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Original Article

Increased Mitochondrial DNA Copy Number and Oxidative Damage in Patients with Hashimoto's Thyroiditis

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Abstract

Background: The purpose of present study was to investigate mitochondrial DNA copy number (mtDNAcn) and mtDNA damage in peripheral blood of patients with Hashimoto's thyroiditis (HT) and healthy controls (HC).

Methods: The relative mtDNAcn and oxidative DNA damage in this case-control study were measured in peripheral blood of 50 patients with Hashimoto's thyroiditis and 50 healthy controls using quantitative real-time PCR. The study was conducted in Tehran University of Medical Sciences hospital, Tehran, Iran in 2018.

Results: HT patients had significantly higher mitochondrial DNA copy number and mitochondrial oxidative damage than the comparison group.

Conclusion: These data suggest the possible involvement of mitochondria and oxidative stress in the pathophysiology of HT.

Keywords: Mitochondrial DNA; Hashimoto's thyroiditis; Oxidative stress; Autoimmunity

Introduction

Hashimoto's thyroiditis (HT) is an autoimmune condition arising from an abnormal immune response to thyroid antigens (1). Although the exact mechanism (s) of induction and maintenance of thyroid autoimmunity is not fully understood, oxidative stress and mitochondrial dysfunction are involved in the pathogenesis of autoimmune thyroid disorders including HT (2-4). Current research also identify a link between altered thyroid state (such as hypo- and hyperthyroidism), thyroid hormones and mitochondrial oxidative stress (5-7). Mitochondria are the main endogenous source of reactive oxygen species (ROS) and contain an independent genome (mtDNA) that is multicopy (8).

The total number of mitochondrial DNA copy number (mtDNAcn) remains relatively constant within the cell, under normal physiological conditions, but changes in these genetic materials have been identified in several human diseases (9, 10). Moreover, oxidative stress may influence the mitochondrial abundance and DNA copy number (11). Therefore, assessment of mtDNAcn in patients with HT provides a reasonably precise estimate of the relationship between mitochondria and disease.

ROS-mediated mtDNA damage is another crucial factor in mitochondrial dysfunction. mtDNA is highly vulnerable to oxidative damage for the following reasons. First, the lack of histones, which have a role in DNA protection (12). Second, mtDNA exists in close proximity to the respiratory chain, a well-known source of ROS. For this reason, it is considered as a good target for damaging effects of ROS (13). Third, mtDNA repairs capacities are less effective than nuclear repair systems. Therefore, overproduction of ROS under stressful conditions lead to various types of mtDNA damage including strand breaks, Abasic sites, base changes, and deletions (14).

Although the four nitrogenous nucleobases of DNA have labile sites for oxidation, the most susceptible site for oxidative damage on DNA is on guanosine. The oxidized form of guanine is a primary oxidative base modification in DNA (15). Therefore, guanine oxidation products becomes important biomarkers for the measurement of free radical-induced damage to nuclear and mitochondrial DNA. Formamidopyrimidine DNA-glycosylase (FPG) is an enzyme that exhibit a high specificity for oxidized purines, including 8oxoGua, 2, 6-diamino-4-hydroxy-5-formamidopyrimidine (FaPyGua), and 4, 6-diamino-5-formamidopyrimidine (FaPyAde) and other ring-opened purines (16, 17). This enzyme has both DNA glycosylase activity and AP lyase activity and generates single-nucleotide gaps with a 3′ phosphate terminus (18). Cleavage of the DNA lesions by FPG can reduce further amplification of this particular region by Taq DNA polymerase (19).

Thus, this method is suitable for measurement of oxidative damage to DNA and has been widely applied on the study of oxidative stress-related disease. The two main objectives of this study were (i) analysis of mtDNAcn in patients with HT and in HCs, (ii) assessment of oxidative DNA damage.

Methods

Study population

In this case-control study, venous blood samples were taken from 50 drug-naïve and newly diagnosed patients with HT and 50 HC in 2018. The diagnosis of patients was performed in Outpatient Endocrinology Clinic, Tehran University of Medical Sciences hospital, Tehran, Iran. It was based on physical examinations and bio chemical laboratory results (thyroid-stimulating hormone (TSH), free T4, and anti-thyroid peroxidase (anti-TPO) antibodies). The features and laboratory results of patients with Hashimoto's thyroiditis and controls are described in Table 1.

Table 1: Clinical and demographic features of the patients with Hashimoto's thyroiditis and controls

Features	*Patients*	*Controls*
Number	50	50
Age(yr)	39.12 ± 12.24	30.44 ± 11.07
Gender(Female/Male)	43/7	41/9
FT4(ng/dl)	0.90 ± 0.16	1.16 ± 0.18
TSH(μIU/ml)	16.27 ± 18.81	1.89 ± 1.17
Anti-TPO(IU/ml)	498.51 ± 349.62	11.69 ± 4.35)

TSH: Thyroid stimulating hormone, FT4: Serum free T4, anti-TPO: anti-thyroid peroxidase antibody

Ethics statement

All aspects of the study protocol have received ethical approval from the Ethics Committee of Tehran University of Medical Sciences (no.IR.TUMS.REC.1397.072). It was performed in accordance with the standards laid down in the Declaration of Helsinki. Written informed consent **was** taken **from** all research participants.

Laboratory assessment of thyroid function

Serum levels of free thyroxine (FT4), Thyroid-stimulating hormone (TSH), and anti-TPO antibody were measured in all patients and HC. Estimation of serum FT4 concentrations (normal reference: 0.8-2.0 ng/dl) were done by a competitive enzyme immunoassay kit (Monobind Inc., Lake Forest, USA). The intra- and inter-assay coefficient of variation were 10.98% and 10.81%, respectively. The detection limit of the test was 0.3 ng/dl. Estimation of Serum TSH concentrations (normal value: 0.2–4 μIU/ml) were examined by immunoradiometric assay (TSH IRMA, Radim, Pomezia, Rome, Italy). Estimation of anti-TPO antibodies was also determined, using the diagnostic enzyme-linked immunosorbent assay (ELISA) kit (Monobind Inc., Lake Forest, USA). Values higher than 40 IU/ml were considered positive.

Assay of mitochondrial DNA copy number

DNA was extracted from whole blood using a DNA extraction kit (GeneAll, Seoul, Korea), according to the manufacturer's protocol. MtDNAcn was measured using a real-time quantitative PCR assay.

Two primer sets were designed for quantitative amplification of mtDNA (ND2, 5'- CCCTTACCACGCTACTCCTA-3' and 5'-GGCGGGAGAAGTAGATTGAA-3') and nuclear DNA (β-actin, 5'-AGACGCAGGATGGCATGGG-3' and 5'-GAGACCTTCAACACCCCAGCC-3'). To calculate the mtDNAcn**,** the mitochondrial to nuclear DNA ratio for each sample was determined separately. Real-time PCR was performed with SYBR Premix EX Taq II kit (Takara, Japan) in Rotor-Gene 6000 apparatus (Corbett Life Science, Australia). PCR conditions used in this study were as follows: 1 min at 95 ºC for the first cycle, 40 cycles of 4 sec at 95 ºC, 30 sec at 62 ºC, 15 sec at 72 ºC and a final extension at 72 °C for 15 sec.

Determination of oxidative DNA damage

Oxidized purine nucleotides are useful markers for the study of DNA damage arising from oxidative stress conditions. Therefore, the existence of oxidized purine bases (mostly 8-oxodGuo) that are FPG-sensitive sites were evaluated in whole blood DNA of patients with HT and normal controls. Oxidative DNA damage was quantified using real-time quantitative PCR. DNA damage was measured after treatment with formamidopyrimidine]-DNA glycosylase (FPG) and was presented as ΔCt. (ΔCT = CT treated - CT untreated). FPG generates apurinic sites by the enzymatic removal of damaged purines from double stranded DNA, and create 1 base gap (AP site) by AP-lyase activity that cleaves both 3´ and 5´ to the AP site. The presence of abasic sites in DNA after treatment of FPG have been shown to reduce the PCR efficiency and therefore increase the Ct value. For FPG digestion, a volume of 2 μl of each DNA sample was treated with 1U of FPG in 1xNEB buffer at 37 °C for 1 hour. Then the digested DNA was amplified by PCR program under the same conditions as mentioned above.

Statistical analysis

Comparison of the results from experimental and control groups was performed by two-tailed t-test. The Pearson correlation coefficient used to evaluate the strength of a linear association between two variables. Data are presented as mean ± standard deviation. If the *P*-value was equal to or less than 0.05, the results were statistically significant. All statistical analysis was performed with SPSS software (ver. 11.0; SPSS, Inc. Chicago, IL, USA).

Results

Increased mitochondrial DNA copy number in peripheral blood of patients with HT

The patients had significantly higher levels of mtDNA copy number than healthy controls (mean 0.57 vs 0.56, *P*=0.04).

There was no correlation between mtDNAcn and age, sex, anti-TPO antibodies, TSH and free T4.

Increased oxidative DNA damage in patients with HT

The mean value of oxidative DNA damage in patients (1.50 ± 0.78) were significantly increased (*P*=0.001) compared to healthy group (0.68±0.57). Moreover, no correlation was found between oxidative DNA damage and age, sex, anti-TPO antibodies, TSH and free T4. There was also no correlation between mtDNAcn and oxidative DNA damage (Fig. 1).

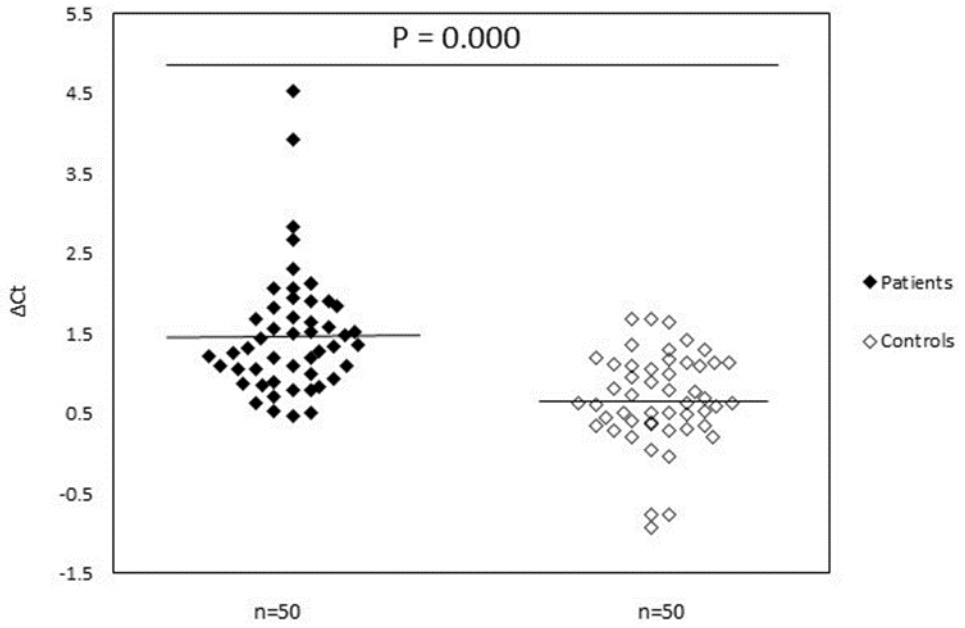

Fig. 1: Increased mtDNA damage in patients with Hashimoto's thyroiditis as compared to normal controls. The ΔCt is the difference between Ct value from DNA sample treated with FPG and Ct value from DNA sample without FPG treatment. Please note that a higher ΔCt value corresponds to comparably higher levels of oxidative base damage

Discussion

This case-control study was conducted to investigate the status of peripheral blood mtDNA copy number and mitochondrial oxidative damage in subjects with HT. Patients with HT had significantly higher mitochondrial DNA copy number and mitochondrial oxidative damage than the comparison group. These findings are consistent with earlier results showing a link between oxidative stress and autoimmune thyroid diseases including HT and have the potential to provide new insights into the pathogenesis of HT. Therefore, further studies toward the identification of oxidative stress markers could improve our understanding of disease pathogenesis. To date, a wide range of oxidative stress biomarkers and laboratory techniques has been recognized but, unfortunately, there is no general agreement on the most appropriate biomarker (20, 21). In re-

cent years, great attention has been paid to investigating the interrelationships between mitochondrial DNA alterations and oxidative stress (22). The mitochondrial genome is a suitable target for laboratory measurement of oxidative stress for the following reasons. First, mtDNA, which is present in multiple copies per cell, is a marker of mitochondrial function and a growing body of evidence shows that quantitative and qualitative changes in mtDNAcn occur in many human disorders, including autoimmune diseases (9). Second, mitochondrial dynamic, and mtDNAcn are sensitive to oxidative stress and the pressure of oxidative stress may contribute to the alteration of mtDNAcn (23). As a result, quantification of mtDNAcn will provide **a** better understanding of the pathogenesis of oxidative stress-related diseases. Third, the increased level of oxidative stress byproducts can lead to several types of DNA damage, including the formation of oxidatively modified guanine bases, which is one of the most common types of mitochondrial and nuclear oxidative DNA damage (24). Therefore, it has been extensively used as a biomarker for oxidative stress.

With reference to the subjects mentioned above, and considering the importance of thyroid hormones in regulation of mitochondrial activities (6), the present study focuses on the quantification of two important measures of oxidative stress: mtDNAcn and formation of DNA lesions such as 8-hydroxy guanine.

qPCR results showed a significant elevation in the copy number of mtDNA and concomitant increased mitochondrial DNA damage in the whole blood of patients with HT. To the best of our knowledge, this is the first study to report the copy number variations of mtDNA in the blood of HT patients. The present results are consistent with those of previous studies, showing increased copy number of mtDNA and defect of respiratory chain complex I within thyroid oncocytes and oncocytic lesions (cells with abundant granular eosinophilic cytoplasm containing many mitochondria) of patients with HT (25, 26). However, some studies have reported no change in (27) or even reduced amount of mtDNAcn (28) in patients with hypothyroidism or animal models of hypothyroidism.

Parallel controversies also exist over the role of oxidative DNA damage in HT patients. The present study provided evidence for increased levels of oxidative DNA base modifications in peripheral blood of patients with HT. These findings are in accordance with the earlier reported studies. For instance, increased levels of 8-OHdG were found in DNA isolated from liver tissues in rats with hypothyroidism (29). Elelevated levels of 8-OHdG have also been reported in the urine and thyroid tissue of patients with HT (3, 30).

Moreover, a positive correlation has been reported between urinary 8-OHdG and thyroglobulin antibodies in the HT patients (30). Contrary to the above presented experiments, several other studies show decreased levels of oxidative DNA base lesions in hypothyroid state. For instance, López-Torres et al. indicated a decreased concentration of 8-oxodG in heart genomic DNA of the animal model of experimental hypothyroidism (31). Additionally, levels of 8-OHdG in supernatant of mononuclear cell cultures did not differ between patients with HT and controls (32). Similar results were also obtained when the urinary levels of 8-OHdG were determined in children with euthyroid congenital hypothyroidism (33).

These contradictory results may be difficult to **i**nterpret for a number of reasons. In our opinion, the main source of uncertainty that could contribute to faulty conclusions is the incomplete knowledge about the role of endocrine and thyroid hormones in regulating of tissue oxidative stress as a dynamic and complex phenomenon. Another major challenge is the lack of clear understanding about the regulatory programs required for the coordinated expression of the nuclear and the mitochondrial genomes of the oxidative phosphorylation (OXPHOS) machinery.

It must be remembered that each cell type has specific capacity of oxidative phosphorylation that is required for its metabolic and energetic requirements (34) and hormones undoubtedly play an important role in mitochondrial biogenesis (35).

The pleiotropic effects of thyroid hormones on mitochondria (36), thyroid status (37-39), and the existence of mechanisms that control mtDNA expression between different tissues (34) are other complicating factors that contribute to a delay in understanding of the mechanism of oxidative stress implication in HT. In addition to those mentioned above, clinical factors such as duration of disease, disease activity, stage of disease and its treatment can also lead to misinterpretation of results. The choice of different methods and normalization criteria seems to be a plausible explanation for part of these discrepancies.

This study, like other researches has some limitations considered. One main limitation is related to variability in copy number of mitochondrial DNA in tissue and cell types (40). Therefore, changes in whole blood mtDNAcn may not reflect the exact copy number of mitochondrial DNA in the thyroid. The second limitation that can affect the conclusion validity is related to the distribution of different blood cell types across samples. This distribution may influence the estimates of mtDNAcn in whole blood and may confound associations under investigation (41). Another relevant issue is the differences between the distribution of immune cell subtypes in the thyroid and the circulation of patients with autoimmune thyroid diseases (42). Therefore, measuring the mtDNAcn and oxidative DNA damage in DNA extracted from thyroid of patients may yield different results. Lack of post-treatment follow-up is considered another limitation of this study because comparison of mtDNA variation before and after treatment is one effective way to evaluate the degree of change resulting from medical interventions.

Conclusion

Hypothyroidism increases the mtDNAcn and oxidative DNA damage. Oxidative damage to mtDNA and quantitative changes in human mitochondrial DNA (mtDNA) copy number may contribute to the cascade of events leading to HT.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interests.

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