA D-loop than in the non-D-loop region in human cancers, the relationship between the mtDNA D-loop and *TP53* in the tumorigenesis of breast cancer is therefore worthwhile for further study. Recently, promoter methylation has

been linked to gene silencing and cancer progression, and aberrant methylation in the *TP53* promoter region was also reported to involve in carcinogenesis of breast cancer [28]. We therefore intended to examine not only mtDNA D-loop mutations but also mutations in the coding region and the promoter methylation status of *TP53* by genotyping the pathology-proven human breast cancers without *BRCA1*/*BRCA2* mutations.

(2) *The gap of the present knowledge we wish to fill*

(a) lines 9 - 21, page 6

In this study, we reassess the correlation between somatic mtDNA D-loop mutations with clinicopathological parameters (*e.g.* age, tumor size, lymph node status, metastasis) with known prognostic relevance in 30 patients with breast cancer without *BRCA1*/*BRCA2* mutations. We wished to know better that in human breast cancers, whether the somatic mutations in the mtDNA D-loop are of prognostic value, and if such correlation works through a mechanism dependent or independent of *TP53* mutations.

(3) *The novel findings of our study*

(a) lines 52-60, page 14 – lines 4-11, page 15

For what appears to be the first time in the literature, we found that not only the presence, but also the number of somatic mutations in the mtDNA D-loop region correlates with a negative PR, and therefore may be an indicator of poor prognosis in breast cancer. Additionally, to address the possible role of somatic mtDNA D-loop mutaions in respective to the *TP53* mutations in tumorigenesis, we report the mutant alleles within these genomic segments (mtDNA D-loop and *TP53* coding region) among the Taiwanese breast cancer patients. We found that the occurrence of somatic mtDNA D-loop and *TP53* mutations (and the *TP53* promoter methylation) seemed to be independent of each other in the breast cancer patients we studied.

2. How the heteroplasmy of mitochondrial mutations was discriminated from the contamination of tumor tissue by normal cells? How quantitative aspects of heteroplasmy were assessed (see Results section), if the methodology of mutation detection was limited to the DNA sequencing?

> Indeed, because the DNA was not derived from a microdissected tumor, we cannot exclude the possibility that contamination of the surrounding non-tumorous tissue contributed to the heteroplasmy we observed. Nevertheless, this heteroplasmic phenomenon may represent the progression of tumorigenesis. Since we only used

sequencing-based technology, the term “heteroplasmy” we described in this study simply denote the coexistence of two alleles in a specific nucleotide position instead of the different percentages of mutant-carrying mitochondria in different tumor tissues, which can only be determined with quantitative methods. We add the paragraph (please see below) in “**MATERIALS AND METHODS**” to show the term “heteroplasmy” we described in the text will simply denote the coexistence of different alleles at a specific nucleotide position without quantification.

(a) lines 33-48, page 7

Notably, in this study, the somatic mutations denoted those mutations found only in the tumor tissue but not in the adjacent non-cancerous part. Sequence variants observed in both tumor and non-tumorous samples without quantitative differences were considered to be germline mutations/variations of human mtDNA. Besides, since we only used sequencing-based technology, the term “heteroplasmy” we described in the text will simply denote the coexistence of different alleles at a specific nucleotide position instead of the different percentages of mutant-carrying mitochondria in different tissues, which can only be determined with quantitative methods.

3. The manuscript has to be critically shortened. Although the English is generally good, there are some language deficiencies which confuse the meaning. For example, the description of p53 mutations (number of types of mutations / number of cases with mutation) is confusing.

> The revised ms has been shortened as suggested. Besides, the description about mtDNA D-loop and TP53 mutations has been rectified (see below).

(a) lines 25-37, page 2

Four *TP53* mutations were found in six tumor samples (20%), of these, 2 (c.437G>A and c.706T>C) appear to be novel. Statistic analyses showed only the status of PR correlated with the **number of somatic mtDNA D-loop mutations** (likehood chi-square test, $p<0.05$). Somatic mutations in the mtDNA D-loop and in *TP53* seemed to be independent to each other (Fisher’s exact test, $p>0.05$). These results suggested that **the number of somatic mtDNA D-loop mutations** may be an indicator of poor prognosis through a mechanism independent of TP53.

(b) lines 28-33, page 11

Statistical significance was observed only between **the number of somatic D-loop**

mtDNA mutations and the status of the progesterone receptor (PR), which was negatively associated with **the number of somatic mtDNA D-loop mutations** ($p<0.05$; Table 3).

(c) lines 6-19, page 13

In addition, a previous similar study from Taiwan did not assess **the number of somatic mutations in the mtDNA D-loop**, but only used the presence or absence of such mutations to correlate with other clinicopathological parameters [8]. We actually observed that patients with two or more somatic mutations in D-loop mtDNA had a much poorer survival rate than those with no or only one somatic mutation of D-loop mtDNA (Figure 1). Therefore, we took **the number of somatic mutations** into consideration when performing our statistical analyses.

(d) lines 43-53, page 13

A correlation between **the number of somatic mtDNA D-loop mutations** and the negative status of the progesterone receptor is established. Since a negative PR means a poor prognosis in breast cancer, we speculate that **the number of somatic mutations in the mtDNA D-loop** may be an indicator for poor prognosis, a finding that echoes well with the report of another study on the same population [8].

(e) lines 53-58, page 14

For what appears to be the first time in the literature, we found that not only the presence, but also **the number of somatic mutations in the mtDNA D-loop region** correlates with a negative PR, and therefore may be an indicator of poor prognosis in breast cancer.

(f) lines 7-9, page 17

Figure 1. Cumulative survival curve of the 30 patients examined with respective to **the number of somatic mtDNA D-loop mutations** detected.

Research Article to Cancer Genetics Cytogenetics

The Relevance of Somatic Mutations in Mitochondrial D-loop Region and Their Relationship to *TP53* Mutations in Breast Cancer

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Running Title: MtDNA D-loop and *TP53* mutations in breast cancer

Abstract

The objective of this study was to investigate whether somatic mutations in the mitochondrial DNA (mtDNA) D-loop region correlate with known prognostic factors (age, tumor size, lymph node status, metastasis, TNM stage, lymphvascular invasion, and statuses of the progesterone receptor (PR), estrogen receptor (ER), Her2/neu and TP53 determined by immunohistochemistry), and their relationship to *TP53* mutations in human breast cancer. Thirty BRCA-unrelated breast tumors as well as the adjacent non-tumorous tissues were genotyped for the mtDNA D-loop region, and the promoter as well as the coding region of the *TP53* gene. Clinicopathological parameters were recorded and assessed. A total of 17 somatic mtDNA D-loop mutations was identified in 13 of 30 tumor samples (43%), of these, 2 (544C>T and 16510A>C) were novel. Four *TP53* mutations were found in six tumor samples (20%), of these, 2 (c.437G>A and c.706T>C) appear to be novel. Statistic analyses showed only the status of PR correlated with the number of somatic mtDNA D-loop mutations (likehood chi-square test, $p<0.05$). Somatic mutations in the mtDNA D-loop and in *TP53* seemed to be independent to each other (Fisher's exact test, $p>0.05$). These results suggested that the number of somatic mtDNA D-loop mutations may be an indicator of poor prognosis through a mechanism independent of TP53.

***Keywords*: Mitochondrial DNA; D-loop; Breast cancer; *TP53*; Prognosis**

1. Introduction

Breast cancer is the most common cancer of women in most industrialized countries [1]. Its incidence has rapidly increased over the past decade in Asia as well as in many areas of the world [2,3]. It is associated with different types of molecular aberrations such as mutations harbored in oncogenes and tumor suppressor genes [4-6]. While, recent studies showed somatic mutations in mitochondrial DNA (mtDNA) may also contribute to the initiation and progression of human breast cancer [7,8].

Mutations in the mtDNA have been identified in various types of cancers, including cancers of colorectal, head and neck, liver, lung, stomach, prostate, and breast [7,9-13]. Of these, a variety of mtDNA defects was identified, such as dimerism in human leukemia leukocytes [14], partial deletions in the tRNA-Tyr and tRNA-Trp genes in rat liver tumor [15] and heteroplasmy in tumor tissues [16]. A survey of the mtDNA in bladder, head and neck, and lung cancers showed 51% (21/41) contained tumor-specific mtDNA mutations [10].

The D-loop region of mtDNA, which is non-coding and is so called because of its structure, is a hotbed of mutation and contains two hypervariable regions [17]. Recent surveys of the D-loop revealed that 48% (15/31) of gastric tumors [18], 39% (24/61) of hepatocellular carcinomas (both studies were from Taiwan) [19], 68% (13/19) of hepatocellular carcinomas (from Japan) [20] and 88% (14/16) of prostate cancer [21,22] harbored tumor-specific mtDNA mutations. High frequency of somatic mutations was also found in the mtDNA D-loop of breast cancer, which was approximately 60 times more susceptible to mutation than the mtDNA coding region [7]. One recent study from Taiwan found that 30% (18/60) of breast cancer patients have somatic mtDNA D-loop mutations and claimed that it is an independent prognostic factor [8]. However, another recent study of early-stage breast cancer in the Chinese population found that only two of ten

patients carried somatic mtDNA mutations, suggesting the prevalence of somatic mutations in breast cancer in the previous literature may have been an overestimate [23].

The fact that mitochondria are vitally important in energy metabolism, generation of reactive oxygen species, aging and apoptosis suggests that mitochondria may serve as a key switch in shifting the cell from death to abnormal cell growth, thus contributing to the neoplastic process [24]. Although mitochondrial genome instability and somatic alterations have been demonstrated in breast cancer, the correlation between the mtDNA mutations and the clinicopathological parameters of breast cancer remains poorly understood.

The dysfunction of another important guardian-angel gene, *TP53,* is one of the most common genetic alterations found in human cancers. Almost 50% of human cancers and 20-30% of breast cancers contain mutations in the *TP53* [25]. This high incidence of mutations suggests that the *TP53* may constitute a useful tumor marker [26]. The TP53 has been shown to have close functional interaction with mitochondria in maintaining normal mitochondrial homeostasis including its genomic stability. The concomitant presence of mutations in *TP53* and mtDNA were predominant in breast cancer with poor prognosis. However, among the tumors with mutations in both the mtDNA and in the *TP53* gene, the non-D-loop mutations contributed more significantly to the poor prognosis than the D-loop mutations [27]. Because the mutation occurs much more frequently in the mtDNA D-loop than in the non-D-loop region in human cancers, the relationship between the mtDNA D-loop and *TP53* in the tumorigenesis of breast cancer is therefore worthwhile for further study. Recently, promoter methylation has been linked to gene silencing and cancer progression, and aberrant methylation in the *TP53* promoter region was also reported to involve in carcinogenesis of breast cancer [28]. We therefore intended to examine not only mtDNA D-loop mutations but also mutations in the coding region and the promoter methylation

status of *TP53* by genotyping the pathology-proven human breast cancers without *BRCA1*/*BRCA2* mutations.

In this study, we reassess the correlation between somatic mtDNA D-loop mutations with clinicopathological parameters (*e.g.* age, tumor size, lymph node status, metastasis) with known prognostic relevance in 30 patients with breast cancer without *BRCA1*/*BRCA2* mutations. We wished to know better that in human breast cancers, whether the somatic mutations in the mtDNA D-loop are of prognostic value, and if such correlation works through a mechanism dependent or independent of *TP53* mutations.

2. Materials and methods

2.1. Patients and tissue samples

A total of 30 female patients with breast cancer were enrolled in this study. Pathological diagnoses of the cancer tissues showed only ductal and/or lobular carcinomas in all samples examined. For each patient, the clinicopathological parameters, including the age at the time of diagnosis (two age groups: <50 and ≧ 50 years old, including 10 and 20 patients, respectively), tumor size (T1-T4), lymph node status (L0-L3), metastasis (M0-M1), TNM stage (I-IV), lymphvascular invasion (Yes/No), and statuses of hormone receptors (including ER and PR), epidermal growth factor (Her2_neu) and TP53 protein accumulation, were scored and the prognostic relevance (*e.g.* impact on survival, effect of systemic therapy) of each was established. Both of the breast cancer tissues and adjacent non-tumorous breast tissues were obtained from the patients after obtaining their informed consent, and these were used for mutation analyses. This work was performed with approval of the Institutional Review Board of Changhua Christian Hospital, Taiwan.

2.2 Mutation analyses

Total cellular DNA from the patients' cancer tissues and adjacent non-tumorous tissues were extracted separately using the Genomic DNA Purification Kit (Gentra Systems, USA). All DNA samples were excluded to include any *BRCA1* and *BRCA2* mutations by a preliminary sequencing analysis and were subsequently used for mtDNA D-loop and *TP53* gene mutation analyses.

2.2.1. Somatic mutation analysis in mtDNA D-loop region

Somatic mutations in the mtDNA D-loop region were analyzed by polymerase chain reaction (PCR) and direct sequencing as described in [19]. Three primer sets: (1) mtF159

(5’-TATCGCACCTACGTTCAATAT-3’) and mtR577 (5’-CTGTGGGGGGTGTCTTTGG-3’), (2) mtR242 (5’-GTTATT ATTATGTCCTACAAGC-3’) and mtF16411 (5’-CGTGAAATCAATATCCCG-3’), and (3) mtF16100 (5’-ATTACTGCCAGCCACCATG-3’) and mtR16544 (5’-ACGTGTGGGCTATTTAGG-3’), were used for the amplifications of three different mtDNA D-loop fragments. The PCR reaction mixture contained 20 ng DNA, 375 μM of each dNTP, 50 nmol of each primer, 1x PCR buffer and 2.5 U Taq DNA polymerase (BD Biosciences, USA) in a final volume of 20 μl. The amplification condition was 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 60 s. PCR products were sequenced using the ABI 3130xl Genetic Analyzer and the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystem, Foster City, CA). For those samples examined showing mtDNA heteroplasmy and/or somatic variations, re-sequencings for newly amplified PCR products were carried out for the PCR products to confirm the sequencing results.

Notably, in this study, the somatic mutations denoted those mutations found only in the tumor tissue but not in the adjacent non-cancerous part. Sequence variants observed in both tumor and non-tumorous samples without quantitative differences were considered to be germline mutations/variations of human mtDNA. Besides, since we only used sequencing-based technology, the term “heteroplasmy” we described in the text will simply denote the coexistence of different alleles at a specific nucleotide position instead of the different percentages of mutant-carrying mitochondria in different tissues, which can only be determined with quantitative methods.

2.2.2. Mutation analysis in TP53 gene in breast cancer

Possible mutations in the exons 5, 7 and 8 of *TP53* gene in the cancer tissues were further screened by PCR and direct sequencing as described in [29]. Three primer sets: (1) *TP53*-5F (5’-GTTTCTTTGCTGCCGTCTT-3’) and *TP53*-5R (5’-AATCAGTGAAGGAATCA GAGG-3’), (2) *TP53*-7F (5’-ATCTTGGGCCTGTGTTAT-3’) and *TP53*-7R (5’-GGAT

GGGTAGTAGTATGGAA-3') and (3) *TP53*-8F (5'-AGGGTGGTTGGGAGTAGAAT G-3') and *TP53*-8R (5'-AGAGGCAAGGAAAGGTGATA-3'), specific for the E5, E7 and E8 of *TP53* were used in the PCR amplifications. The PCR reaction mixture and cycling profile were similar to that used in mtDNA D-loop mutation screening (see above), except for the primer sets and the Tm (60°C) used. PCR products were sequenced on the ABI 3130 Automatic DNA Sequencer (Applied Biosystems).

2.3. Methylation analysis of TP53 promoter region

The methylation status of CpG islands in the *TP53* promoter region were assessed by DNA bisulfite treatment followed by PCR and DNA sequencing. DNA bisulfite treatment was performed with MethylSEQr^{TM} Bisulfite Conversion Kit (Applied Biosystem, Foster City, CA) according to the manufacturer's instructions. The *TP53* promoter region of the bisulfite-treated DNA was then amplified by a nested PCR described by Kang *et al*. [28] with some modifications. Briefly, the PCR reaction mixture contains 0.05 mM deoxynucleotide triphosphate, 1.25 U Fast Start Taq (Roche Molecular Biochemicals), 5 pmol of each primer, 1x PCR buffer, 2.5 µl bisulfite converted DNA and 1 µl primary PCR product in a final volume of 25 µl. The PCR profile consisted of initial denaturation at 94°C for 5 min, followed by 45 cycles (94°C for 1 min, 52°C for 1 min, 72°C for 1 min), with a final extension at 72°C for 2 min. The PCR products were purified with a QIAEX II PCR purification kit (Qiagen) and then sequenced on the ABI 3130 Automatic DNA Sequencer (Applied Biosystems).

2.4. Immunohistochemistry

Immunohistochemistry for estrogen receptor (ER), progesterone receptor (PR), TP53 and Her2/neu were performed on 4 mm-thick sections on slides, as described in [30]. Briefly, the

procedure was as follows. After deparaffinization, endogenous peroxides were inactivated with 3% H_2O_2 in methanol for 15 minutes at room temperature. This was followed by incubation with protein blocking solution (Dako, Carpinteria, CA) for 30 min at room temperature. The slides were then incubated with primary monoclonal antibody to the ER, PR, TP53 or Her2/neu (Thermo Fisher Scientific Co., Pittsburgh, PA) at 4 °C for 7 hr. The sample was further incubated with a peroxidase-conjugated goat anti-mouse immunoglobulin for 1 hr. Finally, a colorimetric reaction was carried out with a solution containing 50 mM Tris HCl, pH 7.6, 0.66 mol/l of 3.3'-diaminobenzine, and 0.06% of H_2O_2, and then counterstaining with hematoxylin stain was performed. For each sample, three specimens/slides were prepared and examined.

2.5. Slide Evaluation

Four areas of each slide were selected at random and photographed for evaluation. The staining intensity was scored semiquantitatively according to percentage of positively stained cells: (1) grade 0, absence of expression; (2) grade 1, low expression (<10% positive cells); (3) grade 2, median expression (10–75% positive cells); (4) grade 3, high expression (≥75% positive cells).

2.6. Statistical Analyses

The likelihood chi-square test was used to compare somatic mtDNA D-loop alterations and clinicopathological parameters. The relationship between somatic mtDNA D-loop and *TP53* mutations was evaluated by Fisher's exact test. The difference between the comparing groups was considered statistically significant when the *p* value was smaller than 0.05. All statistical analyses were performed on the Statistical Program for Social Sciences program package (SPSS inc.).

3. Results

3.1. Somatic mutations in MtDNA D-loop

A total of 17 somatic mtDNA D-loop mutations (*i.e.* mutations found in breast cancer but not in paired adjacent normal tissue) at 10 distinct nucleotide positions were found in 13 (43%) of the 30 breast cancer samples examined (Table 1). Among the mutations, seven were substitutions (A189G, C489T, C544T, A16274G, T16298C, G16335A and A16510C) and ten were small deletions or varieties of short sequence repeats (514delC, poly C varieties at nucleotide position (np) 303-309 and CA dinucleotide changes at np 514-523). All of the mutations are transitional substitutions and become to heteroplasmic states, except for a cytosine copy number change (9C→8C) at np 303-309 in three samples examined (Table 1). The mononucleotide repeats at np 303-309 is a well known mutational hotspot and prone to instability; somatic mutations at this position were found in 10 (33%) of 30 cancer samples examined. Of the described mutations, C544T and A16510C are two newly identified D-loop variations that have not been previously reported in the literature and the public mtDNA mutation databases (mtDBase: http://www.genpat.uu.se/mtDB/index.html; MITOMAP: http://www.mitomap.org/cgi-bin/tbl6gen.pl). Multiple somatic mutations in the D-Loop region were observed two cancer samples: one has four heteroplasmic alterations and one has two heteroplasmic varieties.

3.2. TP53mutations in breast cancer

Of the 30 examined breast cancer samples, four different (three missense and one nonsense) *TP53* mutations were found, including c.437G>A (p.W146X), c.524G>A (p.R175H), c.706T>C (p.Y236H), and c.853G>A (p.E285K) (Table 2). Among these mutations, c.437G>A (p.W146X)

and c.706T>C (p.Y236H) had not been reported in the literature or the accessible gene mutations database (HGMD: http://www.hgmd.cf.ac.uk/ac/index.php).

3.3. Methylation of the TP53 promoter region in breast cancer and paired normal tissues

There was no apparent difference between paired breast cancer and normal tissue regarding the methylation status in the *TP53* promoter region. Of the 16 potential methylation sites in the 400 bp promoter region of *TP53*, no methylated CpG sites were identified.

3.4. Correlation between somatic mtDNA D-loop mutations and clinicopathological parameters with known prognostic significance

Statistical significance was observed only between the number of somatic mtDNA D-loop mutations and the status of the progesterone receptor (PR), which was negatively associated with the number of somatic mtDNA D-loop mutations ($p<0.05$; Table 3). Somatic mutations occurring in the mtDNA D-loop region and mutations in the *TP53* gene are independent of each other ($p>0.05$; Table 4). In addition, patients with two or more somatic mtDNA D-loop mutations appeared to have a much poorer survival rate than the patients with no or one somatic mtDNA D-loop mutation (Figure 1).

4. Discussion

The importance of D-loop region has been described as comprising other origins of mitochondrial genome [31]. It is believed that mutations in this region result in mitochondrial genome instability and contribute to the process of carcinogenesis. Two previous studies of scanning the entire mitochondrial genome in American population showed 93% [13] and 74% of breast tumors displayed at least one somatic mtDNA mutation while 63.2% of the cases had mutation in the D-loop region [7]. Focusing on the D-loop region analyzing, 10.8% of breast ductal adenocarcinomas have also been described with the existence of somatic mutation [32]. A recent study of the D-loop region in 94 pairs of breast normal/cancer tissues showed 40.42% somatic mtDNA mutation in cancer tissues [33], nearby our present D-loop mutation frequency (43%). Although the mutation frequencies from literatures appear divergence from 10.8% to 65%, it is high enough to believe that D-loop mutation is not a sporadic phenomenon of breast cancer.

A recent study from Taiwan reported that somatic mutation in the mtDNA D-loop region is a new prognostic indicator related to poor prognosis [8]. They found that these breast cancer patients from Taiwan had mtDNA depletion and a higher rate of somatic mtDNA D-loop mutations. However, the 4,977 bp deletion commonly found in studies from Western countries failed to be elucidated in this series from Taiwan [8]. Such a finding caused us to postulate that not only does the presence or absence of mtDNA mutations matter, the genotype and number of mutations may also be important. Different ethnic backgrounds and different tissue types may associate with different mutant alleles, which may contribute to the inconsistent findings regarding their relevance in the previous literature. In this study, 17 somatic mtDNA D-loop were identified (Table 1), while it is interesting to note that some mutations, for example, 16274G->A and 16298 T->C, are originally reported in other cancers, rather than breast cancer. Future studies are needed

to elucidate the biological meaning of these different mutant alleles in the process of tumorigenesis in different tissues. In addition, a previous similar study from Taiwan did not assess the number of somatic mutations in the mtDNA D-loop, but only used the presence or absence of such mutations to correlate with other clinicopathological parameters [8]. We actually observed that patients with two or more somatic mutations in D-loop mtDNA had a much poorer survival rate than those with no or only one somatic mutation of D-loop mtDNA (Figure 1). Therefore, we took the number of somatic mutations into consideration when performing our statistical analyses.

Older age, advanced TNM stage, positive lymphovascular invasion, and the accumulation of defective p53 protein in the cancer cells are definite poor prognostic indicators in breast cancer. Positive staining of the estrogen and/or progesterone receptor in breast cancer is associated with a favorable response to hormone therapy, which represents a favorable outcome. Strong staining of the Her2/neu protein is also an established prognostic indicator after targeted therapy in breast cancer [1,23]. We admit that our case number is small (n = 30) but a trend of poor prognosis by observing a much shorter survival time in our patients who carried two or more somatic mutations in the mtDNA D-loop than those with no or only one somatic mutation is evidenced (Figure 1). We assessed the correlation between the somatic mtDNA D-loop mutations and those clinicopathological parameters with recognized prognostic relevance. A correlation between the number of somatic mtDNA D-loop mutations and the negative status of the progesterone receptor is established. Since a negative PR means a poor prognosis in breast cancer, we speculate that the number of somatic mutations in the mtDNA D-loop may be an indicator for poor prognosis, a finding that echoes well with the report of another study on the same population [8].

Recently, growing evidence has shown that TP53 has a close functional interaction with the mitochondrion during tumorigenesis. It is believed that TP53 has inseparable functional interaction with mitochondria in maintaining normal mitochondrial homeostasis. Concomitant *TP53* and

somatic mtDNA D-loop mutations were found to associate with a poor prognosis in breast cancer in a study from India [27]. Somatic D-loop mtDNA mutations were found to be more prevalent in *TP53*-mutated colorectal cancers than in those without *TP53* mutations. However, by Fisher's exact test, we found no correlation existed between the *TP53* and somatic mtDNA D-loop mutations (Table 4). In addition to the DNA level, we also assessed the promoter region of *TP53*, as well as its receptor status in the cancer cells. None of them showed any significant correlation with D-loop mtDNA mutations. The discrepancy between our present study and the previous studies may originate from different ethnic groups (Taiwan vs. India) or from different tissue types from the same population (colorectum vs. breast). However, looking at the different mutation spots and patterns may provide some insights into the mechanisms that underlie these discrepancies.

Among the *TP53* mutations we found, the nonsense p.W146X mutation would lead to truncated protein and therefore may be nonfunctional. To our knowledge, a truncated TP53 protein or null mutations were related to early, distant metastases [34]. The missense p.Y236H mutation would result in an amino acid substitution of tyrosine to histidine at position 236, which has been described in another missense mutation (p.Y236C) in a patient with Li-Fraumeni syndrome [35]. It was hypothesized that a mutation at this position could result in the hydrogen bond conflicts [36]. The remaining two missense mutations, p.R175H and p.E285K, have been reported in osteosarcoma [37] and multiple myeloma [38], respectively. These missense mutations would not be expected to be functionally equivalent to the wild-type allele and are considered to be pathogenic.

For what appears to be the first time in the literature, we found that not only the presence, but also the number of somatic mutations in the mtDNA D-loop region correlates with a negative PR, and therefore may be an indicator of poor prognosis in breast cancer. Additionally, to address the possible role of somatic mtDNA D-loop mutaions in respective to the *TP53* mutations in

tumorigenesis, we report the mutant alleles within these genomic segments (mtDNA D-loop and *TP53* coding region) among the Taiwanese breast cancer patients. We found that the occurrence of somatic mtDNA D-loop and *TP53* mutations (and the *TP53* promoter methylation) seemed to be independent of each other in the breast cancer patients we studied.

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Figure Legends

Figure 1. Cumulative survival curve of the 30 patients examined with respective to the number of somatic mtDNA D-loop mutations detected.

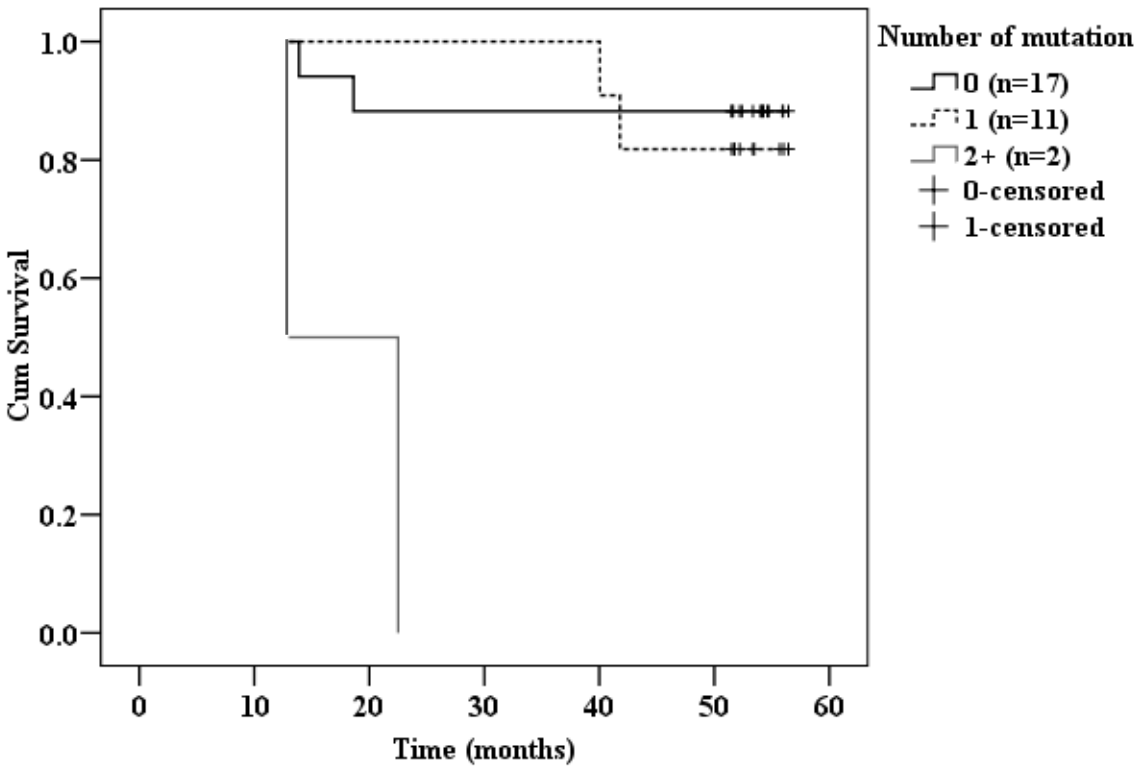
Number of mutation
0 (n=17)
1 (n=11)
2+ (n=2)
0-censored
1-censored
Cum Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
Time (months)

Figure 1

Table 1 The 17 Somatic mtDNA D-loop mutations identified in 30 breast cancer samples

np	Somatic mutation	Cambridge sequence	Normal to tumor pattern [a]	Function	No. of patients	Previously reported in tumor	Reference
189	A → G	A	homo → hetero	H-strand origin	1	elderly muscle	[40]
	8C/9C → 7C/8C/9C		hetero → hetero		1		
	8C/9C/10C → 7C/8C/9C/10C		hetero → hetero		1		
	8C/9C/10C → 8C/9C		hetero → hetero		1		
	9C → 8C		homo → homo	conserved sequence block	3		[7],[8],[12],[41],[42],[43]
	7C → 7C/8C		homo → hetero		1		
	7C/8C/9C → 8C/9C		hetero → hetero		1	colorectal, gastric,	
	8C/9C → 8C/9C/10C		hetero → hetero		1	esophageal, ovarian,	
303-309	8C/9C/10C → 9C/10C/11C	7C	hetero → hetero		1	and breast	
489	T → C	T	hetero → hetero	hypervariable segment 3	1	ovarian	[42]
514	C loss	C	homo → hetero	hypervariable segment 3	1		
514-523	7(CA) → 6(CA)	5(CA)	homo → hetero	hypervariable segment 3	1	cervical, endometrial, ovarian,	[43],[44]
544	C → T	C	homo → hetero	mtTF1 binding site H	1	novel	Present study
16274	G → A	G	hetero → hetero	hypervariable Segment 1	1	prostate, oral	[21],Unpublished data [b]
16298	T → C	T	hetero → hetero	hypervariable Segment 1	1	prostate, oral	[21],Unpublished data [c]
16335	A → G	A	hetero → hetero	hypervariable Segment 1	1	Polymorphisms	Unpublished data [d]
16510	A → C	A	homo → hetero		1	novel	Present study

[a]hetero: heteroplasmic ; homo: homoplasmic.
[b]Saranath, Dhananjaya ; Basak, Subhankar, 2003. MITOMAP mtDNA Sequence Data, http://www.mitomap.org/cgi-bin/tbl15gen.pl#20030527015.
[c]Saranath, Dhananjaya ; Basak, Subhankar, 2003. MITOMAP mtDNA Sequence Data, http://www.mitomap.org/cgi-bin/tbl15gen.pl#20030528109.
[d]Abu-Amero, Khaled K , 2005. MITOMAP mtDNA Sequence Data, http://www.mitomap.org/cgi-bin/tbl15gen.pl#20050919029.

Table 2 The four TP53 mutations identified in 30 breast cancer samples

Nucleotide change[a]	Amino acid change[b]	Exon	Mutation type	No. of patients	Other reported mutations at the same site	Reference
c.437G>A	p.W146X	5	Nonsense	1	—	Present study
c.524G>A	p.R175H	5	Missense	2	p.R175L and p.R175G	[37]
c.706T>C	p.Y236H	7	Missense	1	p.Y236C	Present study
c.853G>A	p.E285K	8	Missense	2	p.E285Q	[38]

[a]Mutations are numbered based on the mRNA sequence (GenBank NM_000546.3). +1 corresponds to the first nucleotide of translation initiation codon.
[b]Amino acid changes are numbered from the Methionine 1 of tumor protein p53 sequence (NP_000537.3).

Table 3 Status of somatic mtDNA D-loop mutations and its correlation with clinicopathological parameters

			Somatic mtDNA D-loop Mutation			
			Negative	Positive		
			0 (n = 17)	1 (n = 11)	≧ 2 (n = 2)	Likelihood chi-square test (*p* -value)
Age		<50	6	2	2	0.058
		≧ 50	11	9	0	
T		T1	8	2	1	0.147
		T2	7	7	0	
		T3	1	0	1	
		T4	1	2	0	
L		L0	12	5	1	0.361
		L1	1	2	1	
		L2	1	3	0	
		L3	3	1	0	
M		M0	17	11	1	0.05
		M1	0	0	1	
Lymphovascular invasion		No	12	7	2	0.442
		Yes	5	4	0	
TNM stage		I	7	1	1	0.054
		II	6	5	0	
		III	4	5	0	
		IV	0	0	1	
ER (IHC)	Negative	0	5	5	2	0.11
	Positive	1	2	4	0	
		2	3	0	0	
		3	7	2	0	
PR (IHC)	Negative	0	10	6	2	0.005*
	Positive	1	0	5	0	
		2	2	0	0	
		3	5	0	0	
HER2_neu (IHC)	Negative	0	12	8	1	0.529
	Positive	1	2	0	0	
		2	1	0	0	
		3	2	3	1	
TP53 (IHC)	Negative	0	9	5	1	0.691
	Positive	1	5	3	0	
		2	1	1	1	
		3	2	2	0	

Age, age at the time of diagnosis; T, tumor size; L, lymph node status; M, metastasis; ER, estrogen receptor; PR, progesterone recptor; IHC, immunohistochemistry; *Statistic significance p<0.05

Table 4 No association between somatic mtDNA D-loop mutations and TP53 mutations

		Somatic mtDNA D-loop DNA		
		Wild-type	Mutant	Total
TP53	Wild-type	14	10	24
	Mutant	3	3	6
	Total	17	13	30

Fisher's exact test: $p > 0.05$

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