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mtDNA copy number/*miR663/AATF* axis in invasive ductal carcinoma of the breast

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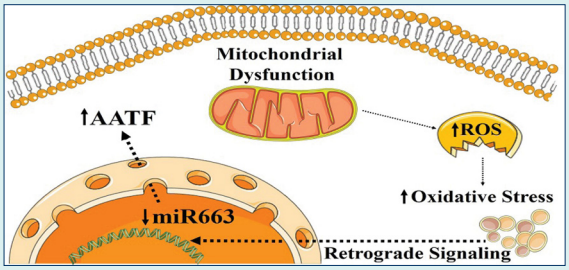
Abstract



Introduction: Mitochondrial DNA (mtDNA) copy number variations have been reported in multiple human cancers. Previous studies indicate that mitochondrial retrograde signaling regulates *miR663*, which plays a key role in tumorigenesis, including regulating apoptosis antagonizing transcription factor (*AATF*). This study investigates the expression of *miR663* and *AATF* in relation to mtDNA copy number in invasive ductal carcinoma (IDC) of the breast.

Methods: Paired primary tumors and adjacent non-tumor tissues were analyzed to assess changes in *miR663* and *AATF* expression using fold-change analysis. The mtDNA copy number was quantified using *COX1* as the mitochondrial gene and *COX4* as the nuclear control gene. To validate the findings, publicly available data from The Cancer Genome Atlas (TCGA) were also analyzed.

Results: A significant reduction in tumor *miR663* expression was observed (fold change = 0.139), with a strong correlation between *miR663* and *AATF* expression. A significant Z-score difference was also detected between *miR663* and mtDNA copy number. *miR663* was predominantly expressed in grade I tumors but significantly downregulated in higher-grade tumors, whereas *AATF* expression increased with tumor grade. In silico analysis of TCGA data confirmed elevated *AATF* expression, with notable variations across breast cancer subtypes.

Conclusion: We observed reduced expression of *miR663* and mtDNA copy number in breast tumors, along with variations in *AATF* levels across subtypes. The decrease in *miR663* could be associated with lower mtDNA copy numbers and impaired retrograde signaling, impacting *AATF* expression and function. Our findings underscore the therapeutic promise of targeting the mtDNA/miR-663/AATF axis, which could lead to advancements in breast cancer treatment.

Introduction

Evidence suggests that breast cancer is not solely a genetic disease but also a metabolic disorder.[1] Defects in mitochondrial metabolism, particularly the reduction of mitochondrial DNA (mtDNA) content, play a crucial role in breast tumorigenesis.[2] Several studies have emphasized the importance of mitochondria in carcinogenesis, including metabolic reprogramming, apoptosis evasion, genomic instability, and tumor metastasis.[3,4]

Mitochondria primarily generate cellular energy through oxidative phosphorylation (OXPHOS) via the electron transport chain (ETC). Disruptions in this process increase reactive oxygen species (ROS) production, leading to oxidative stress, a major contributor to DNA damage and tumor formation.[4] Mitochondria contain multiple copies of mtDNA to ensure efficient ETC protein production. Variations in mtDNA copy number contribute to oxidative stress and are implicated in the progression of multiple tumors, including breast cancer.[5,6]

Studies indicate that somatic mtDNA mutations accumulate in primary breast tumors with age. Low mtDNA levels have also been associated with tumor invasion and chemotherapy resistance.[2] A recent study analyzed somatic mutations in 205 genes involved in glycolysis and OXPHOS pathways using The Cancer Genome Atlas (TCGA) dataset to investigate

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mitochondrial dysfunction in breast cancer. Among 968 patient samples, mutations were identified in 132 genes, with seven genes recognized as potential biomarkers for breast cancer.[7]

Mitochondria regulate nuclear gene expression via mitochondrial retrograde signaling pathways.[8] This retrograde signaling plays a fundamental role in cellular function, influencing a broad spectrum of genes, including microRNAs (miRNAs). As tissue-specific regulators, miRNAs are integral to tumorigenesis. Specific miRNAs support tumor cells by shifting metabolism from OXPHOS to aerobic glycolysis, a phenomenon known as the Warburg effect.[9]

The *miR663* gene on chromosome 20p11.1 is among the miRNAs associated with mitochondrial function in breast cancer. Carden et al demonstrated that *miR663* expression decreased in MCF-7 and MDA-MB-231 breast cancer cells lacking mtDNA. Restoration of mtDNA in these cells reversed this effect, suggesting that *miR663* is epigenetically regulated through retrograde signaling. Bisulfite sequencing further revealed that *miR663* promoter activity is suppressed via methylation induced by ROS. The study also indicated that *miR663* regulates nuclear respiratory chain subunits in ETC complexes I, II, III, and IV.[10]

One of the primary targets of *miR663* is the apoptosis antagonizing transcription factor (*AATF),* whose degradation is a key step in cellular apoptosis. *AATF*, predominantly a nuclear protein, exerts pro-proliferative effects by regulating G1/S and G2/M checkpoints in the cell cycle.[11] Increasing evidence suggests that *AATF* protein is also localized in mitochondria, where it plays an active role in the cellular response to oxidative stress.[12]

Benakanakere et al identified *miR663* as an apoptosis-inducing factor by targeting *AATF* mRNA in oral epithelial cells.[13] The *AATF* (*Che-1*) gene on chromosome 17q12 encodes a 558-amino acid protein featuring an acidic N-terminal region, a leucine zipper motif, phosphorylation sites for kinases, and three nuclear receptor binding motifs. Given its role in cell cycle arrest, *AATF* has been investigated in hepatocellular carcinoma and identified as a potential driver of hepatocarcinogenesis.[14] A 2024 study proved that TNF-α converting enzyme inhibition through *AATF* reduction could be a novel targeted therapy.[15] However, analysis of the *AATF* gene in over 100 breast cancer families failed to identify coding sequence mutations linked to cancer susceptibility, shifting research focus toward miRNA-mediated silencing mechanisms.[16]

Based on these findings, this study investigates the functional axis of mitochondria/*miR663*/*AATF* in breast cancer. To minimize confounding variables, invasive ductal carcinoma (IDC) was selected as the study focus, as it represents 80% of all breast cancer cases.

Materials and Methods

Patients and samples

This study was approved by the Ethics Committee at Tarbiat Modares University, Tehran, Iran (IR.MODARES.REC.1399.131), and all patients provided informed consent. A total of 22 fresh frozen breast tumor tissues and paired adjacent non-tumor tissues were collected from Sina and Farmaniyeh hospitals in Tehran. Demographic data, including tumor grade, lymphatic invasion, family history, and hormone receptor expression, are presented in Table 1.

All samples were histologically confirmed as IDC of the breast. Patients had no prior history of chemotherapy, radiotherapy, or hormone therapy. Clinical and pathological data were documented, including tumor size, grade, and the status of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2/neu).

Table 1. Demographic information of the patients

Characteristics	Sub-groups	Frequency (%)
Histology grade	Grade I (low-well differentiated)	4 (18.2)
	Grade II (intermediate-moderately differentiated)	12 (54.5)
	Grade III (high-poor differentiated)	5 (22.7)
	Missing	1 (4.5)
	Total	22 (100)
Site of primary tumor	Left breast	9 (40.9)
	Right breast	12 (54.5)
	Bilateral	1 (4.5)
	Total	22 (100)
Lymphatic invasion	Positive	13 (59.1)
	Negative	8 (36.4)
	Missing	1 (4.5)
	Total	22 (100)
Estrogen receptor	Positive	18 (81.8)
	Negative	3 (13.6)
	Missing	1 (4.5)
	Total	22 (100)
Progesterone receptor	Positive	15 (68.2)
	Negative	6 (27.3)
	Missing	1 (4.5)
	Total	22 (100)
Her-2	Positive (1-2)	4 (18.2)
	Positive (3 +)	3 (13.6)
	Negative	14 (63.6)
	Missing	1 (4.5)
	Total	22 (100)
Family history	Positive	8 (36.3)
	Negative	14 (63.6)
	Total	22 (100)

Total DNA extraction and determination of mtDNA content

Total DNA was extracted from tissue samples using the DNrich Tissue Kit (Azma Elixir Pajooh, Iran). The quality and quantity of the extracted DNA were assessed via electrophoresis and a NanoPhotometer (NP80, Germany).

Real-time PCR was performed on a StepOne apparatus (Applied Biosystems), and cycle threshold (Ct) values were recorded. Each tumor mtDNA copy number was compared with its paired adjacent non-tumor tissue.

To quantify the mtDNA copy number, the following formula was applied:

$$2^{[Ct\ (nuclear\ gene)\ -\ Ct\ (mitochondrial\ gene)]}$$

As previously described.[17] The relative amplification of the nuclear cytochrome c oxidase subunit IV (*COX4*) gene was compared to that of the mitochondrial cytochrome c oxidase subunit I (*MT-COXI*) gene.[18] Standard curves were generated for each primer, and downstream assays were performed at a 10 ng/µL DNA concentration.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from tissue samples using TRIzol reagent (Yektatajhiz, Iran). RNA precipitation was performed using chloroform and isopropyl alcohol, followed by solubilization in DEPC-treated water and storage at −80 °C until use. The quality and quantity of extracted RNA were assessed, and samples with an A260/A280 ratio of 1.8–2.0 were selected for downstream analyses.

cDNA synthesis was performed using the cDNA Synthesis Kit (Yektatajhiz, Iran). For *miR663*, cDNA synthesis was conducted using a stem-loop primer (Table 2). A 1000 ng/µL concentration of RNA was the favored concentration for cDNA synthesis.

Table 2. Primers and stem-loop sequences

Primer	Sequence (5'-3')
MT-COX1	Forward: TGATCTGCTGCAGTGCTCTGA Reverse: TCAGGCCACCTACGGTGAA
COX4	Forward: GAAAGTGTTGTGAAGAGCGAAGAC Reverse: GTGGTCACGCCGATCCAT
AATF	Forward: TCGGTTTCATGTCCTTAGCAAGC Reverse: GGAGGTGGGCGATGTCAATC
PUM1	Forward: TCGGAAGTAGCAGTTCTCTCG Reverse: CTGTGTCCAATGGGGGTCAA
miR663	Forward: AGGCGGGGTGCTGCGGGA
u47	Forward: CCACTGCTGTAATGATTCTGC
Universal reverse	Reverse: CCAGTGCAGGGTCCGAGGTA
miR663 Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCG-CACTGGATACGACGCGGTC
U47 Stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCG-CACTGGATACGACAACCTCAG

Note: The MT-*COX1* and *COX4* genes were selected as the target and reference genes to quantify the mtDNA copy number. For expression analysis of *AATF* and *miR663*, the *PUM1* and *U47* genes were used as reference genes. A stem-loop primer was applied for *miR663* cDNA synthesis.

Quantitative real-time PCR (qRT-PCR) assay

The *PUM1* and *U47* genes were selected as internal reference genes (Table 2). According to prior research, these are among the most stable housekeeping genes in normal and tumor breast tissues. PUM1 may play a greater role in regulating cellular functions under hypoxia than other reference genes.[19]

The efficiency of qRT-PCR primers was evaluated using LinRegPCR software (Academic Medical Center, Amsterdam, the Netherlands). qRT-PCR reactions were performed using SYBR Green Master Mix High ROX (Ampliqon) under the following conditions: initial denaturation at 95°C for 15 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 45 seconds. All experiments were duplicated, and a non-template control (NTC) was included in each reaction.

TCGA analysis

The expression of *AATF* was analyzed across various stages and subtypes of breast cancer using TCGA data. A total of 114 normal samples, 183 stage I samples, 615 stage II samples, 247 stage III samples, and 20 stage IV samples were examined. For breast cancer subtype analysis, 566 samples were from the luminal subgroup, 37 were from the HER2+ subgroup, and 116 were from the triple-negative breast cancer (TNBC) subgroup. All subtypes were compared with 114 normal samples.

Statistical analysis

Real-time PCR data were analyzed using GraphPad Prism 8.0.2 and REST 2009 software (Qiagen, Technical University Munich). Fold-change calculations were based on the $2^{-\Delta\Delta Ct}$ method. The correlation between *miR663* expression mtDNA copy number and *AATF* expression was assessed using IBM SPSS (version 16.0).

Due to the limited sample size and non-normal data distribution, nonparametric tests were employed, including Wilcoxon, Mann-Whitney U, and Kruskal-Wallis tests. Correlations between gene expression levels were evaluated using Spearman's Rank Correlation Coefficient. A P value < 0.05 was considered statistically significant in all analyses.

Results

Relative expression analysis

The melt curves of *AATF* and *miR663*, along with those of the internal control genes, are presented in Fig. 1. According to LinRegPCR software, the PCR efficiency was 1.93 for *AATF* and 1.81 for *miR663*.

Using GraphPad Prism and REST software, the relative gene expression of *miR663* was calculated after normalization to *U47*. The results demonstrated

a significant decrease in *miR663* expression in breast tumors compared to paired adjacent non-tumor tissues (fold change = 0.139, $P = 0.000$), as illustrated in Fig. 2A.

For *AATF* mRNA quantification, *PUM1* was used as the internal control. The analysis showed no significant change in *AATF* expression (fold change = 0.886, $P = 0.668$) (Fig. 2A). Although REST software did not indicate a significant increase, mean fold-change calculations suggested *AATF* overexpression. To further investigate *AATF* mRNA levels in breast cancer, TCGA

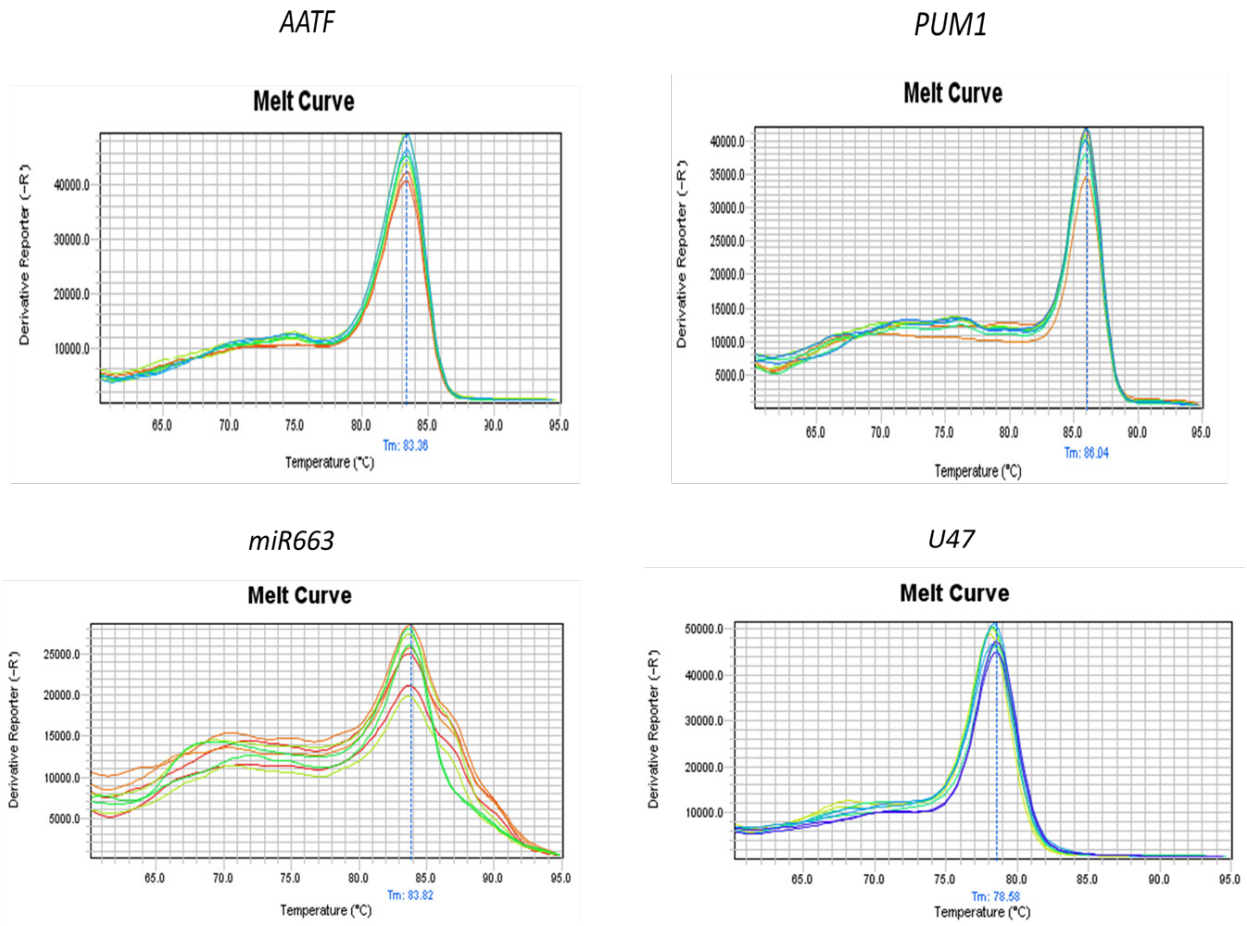

Fig. 1. Melt curve analysis. Top: *AATF* and its internal control *PUM1*. Bottom: *miR663* and the *U47* control gene. The lack of sharpness in the melt curve of *miR663* results from unavoidable primer-dimer interference. No amplification was detected in the NTCs.

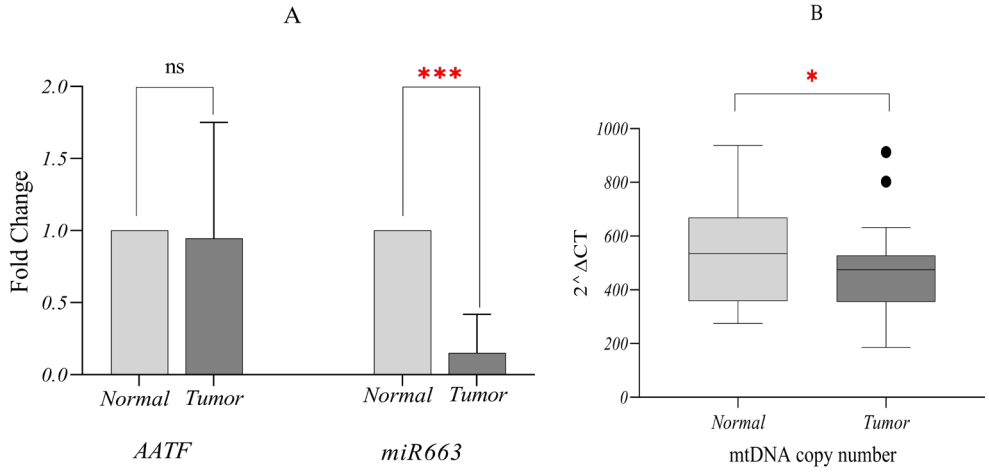

Fig. 2. Relative expression analysis normalized to internal controls. (A) Significant decrease in the median expression of *miR663* in tumor samples compared to adjacent non-tumor tissues. No statistically significant difference was observed in *AATF* median expression between tumor and adjacent non-tumor tissues. (B) Differences in median mtDNA copy number between tumor and adjacent non-tumor tissues. The median mtDNA copy number in tumor tissues was approximately 474.6 copies per cell, showing a reduction compared to adjacent non-tumor tissues, which had 534.9 copies per cell. *$0.01 < P \leq 0.05$, *** $0.0001 < P \leq 0.001$.

data were analyzed, and the findings are presented in the following sections.

Investigation of mtDNA copy number variations

The standard curves for DNA concentrations and the melt curves for *COX1* and *COX4* amplification are presented in Fig. 3. *COX1* exhibited a higher amplification rate than the *COX4* nuclear control gene.

Using the $2^{\Delta Ct}$ method, the median mtDNA copy number in tumor tissues was 474.6 copies per cell, compared to 534.9 copies per cell in adjacent non-tumor tissues. The median mtDNA copy number in tumor samples was analyzed using GraphPad Prism software, revealing a significant difference when outlier points were excluded. These findings align with previous studies reporting a reduction in mtDNA copy number in breast tumor tissues (Fig. 2B).

Association between miR663, AATF expression changes, and mtDNA copy number

Spearman's correlation analysis revealed a significant association between *miR663* and *AATF* expression ($P=0.012$). Additionally, the nonparametric Wilcoxon test indicated a statistically significant difference between *miR663* and *AATF* expression ($P=0.007$, $Z=-2.711$). A significant difference was also observed between miR663 expression and mtDNA copy number ($P=0.017$, $Z=-2.386$).

However, based on Spearman's correlation and Wilcoxon tests, no statistically significant correlation was detected between mtDNA content and *AATF* mRNA expression. Although the two components at the beginning and end of the proposed mtDNA/*miR663*/*AATF* axis did not exhibit a direct statistical correlation, it is plausible that while the decrease in *miR663* did not lead to a significant increase in *AATF* mRNA, it may have contributed to an increase in AATF protein levels. Further research should investigate this potential regulatory mechanism at the AATF protein level.

Correlation between tumor grades and miR663, AATF expression

Clinical data indicated that most tumors were classified as grade II. The correlation between tumor grade and the expression of *miR663* and *AATF* was assessed using the Kruskal-Wallis test in SPSS software. The results demonstrated a progressive decrease in *miR663* mean fold-change levels and a corresponding increase in *AATF* mean fold-change with increasing tumor grade (Fig. 4).

Consistent with overall findings, *miR663* mean fold-change was significantly reduced in grade II and grade III tumors, whereas grade I tumors increased *miR663* expression, reaching levels comparable to non-tumor tissues or even higher.

Based on available evidence, as tumor cells progress to higher grades and lose differentiation, the inhibition of *miR663* intensifies, weakening its tumor-suppressive effects. This reduction in *miR663* removes its inhibitory influence on *AATF*, allowing the anti-apoptotic properties of tumor cells to develop.

Analysis of TCGA data

The analysis of TCGA data revealed that *AATF* expression was significantly higher in tumors than in normal tissues, slightly increasing as the disease stage progressed. The median *AATF* expression in normal samples was 38.5 transcripts per million (TPM). However, expression levels increased to 45.6, 49.3, 49.1, and 50.0 TPM in stages I, II, III, and IV, respectively (Fig. 5A).

In addition, *AATF* expression varied across breast cancer subtypes. While *AATF* levels were reduced in HER2+ tumors, they were elevated in luminal and TNBC tumors compared to normal tissues. The median *AATF* expression was 48.3 TPM in luminal tumors, 33.07 TPM in

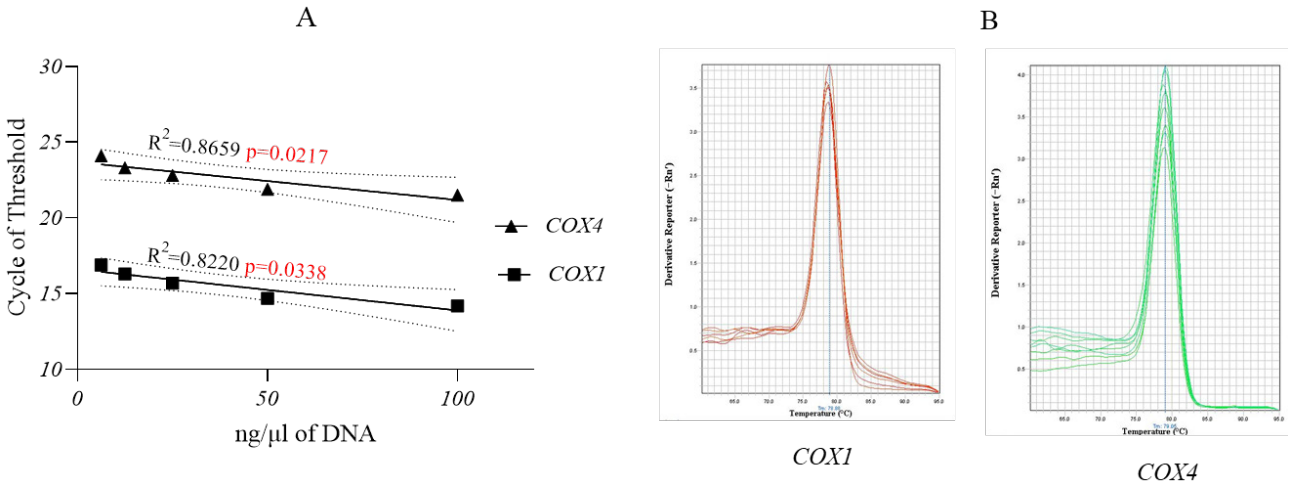

Fig. 3. mtDNA copy number analysis. (A) Standard curves of five DNA serial dilutions tested for the *COX1* and *COX4* genes. The *P-value* was 0.021 for *COX4* and 0.033 for *COX1* indicating a strong link between the DNA concentration and amplification rate (B) Melt curves of *COX1* and *COX4* gene amplifications.

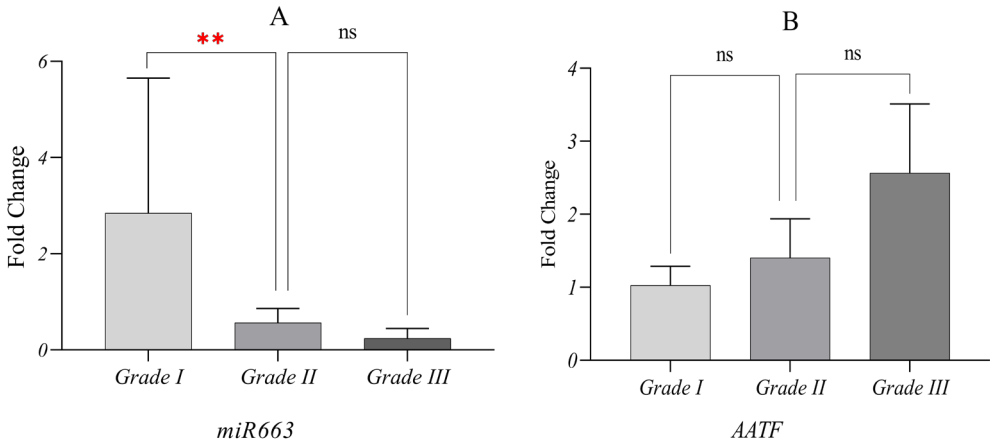

Fig. 4. Mean fold-change of miR663 and AATF based on tumor pathological grade. The significance of group differences was analyzed using the Kruskal-Wallis test, and standard errors of the mean (SEM) are presented. (A) With decreased tumor cell differentiation, *miR663* expression declined. The *P-value* was 0.00 for the comparison between Grade I and Grade II. The difference between Grade I and Grade III also yielded the same rate with a *P-value* of 0.00 (not indicated). The difference between grade II and grade III was not statistically significant (P = 0.42) (B) No statistically approved link between *AATF* expression and tumor grade was observed. However, it was found that as tumor cell differentiation decreased, *AATF* was overexpressed. ** $0.001 < P \leq 0.01$, ns; not significant $P > 0.05$.

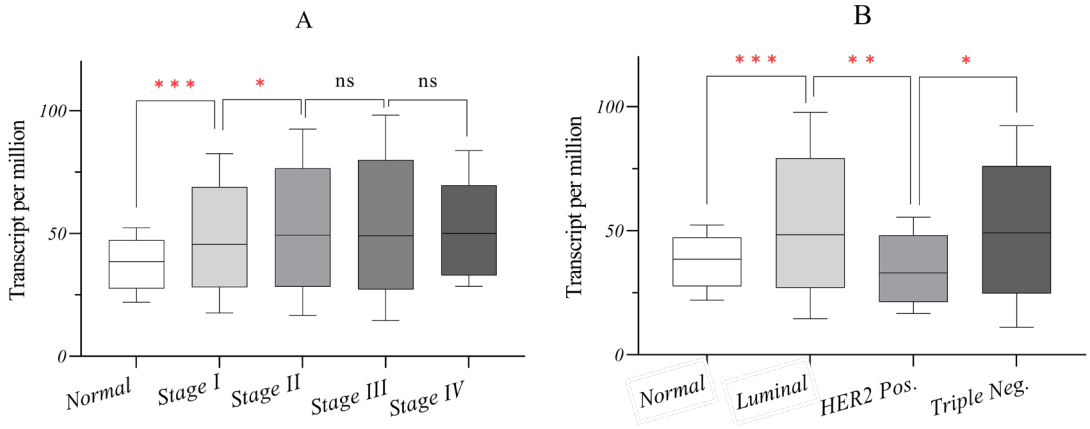

Fig. 5. In silico analysis of AATF expression in breast cancer based on TCGA data. (A) *AATF* expression significantly increases in tumors compared to normal breast tissues. (B) *AATF* expression is reduced in HER2 + tumors, whereas luminal and TNBC tumors exhibit elevated expression levels. * $0.01 < P \leq 0.05$, ** $0.001 < P \leq 0.01$, *** $0.0001 < P \leq 0.001$, ns; not significant $P > 0.05$.

HER2 + tumors, and 49.1 TPM in TNBC tumors (Fig. 5B).

These findings are noteworthy, as when all subtypes are analyzed collectively, the data appear normalized, reflecting a relative increase in *AATF* expression. However, when analyzed separately, a significant increase in luminal and TNBC subtypes becomes evident despite their distinct pathogenesis. Conversely, *AATF* expression in HER2 + tumors is markedly reduced, reaching levels comparable to or even lower than those in normal tissues.

Discussion

Identifying *miR663* as a target of mitochondrial retrograde signaling in breast tumor cells has introduced new insights into mitochondrial–nuclear crosstalk.[10] This study demonstrated the downregulation of mtDNA/*miR663* in primary breast cancer tissues. The reduction in *miR663* expression was substantial, nearly diminishing in tumors with higher pathological grades. These findings, consistent with previous studies, suggest that mitotherapy and miRNA-based therapies could be potential strategies for breast cancer treatment.[10,20]

A comprehensive study by Carden et al established *miR663* activity in retrograde signaling and revealed clinically relevant findings. In silico analysis of TCGA data showed a significant reduction in *miR663* expression in metastatic tumors compared to primary tumors. Furthermore, an increase in *miR663* expression was observed in stage II tumors compared to stage I, followed by a significant decline in stages III and IV. Kaplan-Meier survival analysis indicated that higher *miR663* expression was associated with more prolonged patient survival.[10]

A 2021 study by Wang et al further demonstrated that reduced *miR663*a expression was significantly associated with lymph node metastasis and poor survival outcomes in breast cancer patients. The researchers proposed *miR663*a as an independent prognostic factor for breast cancer.[20]

Experimental evidence has established a correlation between mtDNA copy number variations and breast cancer; however, findings across blood and tumor tissue studies remain inconsistent. Several studies in breast cancer patients have reported increased mtDNA copy numbers in blood samples compared to matched controls.[21-23] A comparative study analyzing breast cancer patient samples found that mtDNA copy numbers in buffy coat and tumor tissues were lower than those in serum and adjacent non-tumor tissue.[24] A recent study identified an inverse correlation between leukocyte mtDNA copy number and breast cancer patient survival.[25] Rai et al conducted a simultaneous analysis of blood and tumor tissue samples from patients with metastatic breast cancer, confirming that mtDNA copy number is regulated independently in blood and tumor tissues. Their findings indicated a decrease in mtDNA copy number in blood but an increase in tumor tissue.[26] A study on Mexican breast cancer patients reported a reduction in tumor mtDNA copy number, along with three deletions at *A249del, A290del, and A291del*, as well as *C16327T* mutations in mtDNA.[27]

Our investigation of *AATF*, a key target of *miR663*, did not reveal any significant changes in mRNA expression. However, TCGA breast cancer data analysis showed that *AATF* expression varied across different subtypes. Specifically, *AATF* expression was markedly reduced in HER2 + tumors while elevated in luminal and TNBC subtypes compared to normal breast tissues.

Early studies on *AATF* function in breast cancer demonstrated its role as a stimulatory factor for ER expression.[28] The immunoblotting analysis further confirmed *AATF* overexpression in the MCF-7 breast cancer cell line compared to the non-tumorigenic MCF-10A cell line.[29] Beyond breast cancer, research in multiple myeloma has identified new roles for *AATF*, including reducing genomic instability and mitigating DNA damage by clearing R-loops (triple-stranded RNA: DNA hybrids).[30] A separate study in non-mammary cell lines showed that *AATF* depletion led to histone deacetylation and reduced cell proliferation.[31]

A direct link between mitochondrial function and *AATF* was established in cisplatin-treated bladder cancer cell lines, where *AATF* inhibited drug-induced apoptosis by reducing cytochrome c levels, a key component of OXPHOS.[32] A recent and interesting study disclosed new localization and function of AATF protein in mitochondria in interaction with HCLS1-associated protein X-1 (HAX1). HAX1 regulates cell migration, a key process in carcinogenesis and metastasis. Furthermore, it supports the anti-apoptotic activity exerted by AATF. They revealed that the HAX1/*AATF* complex was located in the mitochondria of the MCF-7 cell line, acting in cellular response to oxidative stress.[12]

Due to the limited sample size, this study had insufficient statistical power to fully elucidate the functional role of the mtDNA/*miR663*/*AATF* axis in breast cancer pathogenesis. Future studies focusing on AATF protein expression, tumor grade, and breast cancer subtypes are necessary to clarify further the impact of this axis on breast cancer progression.

Conclusion

We identified a significant reduction in *miR663* and mtDNA copy number in breast tumors. A strong correlation was also observed between *miR663* and *AATF* expression. During the experimental phase of our study we noticed that *miR663* was significantly reduced in Grade II and III tumors compared to Grade I. In the bioinformatics analysis we found a significant increase in *AATF* in luminal and TNBC subtypes and a reduction in *AATF* in HER2 + tumors.

These results, combined with emerging evidence on the functional coordination of the mtDNA/miR663/AATF axis, suggest that mitotherapy and miRNA-based therapies could serve as potential strategic approaches for breast cancer treatment. This may contribute to the advancement of personalized management strategies for specific breast cancer subtypes.

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Authors' Contribution

Conceptualization: Shirin Shahbazi, Farzaneh Dahi.
Formal analysis: Shirin Shahbazi, Farzaneh Dahi.
Investigation: Shirin Shahbazi, Farzaneh Dahi.

Research Highlights

What is the current knowledge?

- mtDNA copy number variation has been reported in various human cancers.
- Mitochondrial retrograde signaling regulates miR663.
- Increased miR663 is associated with improved breast cancer patient survival.
- The AATF gene is a target of miR663.
- AATF is primarily a nuclear protein but relocates to mitochondria in response to oxidative stress.

What is new here?

- A significant reduction in miR663 expression and mtDNA copy number was identified in breast tumor tissues.
- A strong correlation was observed between miR663 and AATF expression in breast tumors.
- miR663 mean fold change was significantly reduced in Grade II and III tumors compared to Grade I.
- AATF expression was reduced in HER2 + tumors but elevated in luminal and TNBC subtypes.

Methodology: Shirin Shahbazi, Loabat Geranpayeh.
Project administration: Shirin Shahbazi.
Writing–original draft: Shirin Shahbazi, Farzaneh Dahi, Loabat Geranpayeh.

Competing Interests
The authors declare that there is no conflict of interests.

Ethical Approval
This study was approved by the Ethics Committee at Tarbiat Modares University in Tehran, Iran (IR.MODARES.REC.1399.131) and the patients signed the informed consent.

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