



## Molecular study of BRAF gene polymorphisms and the association with mtDNA copy number in thyroid cancer

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### ABSTRACT

TC is one of the most common types of endocrine cancer in the world, the incidence of thyroid cancer has increased recently, ranking 13th among the most common cancers and the most sixth among women. Females had the highest incidence of illness and death compared to males. Thyroid cancer has been associated with genetic changes where mtDNA and its relationship to the Cancer have been studied, the morphological variations in the BRAF gene and their effect on TC patients have been studied.. The study samples were collected from patients of Warith International Cancer Institute in Kerbala / Iraq, the study included (100 samples) were 50 for patients and 50 for control , DNA measured inside mitochondria by Quantitive Polymerase Chain Reaction (QPCR) , and morphological variations of the BRAF gene were detected on 5 samples of patients against one of control sample with the sequencing the result of this study was shown that there is two new SNPs (rs2128998142 and rs2128998351) were detected in the BRAF gene and the current study found an association with thyroid cancer. The mtDNA content was associated with the risk of thyroid cancer, copy number of mitochondrial DNA for the ND1 gene compared to the normal HGB gene increased by a very large percentage in patients with thyroid cancer for the control group. Our findings lead us to hypothesize that an increase in the number of mitochondrial DNA copies in leukocytes may be associated with oxidative DNA damage and operate as a separate risk factor for thyroid cancer ( TC )

**Keywords:** *Polymorphism , mtDNA , BRAF , ND1 , TC , SNP*

### INTRODUCTION

Thyroid cancer was the 13th most prevalent malignancy overall and the sixth most prevalent in women. According to (Kitahara, C. M., & Schneider, A. B., 2022), different countries around the world had varying rates of thyroid cancer discovery. In China, between 200 and 700 new cases of thyroid cancer were identified in 2015, accounting for 5.11% of all cancer cases and 7900 thyroid cancer-related deaths.

Compared to men, women experienced the highest rates of morbidity and death. )Lam, A. K., 2022( (He J, et al., 2019) TC is the most common in non-Hispanic whites, Asians, and Pacific Islanders in compartion to other races (Weeks, K. S., et al., 2018). In Iraq, the incidence of thyroid cancer increased significantly since 2007 from 0.62 to 2.96, the ratio of males to females was 1:2.5. (Hussain, A. M., & Lafta, R. K., 2021).

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There are numerous risk factors for thyroid cancer, including hormones, dietary iodine, family history, age, obesity, radiation exposure, environmental contaminants, and gender (Jawad.Z.N and, Awad, W.2020)

There are several different types of thyroid cancer; the most prevalent types are papillary and follicular thyroid cancer, which account for 95% of thyroid cancer cases (Anari, S., et al., 2022), while the other types, including myeloid and non-myeloid thyroid cancer and the rare Horthel cell carcinoma, around 5% of thyroid cancer cases.

mtDNA-CN is correlated to the body health, as its decreasing below the normal limit causes chronic kidney disease, diabetes, cardiovascular disease (He, W. J., et al., 2022) and some types of cancers such as bladder, breast, esophagus, kidney, liver and breast cancer (Tuchalska-Czuroń, J, et al., 2019) (Jawad, Z.N.2023), while increasing in copy number above the normal limit causes many diseases, such as thyroid cancer (Esfahanian, F., et al., 2021), the study of polymorphisms helps diagnoses of a different genotypes of genes.

As the human genes have a large morphological multiplicity in different pieces of the gene in DNA, one of the important genes associated with the risk of thyroid cancer is the (BRAF) gene, as it is one of the genes that regulate cell growth, any defect in this gene leads to uncontrolled growth and formation of the tumor. mutations and morphological variant in BRAF gene are closely related to thyroid cancer.

SNPs are the most common genetic variations in genome (Stratton MR, 2009) and some heterogeneities in nucleotides can push the sequence of genes into a malignant genotype leading to formation of cancer cells. (Jawad.Z.N and, Awad, W.2020) (Lim LM, et al. 2022) (Lence-Anta JJ, et al. 2014).

Morphological heterogeneities increase the likelihood of thyroid cancer and its progression, as the study found that it has a significant impact on patients with all types of thyroid cancer (Kaubryte J and Lai AG, 2022) (Kyrodimos, E., et al. 2023)

The morphological variations in BRAF have an important impact on the formation of tumors and

the incidence of different types of cancers as well as thyroid cancer, also its associated with rectal cancer and colon cancer, and affect the size and seriousness of the tumor as well as increase the mortality rate. (Shalan, A., et al. 2022)

## MATERIALS AND METHODS

### *Patients and Control*

The study samples were collected from Warith International Cancer Institute in Karbala / Iraq, the study included (100 samples) after being surgically diagnosed and the samples were divided into 50 patients with malignant thyroid cancer before taking treatment compared to 50 samples of healthy people outwardly as a control group by drawing 5 ml of venous blood.

### *Sample collection/ storage*

As part of the DNA extraction, we collected the blood of (100 samples) in EDTA tubes. Using the Accupower® Genomic DNA Extraction Kit (Bioneer. Korea), genomic DNA extracted from EDTA blood leukocytes and stored at -20 C until the day of usage. QPCR then used to determine the copy number of mitochondrial DNA.

### *Polymerase chain reaction*

Primer 7.0 was used to create the primers for the amplification of the BRAF gene and BRAF mutant genes (Bioneer. Korea). The two primers used were 5'-TCATAATGCTTGCTCTGATAGGA-3' and 5'-GGCCAAAATTTAATCAGTGGA-3', respectively. The amplified fragment was (224 base pare) in length. 5µl of template DNA, 12.5µl of GoTaq®Promega Green Master Mix 2X, 2 µl of primers (foreword and reverse), and 3.5 l of nuclease-free water were all included in the reaction mix, which was carried out in 25 µl volumes (Applied PCR system, USA). The following PCR conditions were used: The following conditions were used for the PCR reaction: initial denaturation at 94°C for 5 min, followed by denaturation at 94°C for 30s, annealing at 60°C for 1 min, extension at 72°C for 1 min, and final extension for 7 min at 72°C. The fragments were visualized by electrophoresis on a 1% agarose gel dyed with 5% ethidium

bromide. The gel was then examined under UV light to determine whether an allele-specific band was present or not using the Cleaver Gel

Documentation System (Cleaver Scientific Ltd., UK).

No.	Steps	Temperature	Time	No. of cycles
1	Initial Denaturation	95C°	5min.	1
2	Denaturation	94C°	30sec.	35
3	Annealing	60C°	1 min.	
4	Extension	72C°	1 min.	
5	Final Extension	72C°	5 min.	1
6	Final hold	4 C°	-	

**DNA Sequencing methods**

Two DNA strands were studied for 5 patient samples and one control sample, which were sent with their primers to MacroGen in Korea.

Geumchen, Seoul, South Korea). MacroGen Inc (specialized in the analysis of sequencing products of those samples, the result of the sequences was compared with similar DNA sequences of the gene previously and recorded globally where the reference database was extracted reference database (GenBank acc. AB082923.1, NC\_000001.11, and NM\_001126118.1) From Genebank website <https://www.ncbi.nlm.nih.gov> PCR sample sequence products were analyzed, refined, lined up and analyzed together with NCBI samples by BioEdit Sequence Alignment Editor Version 7.

**Measurement of Leukocyte mtDNA-CN by Quantitative Polymerase Chain Reaction**

The gsync™ Blood DNA Extraction Kit (Bioneer Korea) was used to extract the genomic DNA from peripheral blood leukocytes. Quantitative PCR (qPCR) was used to determine the relative amount of mtDNA-CN. A seven-point standard curve was used to quantify the

copy number ratio of the mitochondrial ND1 gene to a singlecopy nuclear gene (HGB) for each sample. A reference DNA sample was diluted by 2-fold serial (range: 0.3125–20 ng/l) to create the standard curve for each batch. Then, Using the 2–ddCt equation, the ratio of each sample was then standardized with a calibrator DNA sample and transformed into the relative amount of mtDNA-CN. The mitochondrial ND1 gene included the following primer sequences: 5' -CCC TAA AAC CCG CCA CAT CT-3' (forward); 5' -GAG CGA TGG TGA GAG CTA AGGT-3'. (reverse). The primers used to amplify the HGB reference gene were 5' -GCT TCT GAC ACA ACT GTG TTC ACT AGC-3' (forward) and 5' -CAC CAA CTT CAT CCA CGT TCA CC-3'. (reverse).

The final volume of each PCR reaction mixture was 10 µL, and it contained 4 ng of genomic DNA, 10 nM of each primer, and 5 µL of 1 SYBR green master solution. For both primers, the thermal cycling settings were 95°C for 10 min, then 40 cycles of 95°C for 15 s, followed by 60°C for 1 min. The tabel below shown the steps of quality control were the R 2 for each standard curve should be >0.99, with standard deviations (SDs) of the Ct values of 1.95.

Steps	Temperature	Time	Cycles
1	95C°	03:00	1
2	95C°	00:15	40
3	60C°	01:00	
1	95C°	02:00	1
2	95C°	00:03	40
3	60C°	00:30	

**Statistical Analysis**

The Statistical Analysis Program (SPSS) Special Packages of Social Science V.22 was used to analyze the results of the study, and the level of significance  $P \leq 0.01$  and  $P \leq 0.05$  was adopted to find out the statistical differences for the study samples.

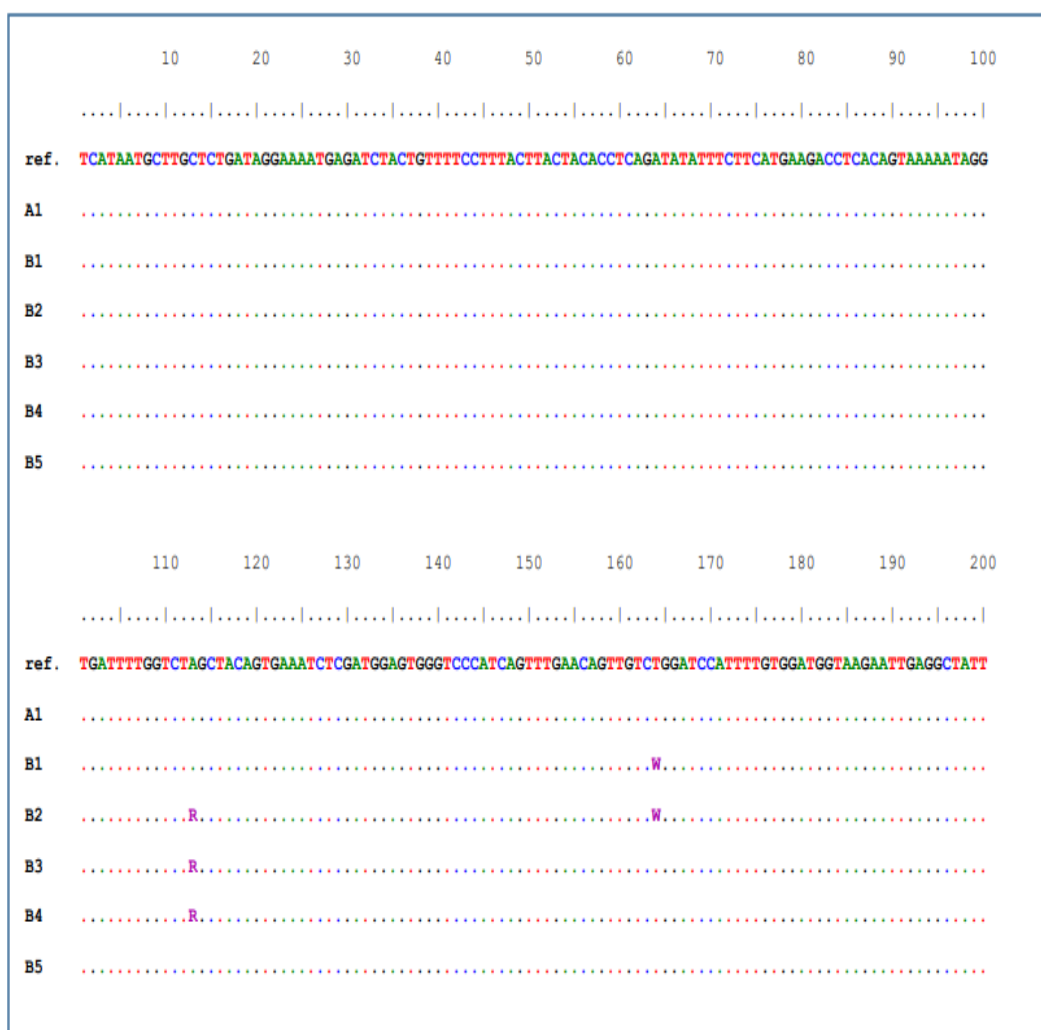
**RESULTS**

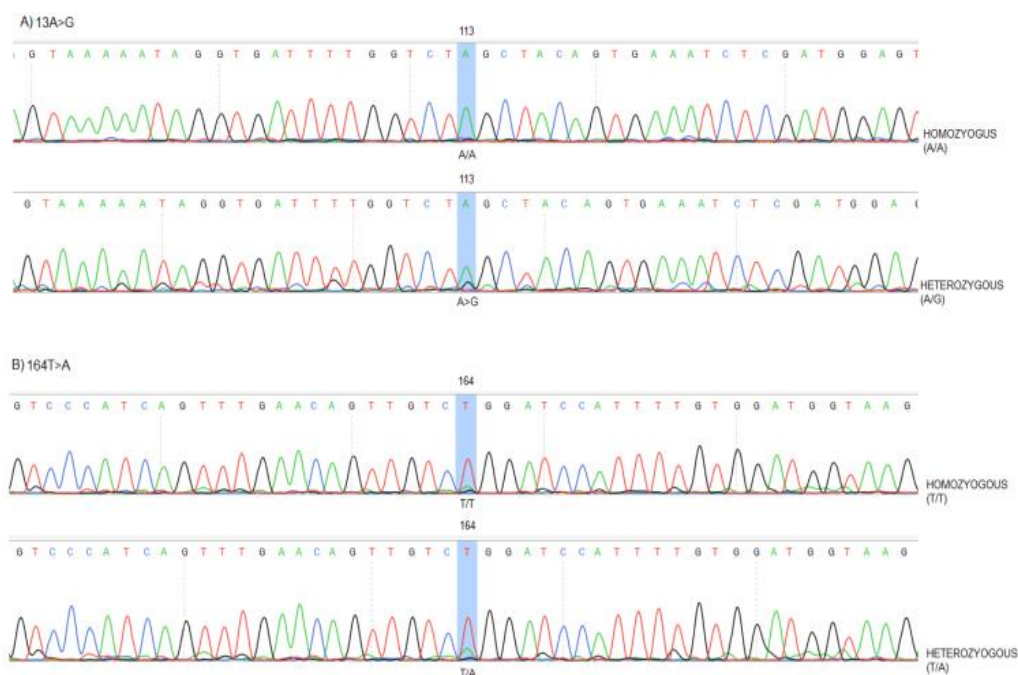
**BRAF sequencing**

Two Morphological Polymorphisms (SNP) were discovered, the first one is rs2128998351 at the site (A113G) by replacing adenine with guanine and the heterozygous samples were heterozygous

and which differed with samples (B1,B5) and (A1) control sample (homozygous). these samples were compared with data obtained from the genebank using the NCBI Blast site , these sample similar to original BRAF.

The second single nucleated polymorphism (SNPs) is rs2128998142 at site T164A by replacing thymine with adenine and heterozygous in the two samples (B1 and B2) with heterozygous genotype which differed with samples (B3,B4,B5) and (A1) control sample (homozygous) which was compared with data obtained from the genebank using the NCBI Blast site, these sample similar to original BRAF.





**Detected single nucleotide polymorphism (SNP) within the chromatogram of the BRAF gene segment**

Although there was no previous study that found an association between rs2128998351 SNP and rs2128998142 SNP with thyroid cancer, this study showed that SNP was associated with the thyroid cancer due to its presence in 2-3 patients, while its occurrence was not observed in the control sample.

BRAF gene plays an important role in the emergence and development of the tumor as it is responsible for cell growth. any mutations or variations in this gene may affect its function and lead to loss of a control over cell growth and thus formation of cancerous cells such as thyroid cancer. (Azhar et al. 2020 (Shimizu, Y. et al. 2022)

BRAF gene has a role in reducing the expression of some important electron transport genes in mitochondria, which inhibits respiration and metabolism in mitochondria, so mtDNA-CN rises in response to mitochondrial dysfunction compensation . (Lee MH., et al. 2011) .

The morphological variations SNPs in BRAF gene have an important impact on the formation of tumors and the incidence of different types of cancers such as thyroid cancer.also they are associated with rectal cancer and colon cancer

and affect the size and seriousness of the tumor as well as increase the mortality rate. (Shalan, A., et al. 2022).

**MtDNA – CN**

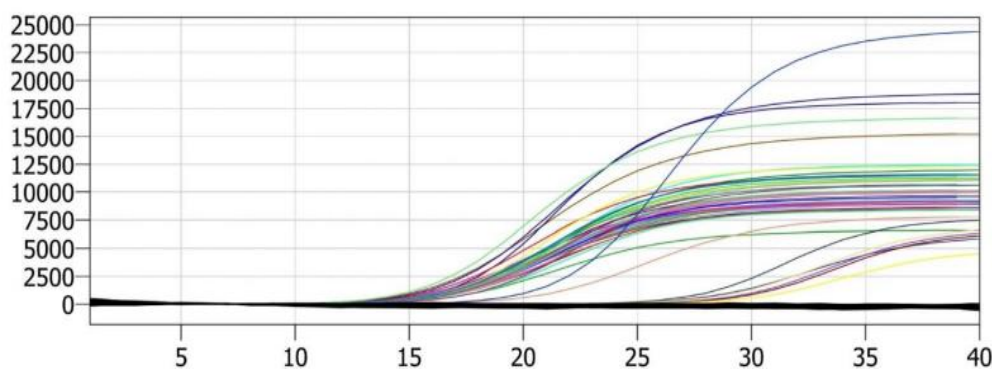
This study found a positive association in the ratio of copy number of ND1 / mtDNA in people with thyroid cancer, and as shown by a study (Zheng, J. et al. 2019) that higher mtDNA-CN levels are a risk factor for thyroid cancer as Papillary thyroid cancer tissue had almost four times the amount of mitochondrial DNA copies compared to normal thyroid tissue, While the study's results disagreed with those of Thakur, N., et al. (2020), who showed in his study that The risk of cancer is not associated with the amount of copies of mtDNA in leukocytes. Another study found a decrease in mtDNA in leukocyte mitochondria in thyroid cancer patients. (Perdas, E., et al.,2019) . Mitochondria produce ROS (Reactive Oxygen Species), which helps in connection between cells and support the immune system, but its increase above the normal level causes oxidative stress (defect or destroy in tissue leads to damage of the body cells ) thus cause tumors, a copy number of mitochondrial DNA increased as the oxidative stress was increase . When oxidative stress increases ,the cellular respiration within the



mitochondria was inhibit while mtDNA in white blood cells was increase . this reaction is to inhibiting mitochondrial functions .

mtDNA is a vital indicator of mitochondrial functions ( Han Y et al., 2013)

pv	Standard Error	Standard Deviation	Mean	sample
0.000	0.118	0.788	0.582	control
	165.52	1110.32	1591.63	patient



**Mitochondrial DNA by Quantitative Polymerase Chain Reaction (Q-PCR) in NDI and HGB genes for thyroid cancer patients and control**

Conclusion: Investigations on thyroid cancer should include genetic studies. Study and diagnose other types of polymorphisms (SNPs) or mutations in BRAF gene by targeting other regions of the gene or other genes associated with the disease using different molecular techniques and expanding the scope of samples for patients and healthy people to improve the relationship between these genes and thyroid cancer .

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