Cytogenetic Analysis Associated with Hashimoto's Thyroiditis in Samples of Iraqi Patients: an in vitro study

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ABSTRACT

Background: One of the most prevalent autoimmune diseases is Hashimoto's thyroiditis (HT), which is characterized by autoantibodies specific to the thyroid but whose precise cause is still unknown. **Objective:** By comparing a sample of Iraqi patients with healthy controls using the comet assay, chromosomal abnormalities, micronucleus production, and mitotic index, the current study aims to clarify the cytogenetic impact of Hashimoto's thyroiditis. **Methodology**: The study included ten Iraqi patients with HT (six males and four females), ages 20 to 75 years old, compared with ten healthy control groups (six males and four females). **Results**: The results showed that only cells in metaphase /1000 were being assessed, and the patient group (9.82 ± 0.0153) was more affected than the healthy control group (5.94 ± 0.170). The micronucleus formation result compared to the proportion of healthy controls was (0.004±0.0018 Mn/cell), while the frequency of Mn formation in HT was higher (0.0068±0.00132 Mn/cell). Three parameters were employed in the comet assay to indicate DNA damage in HT patients and healthy controls: tail length (9.2±6.016 and 25.5±10.607 px), tail moment (2.047±2.687 and 11.32±15.058), and DNA damage in the tail (20.892±11.225 and 35.153±44.429%). **Conclusion:** The percentage of mitotic index, micronucleus formation, and DNA damage detected by the comet assay in HT patients was higher than in healthy control.

Keywords: Hashimoto's thyroiditis, Mitotic index, Micronucleus, and Comet assay.
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1-INTRODUCTION

The etiopathogenesis of Hashimoto's thyroiditis (HT), sometimes referred to as chronic lymphocytic thyroiditis or autoimmune thyroiditis (AIT), is still unknown. It is characterized by persistent thyroid gland inflammation. In places where iodine is plentiful, hypothyroidism is primarily caused by HT, which is also the most prevalent autoimmune illness and endocrine problem (1,2). The diagnosis of HT is based on imaging tests (hypoechogenic inhomogeneous thyroid structure in ultrasonography) and biochemical testing (positive circulating thyroid autoantibodies) with distinctive clinical symptoms (3). The incidence of HT is on the rise. Anti-thyroid peroxidase antibodies (TPOAb) and anti-thyroglobulin antibodies (TGAb) are produced in HT patients. About 90% of HT patients have circulating TPOAb, but TGAb is less specific and less sensitive than TPOAb (positive in 60–80% of patients) (2,4). Women are almost eight times more likely to get HT than men. Furthermore, Whites and Asians are more likely to have it than African Americans (1). All age groups of women are mostly affected by HT, although middle-aged women are more likely to be affected (5).

Our body is made up primarily of somatic cells. It's possible that for our organism to maintain normal balance, these cells will need to proliferate. The cell cycle is a highly regulated process that drives growth. A cell eventually divides into two identical cells during this process, which takes one or two days. Cell death or

unchecked cell proliferation that leads to cancer can be the outcome of cell cycle dysregulation (6). Furthermore, cell development or death may be altered as a result of exposure to hazardous substances (7). Since bone marrow is the source of all blood cells, many studies that assess the immune system's activity and how various agents affect it rely on lymphocytes' capacity to proliferate in lymphoid organs and/or divide bone marrow cells (8). The ratio of cells in a population going through mitosis to all cells is known as the mitotic index (MI) assay (10). Consequently, this assay allows for detecting the effects of various physical and chemical agents on the mitotic response. Prior research has shown that chemicals, radiation, medications, and medicinal plants can all positively or negatively impact MI (10, 11, 12).

Micronuclei (MN), originating from acentric chromosomal fragments or chromosome lagging during anaphase, are cytoplasmic chromatin masses resembling tiny nuclei (13). Cells containing two or more nuclei during interphase result in chromosomal damage and micronuclei. This indicates that binucleate human lymphocytes, which would have caused in vivo aging (14). Micronuclei (MNs) are small additional nuclear entities that can be detected using light microscopy. They are formed in dividing cells from complete chromosomes or chromatids that lag behind in anaphase or from segments of chromosomes without centromeres. Numerous factors, such as tubulin mis-attachment, kinetochore protein or assembly abnormalities, late replication, histone epigenetic alterations, nucleoplasmic bridge creation, and gene amplification, can cause MN (15). They could develop on their own as a means of eliminating excess DNA (16). They could also be the consequence of being around aneugens or clastogens. The MN frequencies have been investigated in erythrocytes, exfoliated epithelial cells, peripheral lymphocytes, and other cell types to monitor human genetic damage (15).

Measuring DNA damage can be done quite simply with the comet test. Its application in many scientific domains, such as mutagenicity testing for chemical and medicinal approval, has increased dramatically (17). The comet assay, known as single-cell gel electrophoresis, can utilize numerous cell types. Comet test analysis can be actually carried out on any eukaryotic cell that can be obtained as a single cell or a nucleus suspension (18). It is commonly used in genotoxicity testing, both in vitro and in vivo, clinical settings, and human biomonitoring studies to investigate the effects of exposure to potentially harmful substances on DNA in the workplace or environment. The strength of the comet tail in relation to the head (19) determines the damage to DNA. The comet assay's key benefits include its low cost, rapidity, simplicity, demand for a very small number of cells without needing cell culture, and broad adaptability (19, 20). In fact, variations on this method enable the quantification of various DNA structural changes (such as oxidation, alkylation, cross-linking, etc.) as well as DNA repair ability (21, 22).

2-Material and Method

2.1 Subjects

Ten patients with Hashimoto's thyroiditis (HT) were referred to the specialized laboratories that served as the subjects of this assay. The laboratory team's clinical examination and laboratory analyses served as the foundation for the diagnosis. The patients were Arabs from Iraq, and they ranged in age from 20 to 75. They were initially identified from January to July 2022. A control group of ten additional healthy people had examinations as well. They were matched for age and ethnicity with patients; they were university staff members and students without a history of HT symptoms. For cytogenetic analysis, each subject had two milliliters of peripheral blood extracted via aseptic venipuncture using a disposable syringe.

2.2 Mitotic Index Assay

The procedure of (23) was followed to estimate the MI and C.A. in HT patients. The percentage rate for only the divided cells was then determined using the formula below:

$$\text{Mitotic index} = \left(\frac{\text{Number of the divided cells}}{\text{Total number of the cells}} \right) \text{x } 100$$

2.3 Micronucleus assay

The procedure of (24) was followed to estimate the MN formation in HT patients. The micronucleus index was scored using the following equation:

$$\text{Micronucleus index (micronucleus/cell)} = \left(\frac{\text{Number of Micronuclei}}{\text{Total Count of Cells}}\right) \text{x } 100$$

2.4 Alkaline comet assay (single cell gel electrophoresis) (SCGE)

Singh and associates created the comet assay's more adaptable alkaline technique in 1988. The comet assay, or the single-cell gel electrophoresis assay, is a simple, fast, and sensitive technique for determining how much DNA damage is present in individual human (and occasionally prokaryotic) cells (25). The fundamental idea of the assay was based on the cells placed on a microscope slide in a thin layer of agarose gel. The DNA was allowed to unravel in an alkaline/neutral environment following the lysis of the cells to remove all cellular proteins. Following chromatin relaxation or fragmented DNA fragments (damaged DNA) migrating away from the nucleus during electrophoresis, the DNA was unraveled, electrophoresed, and then fluorescently colored. There is a clear correlation between the degree of DNA damage and the amount of DNA released from the comet's head (26).

3-Results

3.1 Mitotic Index Assay

The percentage of MI in HT was (9.81±0.007%) compared with the percentage of MI in healthy control, which was 5.94±0.170%, in which only cells at metaphase/1000 were scored, as shown in Table (3-1).

Table (3-1): Mitotic index percentage in HT patients and healthy control

Groups	Mitotic index (MI) %
	Mean ± S.E.
Patients	9.81±0.007
Healthy controls	5.94±0.170
P value	<0.0001**
**P-value ≤0.01	

3.2 Micronucleus formation

The frequency of micronucleus formation in Hashimoto's thyroiditis was 0.0068±0.00132 mn/cell, in contrast to the MN frequency of a healthy control group of 0.004±0.0018 mn/cell. To determine the frequency of MN, at least 1000 cells were scored and divided into four groups: mononucleate, binucleate, trinucleate, and tetranucleate; however, only mononucleate showed in HT patients more than healthy controls, which has significant differences between groups at P- value≤0.05, as shown in Table (3-2).

Table (3-2): The frequency of micronucleus formation in Hashimoto's thyroiditis patients and healthy control

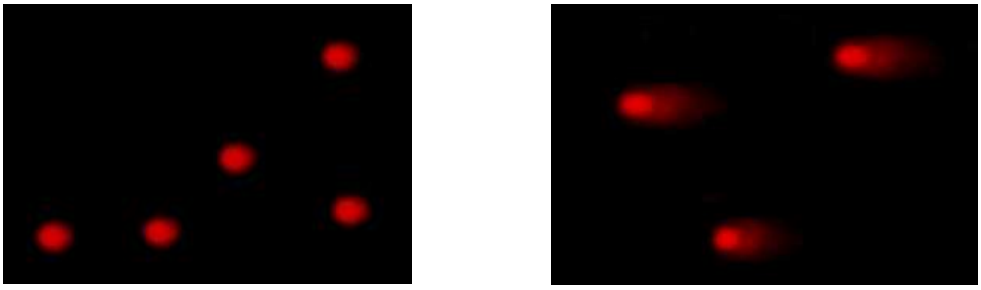
Groups	No. of MN/1000 cells	Distribution of MN in cells (Mean±S.E.)				No. of cells with MN
		0 MN	1 MN	2 MN	3 MN	
Patients	0.0068±0.00132	994±0.663	4.60±0.510	0.800±0.374	0.200±0.200	5.60±0.678
Healthy control	0.004±0.0018	996.24±1.20	2.88±0.0781	0.88±0.0781	0.00±0.00	3.76±1.0208
P-value	0.25	0.14	0.01*	0.84	0.35	0.17
*P-value ≤0.05						

3.3 Comet assay

In healthy control, the results showed that the percentage of tail length, tail moment, and the percent of DNA in the tail were 9.2±6.016, 2.047±2.687 and 20.892±11.225%, respectively, while in the HT patients the percentage increased to 25.5±10.607, 11.32±15.058 and 35.153±44.429%, respectively as represented in Table (3-3) and Figure (3-1).

Table (3-3): Tail length, tail moment, and DNA% in the tail of Hashimoto thyroiditis patients and healthy control (comet assay)

Groups	Tail length px	Tail moment	% DNA in tail
Patients	9.2 ± 6.016	2.047 ± 2.687	20.892 ± 11.225
Healthy control	25.5 ± 10.607	11.32 ± 15.058	35.153 ± 44.429



(A) **(B)**

Figure (3-1): Comet assay in Hashimoto's thyroiditis patients examined by fluorescent microscope (400X) of the control group (A), showing fluorescent spheres without DNA damage (no tail), HT patient (B), showing a lot of fluorescent heads with tails indicating DNA damage (ethidium bromide stain).

4-Discussion

A tissue sample's rate of cell division is gauged by its mitotic index. It is computed by dividing the total number of cells in a high-power field (HPF) by the number of mitotic figures, or cells that are dividing, in the HPF. Hashimoto's thyroiditis typically elevates the mitotic index (27). This is because the inflammation and immune response associated with the disease lead to increased cell turnover. However, the mitotic index in Hashimoto's thyroiditis is still lower than in malignant thyroid tumors (28).

Alzumaili *et al.* (2020) (29) found that patients with medullary thyroid carcinoma (MTC) possessed a high mitotic index that had a significantly worse prognosis than patients with MTC and a low mitotic index. Numerous studies found that the MI is elevated in HT, but they also concluded that the MI is not a sign of malignancy. The MI may be a useful prognostic marker in HT-associated thyroid cancer. Still, more research is needed to confirm this, which summarizes their findings as Moon *et al.* (2018) (30) found that the MI in HT was significantly higher than in normal thyroid tissue. However, they also found that the MI did not correlate with the presence of thyroid cancer; another study by Lee *et al.* (2015) (31) found that the MI was a significant predictor of disease recurrence in patients with HT-associated papillary thyroid carcinoma. Boi *et al.* (2018) (32) found that the MI was significantly higher in patients with HT-associated papillary thyroid carcinoma than in patients with HT without cancer. All things considered, the data points to a higher MI in HT. However, they also discovered that the MI did not correspond with the cancer's aggressiveness.

It is significant to remember that other illnesses like Hashimoto's thyroiditis can also cause a high mitotic index. As such, it is crucial to consider the pathologic and clinical context while interpreting the mitotic index. The increased cell turnover brought on by the inflammatory and immune response, the stimulation of cell

division by thyroid-stimulating hormone (TSH), and autoantibodies against thyroid peroxidase (TPO) and thyroglobulin (Tg) are considered to be the leading causes of the elevated mitotic index in Hashimoto's thyroiditis (33).

The results were recorded by the percentage of cells with MN relative to all counting cells. Table (3-2) findings demonstrated a significant ($P \leq 0.05$) increase in the amount of MN that might form compared to the control group, which had 0.0068 vs. 0.004 mn/cell, respectively. Our findings corroborated those of Karaman *et al.* (2011) (34), who reported that in patients with rheumatoid arthritis disorders, MN frequencies were considerably greater than in controls.

An increasing body of research suggests that MN could play a role in the onset and course of HT. According to a number of studies, blood cells from HT patients have more MN than those from healthy controls. In contrast to the current study, Al-Faisal *et al.* (2014) (35) reported that the frequency of MN was considerably ($p < 0.05$) lower in hypothyroidism compared to other thyroid disorders with no difference relative to the healthy group.

However, the relaxation of supercoiling, which requires a break and takes place independent of pH, is crucial in deciding whether a DNA segment appears in the head or tail of the comet. Therefore, it is a matter of common observation that, rather than tail length, the relative intensity of DNA staining in the tail increases with increasing damage of DNA, finding that it is completely compatible with a rising number of loops becoming relaxed (36). The current study differed from a previous study that found that patients with rheumatoid arthritis had DNA damage rates much greater than those of the controls (34). According to Signore *et al.* (2022) (37), a similar number of DNA breaks were examined.

5-Conclusion

In the current study, the results of MI indicated that the percentage of MI in HT patients was higher than in the healthy control. Also, DNA damage was detected by the comet assay and MN test in HT patients, which estimated that the DNA damage with this disorder was increased compared with the healthy control.

Recommendation:

In this study we recommend increase in samples to cover the remaining provinces, Take another Criteria like: number of pregnancies, presence of multiple partners, Smoking, presence suppressive disease and immune suppressive drug use.

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التحليل الوراثي الخلوي المرتبط بالتهاب الغدة الدرقية داء هاشيموتو في عينات من المرضى العراقيين: دراسة مختبرية

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الخلفية: يعد التهاب الغدة الدرقية داء هاشيموتو (HT) الذي يتميز بوجود أجسام مضادة خاصة بالغدة الدرقية، أحد أكثر اضطرابات المناعة الذاتية شيوعًا، على الرغم من أن المسببات الدقيقة لم يتم توضيحها بالكامل. **الأهداف:** صممت الدراسة الحالية لتسليط الضوء على التأثيرات الوراثية الخلوية الناجمة عن التهاب الغدة الدرقية داء هاشيموتو في عينة من المرضى العراقيين مقارنة مع الاصحاء والتي تم تقديرها بواسطة مؤشر الانقسام الفتيلي والانحرافات الكروموسومية وتكوين النواة الصغيرة وفحص المذنب. **الطرق:** شملت الدراسة عشرة مرضى عراقيين HT تراوحت أعمارهم بين 20 - 75 سنة. وشملت ستة ذكور وأربع إناث مقارنة بستة ذكور وأربع إناث اشخاص اصحاء. **النتائج:** تم الحصول على النتائج التالية والتي تم فيها تسجيل الخلايا فقط في الطور الاستوائي/1000 والتي كانت مجموعة المرضى الملاحظين (9.82 ± 0.0153) أكثر تأثراً من مجموعة الأصحاء (5.94 ± 0.170). أشارت نتيجة تكوين النواة الصغيرة إلى أن تكوين النوية في HT كان أعلى (0.0068±0.00132 نوية/خلية) بالمقارنة مع النسبة المئوية في الأصحاء (0.004±0.0018 نوية/خلية). في اختبار المذنب، تم استخدام ثلاثة عوامل كمؤشر لتلف الحمض النووي في مرضى HT والأصحاء حيث كان طول الذيل (9.2 ± 6.016 و 25.5 ± 10.607)، بينما في تلف الحمض النووي في الذيل كان (20.892 ± 11.225 و 35.153 ± 44.429). ولحظة الذيل (2.047 ± 2.687 و 11.32 ± 15.058) على التوالي .**الأستنتاج:** نسبة فحص مؤشر الأنقسام الخلوي، التشوهات الكروموسومية وتلف الحمض النووي بطريقة الكومنت للمرضى المصابين بداء هاشيموتو اعلى من الاشخاص السليمين.

الكلمات المفتاحية: التهاب الغدة الدرقية هاشيموتو، مؤشر الانقسام الفتيلي، النواة الصغيرة، واختبار المذنب.