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Molecular Analysis by Gene Expression of Mitochondrial ATPase Subunits in Papillary Thyroid Cancer: Is ATP5E Transcript a Possible Early Tumor Marker?

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Manuscript Preparation E
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Background: Cancer development involves an “injury” to the respiratory machinery (Warburg effect) due to decreased or impaired mitochondrial function. This circumstance results in a down regulation of some of the ATPase subunits of the malignant tissue. The objective of this work was to assess and compare the relative expression of mRNA of mitochondrial ATPase subunits between samples of thyroid cancer and benign nodules.





Material/Methods: Samples from 31 patients who had an operation for PTC at the General Hospital of Mexico were snap-frozen and stored at -70°C . Thirty-five patients who had an operation for benign tumors were also included in the study. mRNA expression levels of alpha, beta, gamma, and epsilon subunits of F_1 and “c12” of subunit F_0 were determined by real-time RT-PCR (by duplicate), in order to determine if abnormal expression of these genes could partially explain the Warburg effect in papillary thyroid cancer (PTC).

Results: ATP5E transcript alteration (down-expression) was highly associated to PTC diagnosis OR=11.76 (95% confidence interval, 1.245–237.98; $p=0.04$).

Conclusions: Relative down-expression of ATP5E transcript was highly associated with PTC diagnosis. This transcript alteration may be used as a tumoral marker in papillary thyroid cancer.

MeSH Keywords: **Head and Neck Neoplasms • Mitochondrial Proton-Translocating ATPases • RNA Polymerase I • Thyroid Neoplasms • Thyroid Nodule**

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Background

The most commonly used tool for diagnosis of non-functional thyroid nodules is fine-needle aspiration biopsy (FNAB) [1–3]. However, FNAB still do not provide a diagnosis in 30–40% of cases [4–11]; consequently, non-diagnosed patients must be subjected to diagnostic surgery, which could impact in recurrent laryngeal nerve and parathyroid gland [12–14] morbidity.

Many groups have used diverse alternate diagnostic methods in attempting to define this situation. These methods include ultrasound [15–18], ultrasonographic elastography [19–21], repeated FNAB [22], determination of mutations of BRAF^{V600E} and rearrangements of RET/PTC [23–27], and assessing genetic panels [28,29]. Despite these efforts, when it is decided not to operate on a patient based on these alternate diagnostic tools, the risk exists of not diagnosing a cancer and leaving it to evolve freely in at least 25–30% of the cases.

In an attempt to resolve this problem, metabolic assessment of the thyroid nodule by gammagraphy with technetium-99m (Tc-99m) and Tc-99m-methoxyisobutylisonitrile (MIBI) was proposed. It was found that all thyroid cancer lesions accumulate MIBI (understood as accumulation of the net result of uptake and outflow of the radio-molecule), obtaining a 100% sensitivity and a negative predictive value of 100% [30]. This negative predictive value has been of great clinical use in ruling-out cancer. This characteristic has been found since use of the first *in vitro* tests of tumor tissue [31], as well as in clinical studies performed on thyroid nodules around the world [32–41]. When this tool is applied to cases with a non-diagnostic FNAB, 39% of the cases can be reclassified to the benign group. When there is no MIBI concentration in the nodule, cancer is ruled-out in 100% of cases. However, in 61% of the cases, no definitive diagnosis can be reached. MIBI's concentration within the lesion (due to metabolic activity) may be found to be high, which occurs in all patients with thyroid cancer, but it may also be found to be high in some benign thyroid nodules [30].

The same diagnostic value (negative predictive value of 97–100% [42–46]) can be achieved in thyroid nodules assessed through non-diagnostic aspiration biopsy by means of 18F-fluorodeoxyglucose positron emission tomography (PET).

MIBI is not retained in benign cells; it enters into the mitochondrial matrix because it is a lipophilic cation and there is a natural negative charge in the mitochondrial matrix, but the moment at which oxidative phosphorylation occurs, MIBI is washed out of the mitochondrial matrix [47–49]. In cancer cells, oxidative phosphorylation and ATP production are altered, since conversion of ADP to ATP by ATPases is inhibited. In contrast, pumping of hydrogen ions (protons) from the mitochondrial

matrix to the intermembrane space continues due to the function of the respiratory chain, which intensifies the negative polarity in the mitochondrial matrix (Warburg effect); in consequence, MIBI retention in the mitochondrial matrix is higher and persistent [50–53]. It has been demonstrated that the electrical gradient difference of the mitochondrial transmembrane potential ($\delta\Psi_m$) increases from –60 mV (normal in the mitochondrial matrix) to between –150 and –170 mV [54–59] in cancerous tissue.

This Warburg effect is the reason why PET also has the above-mentioned diagnostic value, because by not producing ATP the cell must consume a large amount of glucose [60–63].

Accordingly, this situation could be related to differences in mitochondrial ATPases between normal and neoplastic cells. The mitochondrial ATPase is characterized as having 2 main multi-protein components: F_0 , immersed in the internal mitochondrial membrane and consisting of 3 types of proteins (subunits) called a_1 , b_2 and c_{12} ; and F_1 , which is oriented towards the mitochondrial matrix and has 5 different polypeptides (subunits) called α_3 , β_3 , δ , γ , and ϵ [65].

Under normal conditions, the flow of protons produced by oxidative phosphorylation is pumped from the mitochondrial matrix to the mitochondrial intermembrane space. Subsequently, these protons return to the mitochondrial matrix by means of the proton channel located in subunit a_1 of component F_0 , inducing rotation of subunit c_{12} of F_0 and this in turn induces rotation of subunits γ and ϵ of component F_1 . This rotation produces changes in the binding affinity of ATPase induced by ADP and P_i , which finally leads to production of 3 ATP molecules for each 360° rotation of subunit γ . In cancer cells this ATP is not produced at the same level (Warburg effect). It is still not known whether any subunit of F_0 or F_1 presents a structural dysfunction. However, some authors have attributed this dysfunction to component F_1 ; protein β has specifically been involved in liver cancer [65–78].

Based on the above, we studied the difference in the relative expression of mRNA of the ATPase subunits between paired malignant lesions and healthy tissues from papillary thyroid cancer patients. Benign tumors of the thyroid gland were analyzed as well. We measured α , β , γ , and ϵ of subunit F_1 and that of the “c12” of subunit F_0 .

The objective of this work was to assess the difference in the relative mRNA expression of the thyroid mitochondrial ATPase subunits between benign and malignant lesions.

Material and Methods

A prospective, observational, descriptive, and comparative study was performed.

The protocol was approved by the Investigation and Ethics Committee of the General Hospital of México. Thirty-one papillary thyroid carcinoma and 35 nodular colloid goiter fresh tissues samples were managed at the Thyroid Clinic of the Hospital General de Mexico and the Instituto Politecnico Nacional.

After a conventional thyroidectomy procedure, a 0.5×0.5×0.5 cm piece of the tumor tissue was resected, as well as a sample from distal healthy thyroid tissue. Both samples were immediately snap-frozen and stored at -70°C . The remnant tissue was sent for histological analysis, which is the diagnostic criterion standard.

Tissue samples (100 mg) were homogenized in 1 ml of TRIzol® (Invitrogen Co., Carlsbad, CA) using a Polytron device (Kinematica Inc., Bohemia, NY).

Total RNA was purified using the PureLink RNA mini kit (Invitrogen Co.) following the protocol provided by the manufacturer. RNA was quantified through spectrophotometry using a Biophotometer device (Eppendorf, Mexico). Integrity of all RNA samples was verified through 1.5% agarose gel electrophoresis and formaldehyde, according to the standard technique [79].

cDNA synthesis was accomplished using the First Strand cDNA Synthesis kit (Thermo-Fisher Scientific Inc., Waltham, MA). Reactions were prepared in 20 μl , containing 5 μg of total RNA and oligo-(dT)18 primers, following the manufacturer's instructions. The obtained cDNA was quantified by spectrophotometer in a Nanodrop 2000 device (Thermo Scientific, Wilmington, DE).

Differential gene expression of the mitochondrial ATPase components between tumor and healthy tissues were assessed by quantitative PCR, using real-time system StepOne Plus (Applied Biosystems Inc., Foster City, CA). All materials used for qPCR were obtained from Applied Biosystems Inc., including the following Taqman assays: Hs00900735_m1 (ATP5A1), Hs00969569_m1 (ATP5B), Hs01101219_g1 (ATP5C1), Hs00829069_s1 (ATP5G1), Hs01086654_g1 (ATP5G2), Hs00266085_m1 (ATP5G3), Hs04194825_s1 (ATP5E). All target probes were marked with FAM fluorophore and covered contiguous exons (so that the genomic DNA would not be detected), except for the probe for gene ATP5A1. As a reference, the constitutive expression gene ACTB was chosen and the Taqman assay (Hs00183.,l533_m1, marked with VIC) was included. Reactions were prepared in 10 μl containing 1 μg

cDNA, 1X Gene Expression Master mix, as well as the specific probes to detect the target and constitutive genes, respectively. Cycling conditions were as recommended by the manufacturer, and the relative values of expression were calculated with the Delta-Delta Ct method [80]. All reactions were performed in duplicate and the reported result is the geometric mean of 2 independent assays.

Relative quantification relates the PCR signal of the target transcript in the malignant nodule to that of healthy tissue from the same patient and in the colloid goiter nodule to that of healthy tissue from the same thyroid gland. Results were normalized to Z-score (Z-scores are expressed in terms of standard deviations from their means).

The basis of our hypothesis is: ATP is not generated by mitochondrial ATPase of malignant cells due to a down-regulation in some of the ATPase subunits studied.

Therefore, odds ratio was calculated using the Z-score, to quantify how strongly the down-regulation, equal or less than $Z=-1.96$ (95%Interval confidence), is associated with malignant cells. A 1-sided Fisher's exact test was done, with a significance level set at $P=0.05$. Finally, clusters of RNA expression were generated using Cluster 3.0 [81].

Patients

Thirty-one PTC patients were included in this study, with an average age of 43.7 years (range 18–78); 29 were women (93.5%), having an average age of 44.2 years.

Thirty-five patients with colloid goiter were studied, with an average age of 45 years (range 20–87); 32 were women (91.4%), with an average age of 44.5 years. Both groups were homogenous in terms of age and sex.

Results

ATP5A1. Eleven patients showed down-regulation of this transcript in PTC. In this group, just 1 patient had equal to or less than $Z=-1.96$. Twenty patients showed up-regulation in PTC; in this group, only 1 had equal to or more than $Z=+1.96$. The colloid goiter group had 12 patients with down-regulated transcript level, of which none had equal to or less than $Z=-1.96$ and 23 had up-regulation, of which none had equal to or more than $Z=+1.96$. In comparing down-regulation (equal or less than $Z=-1.96$), the 1-sided Fisher's exact test result was $p=0.478$.

ATP5B. In the papillary cancer group, 7 patients had down-regulated transcript level, of which none had equal to or less than $Z=-1.96$. Twenty-four patients had up-regulation, of which none

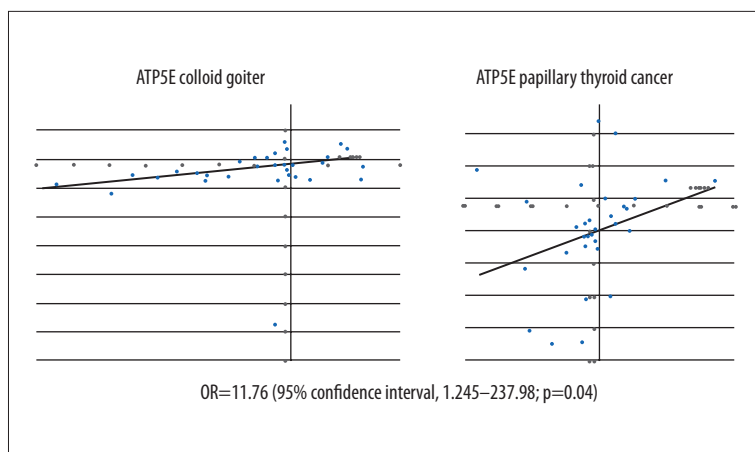


Figure 1. Relative expression of ATP5E in colloid goiter and papillary thyroid cancer.

had equal to or more than $Z=+1.96$. In the colloid goiter group, 3 patients had down-regulated transcript level, of which none had equal to or less than $Z=-1.96$ and 32 patients had up-regulation, of which none had equal to or more than $Z=+1.96$. In comparing down-regulation (equal or less than $Z=-1.96$), the 1-sided Fisher's exact test score was $p=0.277$.

ATP5C1. The papillary cancer group had 12 patients with down-regulated transcript level, of which none had equal or less than $Z=-1.96$ and 19 had up-regulation, of which no patients had equal to or more than $Z=+1.96$. The colloid goiter group had 12 patients with down-regulated transcript level. In this group no patients had equal to or less than $Z=-1.96$; 23 had up-regulation, of which no patients had equal to or more than $Z=+1.96$. In comparing down-regulation (equal to or less than $Z=-1.96$), the 1-sided Fisher's exact test score was $p=1$.

ATP5E. Sixteen patients with PTC showed down-regulation of this transcript, of which 6 patients had equal to or less than $Z=-1.96$ and 15 showed up-regulation, of which none had equal to or more than $Z=+1.96$. The colloid goiter group had 9 patients with down-regulation of the transcript, of which none had equal to or less than $Z=-1.96$; 26 patients had up-regulation, of which none had equal to or more than $Z=+1.96$. The OR was 11.76 (95% confidence interval, 1.245-237.98) in comparing down-regulation (equal or less than $Z=-1.96$) and the 1-sided Fisher's exact test score was $p=0.045$ (Figure 1).

ATP5G1. In the papillary cancer group 21 patients had down-regulated transcript level, of which 5 patients had equal to or less than $Z=-1.96$ and 8 had up-regulation, of which none had equal to or more than $Z=+1.96$. The colloid goiter group had 23 patients with down-regulated transcript level, of which 2 patients had equal to or less than $Z=-1.96$ and 12 up-regulation, of which none had equal to or more than $Z=+1.96$. OR=3.28 (95% confidence interval, 0.56-19.15). In comparing down-regulation (equal or less than $Z=-1.96$), the 1-sided Fisher's exact test score was $p=0.17$.

ATP5G2. In the papillary cancer group 16 patients had down-regulated transcript level, of which only 1 patient had equal to or less than $Z=-1.96$ and 15 had up-regulation, of which none had equal to or more than $Z=+1.96$. The colloid goiter group had 15 patients with down-regulated transcript level, of which none had equal to or less than $Z=-1.96$ and 20 had up-regulation, of which none had equal to or more than $Z=+1.96$. The OR was 3.0 (95% confidence interval, 0.11-79.49). In comparing down-regulation (equal or less than $Z=-1.96$), the 1-sided Fisher's exact test score was $p=0.51$.

ATP5G3. In the papillary cancer group 13 patients had down-regulated transcript level, of which 4 had equal to or less than $Z=-1.96$, and 18 patients had up-regulation, of which none had equal to or more than $Z=+1.96$. In the colloid goiter group 21 patients had down-regulated transcript level, of which 4 had equal to or less than $Z=-1.96$ and 14 patients had up-regulation, of which none had equal to or more than $Z=+1.96$. The OR was 1.88 (95% confidence interval, 0.37-9.39). In comparing down-regulation (equal or less than $Z=-1.96$), the 1-sided Fisher's exact test result was $p=0.35$.

According to several distance-based clustering algorithms (Figure 2), at the level of genes (Y axis), the behavior of ATP5G1 and ATP5G2 was similar since they are located in the same branch. The order of similarity was ATP5E, ATP5C1, and finally ATP5G2, all of which belong to 1 major cluster. The other cluster includes ATP5A1 and ATP5B, which segregate in a separate branch. This may represent biological coherence, as each major branch corresponds to F_0 and F_1 complexes, respectively.

Discussion

It is well established that during cancer progression, alteration of the mitochondrial respiratory chain occurs. Some of the changes that the mitochondria may exhibit during the development and progression of most cancer cells are the differential

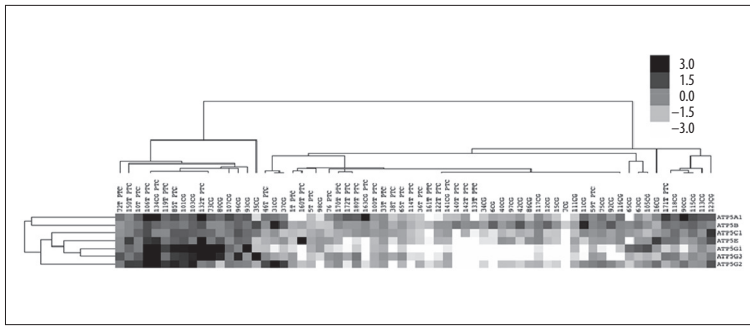


Figure 2. Clusters. At the level of diagnosis (X axis) all groups or clusters formed include both cancer (PTC) and goiter (CG).

expression of respiratory chain subunits and of glycolytic enzymes. It is important to remember that elevated glycolysis in cancer cells does not necessarily mean that these cells only use the glycolytic pathway for ATP generation. However, this peculiar aerobic metabolism of cancer cells, now called the “seven hallmarks” of the cancer phenotype, may in the future help in the diagnosis, prognosis, and treatment of the disease [82].

Under aerobic conditions, normal cells utilize glucose, which is converted to pyruvate and then to CO₂, and water through the Tricarboxylic acid cycle (TCA) and oxidative phosphorylation in mitochondria. Oxygen acts as an electron acceptor, allowing the recycling of NADH to NAD⁺. When the cells have a limited supply of oxygen or have an impairment that impedes oxidation of pyruvate, this molecule then is reduced to lactate in the cytoplasm. This is an inefficient process producing 2 moles of ATP for every mole of glucose consumed. Cancer cells convert most of their glucose into lactate (regardless of presence of oxygen), aiding their own natural selection [83]. In accordance with these findings, it has been shown that most cancer cells have mutations in the TCA cycle enzymes, such as succinate dehydrogenase and fumarate hydratase, leading to metabolic changes that have already been observed at the RNA level [84,85]. Transcriptome changes in carcinogenesis have been widely studied and a great amount of data has been published [86–89]. However, most of these data require confirmation by means of a highly accurate method of quantitative real-time PCR.

Finding a considerable differential gene expression of mitochondrial F₀ and F₁ ATPase subunits between papillary thyroid cancer and a benign condition would be of great help because the diagnosis of malignancy in the thyroid nodule has not yet been resolved. Genes encoding alpha, beta, gamma, and epsilon of F₁, and “c12” of subunit F₀, were analyzed at the RNA level (qPCR by duplicate) to determine if abnormal expression of these genes could partially explain the Warburg effect occurring in PTC.

We propose that transcriptional analysis of several ATPase genes expressed by thyroid tumors should provide a means to define a signature fingerprint of molecular mechanisms underlying this type of cancer and might progressively improve conventional diagnosis parameters. Simultaneous expression levels of several genes offer better possibilities to understand and characterize the molecular mechanisms underlying PTC. Other studies have found correlations among mRNA expression and clinical characteristics of the disease. For example, in breast cancer Sorlie found certain expression patterns of mRNA related to tumor subclasses with clinical implications [90].

Few alterations have been reported to be associated with PTC at the molecular level. To the best of our knowledge, assessment of mitochondrial RNA expression of the ATPase subunits from thyroid tissue has not been analyzed by qPCR.

It is very clear from the results of the present study that there is a high degree of individual variability of the ATPase subunits expression for each patient (regardless of the type of illness), showing a unique profile of mRNA expression for each of the 6 genes measured.

From all 6 genes included in the present study, ATP5E transcript down-expression was highly associated only with PTC diagnosis OR=11.76 (95% CI 1.245–237.98, p=0.04). Many PTC patients displayed deregulation of this transcript. We recognize the limitation of having a very wide confidence interval, which means that we must confirm this initial information with a larger sample. This deregulation in PTC may explain why the rotation of gamma and epsilon F₁ subunits stops, thus, avoiding changes in the binding affinity of the catalytic subunits for ADP and P_i, and finally altering the ATP production [91,92].

Conclusions

ATP5E down-regulation transcript expression was highly associated only with PTC diagnosis and not with colloid goiter.

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