CLINICAL STUDY

Upregulation of thyroid transcription factor-1 and human leukocyte antigen class I in Hashimoto’s disease providing a clinical evidence for possible triggering autoimmune reaction

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Abstract

Objective: An increase in the expression of autoantigens and their presenting molecules human leukocyte antigen (HLA) class I has been demonstrated to be responsible for autoimmune diseases. Thyroid transcription factor-1 (TTF-1 or NKX2-1) synchronously upregulates both HLA class I and thyroid-specific autoantigen, which may be involved in the pathological process of autoimmune thyroiditis. In this study, the expressions and potential role of TTF-1 and HLA class I in Hashimoto’s disease (HT) were examined.

Patients: In this study, 22 resection specimens clinically and histopathologically confirmed to have Hashimoto’s disease and 30 normal thyroid specimens from adjacent tissues of thyroid adenoma were used.

Measurement: Western blot, real-time PCR, and immunohistochemistry were performed to assay TTF-1 and HLA class I in the thyrocytes of Hashimoto’s disease as well as in the normal thyroid from adjacent tissues of thyroid adenoma.

Results: The TTF-1 and HLA class I in Hashimoto’s disease were significantly higher than those in the controls.

Conclusion: Upregulation of TTF-1 and HLA class I in Hashimoto’s disease provide a clinical evidence for possible triggering of autoimmune reaction.

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Introduction

Human leukocyte antigen (HLA) class I autoantigen-presenting molecules play an important role in immune tolerance and immune surveillance (1, 2). An increase in the expression of HLA class I is related to the pathogenesis of organ-specific autoimmune diseases such as pancreatic islet cells of type 1 diabetes mellitus, autoimmune hepatitis, and Graves’ disease (1, 3, 4). It has also been demonstrated to be responsible for immune cell infiltration (5) and can be considered as one of the earliest features of autoimmune attack in these diseases (4, 6–11). On the other hand, inhibition expression of HLA class I can prevent the development of experimental systemic lupus erythematosus, autoimmune blepharitis, and uveitis (12–18). Graves’ disease is one of the most common autoimmune thyroid diseases and is the archetype for organ-specific autoimmune disorders. Accumulated evidence proves that upregulation of HLA class I expression is involved in the pathological process of this disease (6, 19–21).

However, the expressions of HLA class I are still poorly understood in Hashimoto’s disease, which is another typical autoimmune thyroid disease.

Thyroid transcription factor-1 (TTF-1 or NKX2-1) is an enhancer of not only the HLA class I (6, 19) but also the positive control thyroid-specific autoantigens, namely, thyroglobulin (TG) and thyroid peroxidase (TPO). Both autoantigens play an important role in the pathological process of Hashimoto’s disease.

In this study, the expressions and the potential role of HLA class I and TTF-1 in human Hashimoto’s disease were investigated.

Materials and methods

Patients

After obtaining the approval of the institutional research ethics board, 22 resection specimens clinically and histopathologically confirmed to have Hashimoto’s
disease with benign nodule and 30 normal thyroid specimens from adjacent tissues of thyroid adenoma as the negative control according to WHO criteria (WHO, 2004) were collected. Among the specimens, 12 of the Hashimoto’s disease samples and 20 of the controls were paraffin-embedded specimens, whereas the others were fresh tissue specimens collected within the recent 2 years. All the patients with Hashimoto’s disease underwent surgery because the benign or malignant nature of the complicated cold nodules could not be determined. For Hashimoto’ disease, all cases were female with 0.2–14 years’ course of the disease and having an enlarged thyroid volume from 1 to 3. All patients were treated with thyroxine or methimazole for 1–2 months in order to euthyroid before surgery, as they were affected by hypothyroidism (17 cases) or transient hyperthyroidism (five cases). In the case of the control group, the patients were euthyroid for 0.1–10 years in course of the disease. The details about clinical parameters at the time of diagnosis are shown in Table 1.

**Western blot analysis**

The Hashimoto’s disease and control protein samples were prepared as follows: 1 g of tissue was frozen in liquid nitrogen and ground to yield tissue powder, and then suspended in ice-cold RIPA lysis buffer (Biyuntian Biosynthesis Biotechnology Co., Ltd, Shanghai, China). The suspension was then sonicated for 20 min at 0 °C and centrifuged at 8000 g for 30 min. The protein concentration was determined by BCA (Sigma) assay. The lysates were then separated electrophoretically in 12% polyacrylamide gels and transferred onto PVDF membranes (Beijing Biosynthesis Biotechnology Co., Ltd, Beijing, China). The membranes were blocked for 1.5 h at room temperature in 5% nonfat milk and incubated overnight at 4 °C with TTF-1 and HLA class I antibodies (the same as those used in immunohistochemistry), respectively. After the triple wash in Tris buffered saline with tween (TBST) for 30 min, the membranes were incubated with HRP-conjugated secondary antibodies for 45 min and then triple washed again in TBST. Immuno-reactive bands were revealed using an ECL detection system. In the negative group, 2% BSA was used instead of the primary antibodies. GAPDH (Beijing Biosynthesis Biotechnology Co., Ltd) was also detected as an internal control. The X-ray film was scanned, and the band density was calculated using ImageJ software (22).

**Fluorescent quantitative real-time RT-PCR analysis**

Thyroid tissue was frozen in liquid nitrogen and ground to yield tissue powder. Total RNA was isolated from this tissue powder using RNAiso Plus kit (TaKaRa, Dalian City, Liaoning Province, China). The RNA precipitate was then dissolved in 10–15 μl of RNase-free water and analyzed for quantity and quality using a spectrophotometer. The integrity of total RNA was determined by 1% formaldehyde agarose gel electrophoresis. A two-step RT-PCR procedure was performed using the PrimeScript RT Reagent kit (TaKaRa) following the manufacturer’s instructions. cDNA was then used in the real-time PCR. For PCR amplification, 2 μl of cDNA was used in a 20 μl reaction mixture. The PCR primers are shown in Table 2. Hot-start real-time PCR was performed using SYBR green-based detection in Rotor gene 6000 and initiated at 95 °C for 30 s; the mixtures were then subjected to 40 cycles of a two-step PCR, comprising 5 s of denaturation at 95 °C and 30 s annealing/elongation at 60 °C.

β-Actin was amplified as the internal control. Data were then analyzed using the relative expression software.

**Immunohistochemistry analysis**

The tissues were fixed in 10% formalin and embedded in paraffin. Sections (4 μm) were taken from the tissues, affixed to 3-aminopropyl triethoxysilane-coated slides, and air-dried overnight at 37 °C. After dewaxing and antigen retrieval, endogenous peroxidase was quenched with 3% hydrogen peroxide for 5 min. After blocking with goat serum, the slides were incubated for 30 min at 37 °C with mouse anti-HLA class I (at 1:50 dilution), rabbit anti-interferon (IFN)-γ (at 1:300 dilution) and rabbit anti-TTF-1 (at 1:50 dilution) polyclonal antibody (Beijing Biosynthesis Biotechnology Co., Ltd, respectively).

**Table 1** Clinical parameters of Hashimoto’s disease and controls at the time of diagnosis.

<table>
<thead>
<tr>
<th></th>
<th>Gender (M/F)</th>
<th>Age (years)</th>
<th>FT3 (pmol/l)</th>
<th>FT4 (pmol/l)</th>
<th>TSH (mU/l)</th>
<th>TG-AB (IU/ml)</th>
<th>TPO-AB (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hashimoto’s</td>
<td>0/22</td>
<td>44.7 ± 12.1</td>
<td>1.7 ± 0.6</td>
<td>3.8 ± 1.5</td>
<td>52 ± 16</td>
<td>460 ± 39</td>
<td>950 ± 75</td>
</tr>
<tr>
<td>Controls</td>
<td>10/20</td>
<td>41.6 ± 13.7</td>
<td>3.2 ± 0.4</td>
<td>11.8 ± 2.1</td>
<td>1.6 ± 0.3</td>
<td>28 ± 9</td>
<td>33 ± 8</td>
</tr>
<tr>
<td>Normal range</td>
<td></td>
<td></td>
<td>2.5–3.9</td>
<td>5.77–16.23</td>
<td>0.34–5.6</td>
<td>0–54</td>
<td>0–115</td>
</tr>
</tbody>
</table>

**Table 2** Sequence of the primers.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequence of the primer (5’ → 3’)</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-A</td>
<td>Forward: GATTACATCGCCTTGAACGAGG</td>
<td>436–457</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGAGACACGGTGTGAGTCAT</td>
<td>657–637</td>
</tr>
<tr>
<td>TTF-1</td>
<td>Forward: GAGGGCCTAGAACATGAG</td>
<td>201–221</td>
</tr>
<tr>
<td></td>
<td>Reverse: TTGTAGCGGTGGTTCTGGA</td>
<td>278–262</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: CATGTACGTTGCTATCCAGG</td>
<td>393–413</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCTCTTAAATGTCAGGAGCAT</td>
<td>642–622</td>
</tr>
</tbody>
</table>
All procedures were performed with a diaminobenzidine (DAB) detection kit (Beijing Biosynthesis Biotechnology Co., Ltd) following the standard protocol. After washing, all slides were counterstained with haematoxylin–eosin for histological evaluation. For the negative control, the step on incubation with a primary antibody was omitted. The sections were examined under a microscope.

**Immunohistochemical evaluation**

The sections were observed randomly in 10 high-power fields. The results were scored semiquantitatively. The intensity of staining was scored as no staining (0), weak staining (1), moderate staining (2), or strong staining (3). The percentage of stained thyroid cells was scored as no stained cells (0), staining in <25% of the cells (1), staining in 25–50% of the cells (2), staining in 50–75% of the cells (3), or staining in more than 75% of the cells (4). The final result of each section was evaluated by averaging the sum of the intensities and percentages of the stained thyroid cells. The average was then scored as 0–2 (negative), 3–4 (positive), and 5–6 (strongly positive).

For the degree of lymphocytic infiltration, the range was scored as no lymphocytic infiltration (0), infiltration 0–15% (1), infiltration 15–30% (2), infiltration 30–45% (3), infiltration 45–60% (4), infiltration 60–75% (5), infiltration more than 75% (6). The result was scored as 0–2 (negative), 3–4 (positive), and 5–6 (strongly positive).

**Statistical analysis**

Student’s t-test was used to determine the differences of the western blot and PCR results whereas the Fisher exact test was used to determine the differences in positive-staining rate. In addition, the Wilcoxon rank sum test was used for determining the differences in stain results and the Spearman rank coefficient was used for determining the correlation between the parameters. Statistical differences were identified and a P value <0.05 was considered significant.

**Results**

**Western blot analysis of HLA class I and TTF-1 protein in Hashimoto’s disease**

Western blot was performed to determine the expression of HLA class I and TTF-1 in 10 Hashimoto’s disease and 10 control thyroid samples. Both HLA class I and TTF-1 in Hashimoto’s disease were significantly higher than those in the control (Fig. 1).

**Real-time PCR analysis of HLA class I and TTF-1 mRNA in Hashimoto’s disease**

Real-time RT-PCR was performed to determine the expression of HLA class I and TTF-1 in 10 Hashimoto’s disease and 10 control thyroid samples. Both HLA class I and TTF-1 in Hashimoto’s disease were significantly higher than those in the control (Fig. 2).

**Table 3** Immunohistochemistry detecting human leukocyte antigen class I in Hashimoto’s disease and control groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cases (n)</th>
<th>Negative (n)</th>
<th>Positive (%)</th>
<th>Strong positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hashimoto’s</td>
<td>22</td>
<td>0</td>
<td>16</td>
<td>100</td>
</tr>
<tr>
<td>Control</td>
<td>30</td>
<td>25</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>22.7*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>16.7*</td>
</tr>
</tbody>
</table>

*P < 0.01.
Immunohistochemistry analysis of HLA class I, TTF-1, and IFN-γ expression and the degree of lymphocytic infiltration in Hashimoto’s disease

Immunohistochemistry was performed to determine HLA class I, TTF-1, and IFN-γ (or IFNG) expression and the degree of lymphocytic infiltration in 22 Hashimoto’s disease and 30 control thyroid samples. HLA class I was localized in the cytoplasm and basolateral membrane, and the immunoreactivities of HLA class I in Hashimoto’s disease (Fig. 3D and Table 3) were significantly higher than those in the control (Fig. 3E and Table 3). TTF-1 was confined to the nucleus of the thyrocytes, and the immunoreactivities of TTF-1 in Hashimoto’s disease (Fig. 3A and B and Table 4) were significantly higher than those in the control (Fig. 3C and Table 4). Lymphocytic infiltration and IFN-γ expression were found in all samples with Hashimoto’s disease but not in any controls (Fig. 3F and Table 5).

The correlation between the parameters of the lymphocytic infiltration, TTF-1, IFN-γ, and HLA class I

The Spearman rank coefficient was performed to determine the correlation between the parameters of TTF-1, HLA class I, IFN-γ, and lymphocytic infiltration in 22 Hashimoto’s disease samples. The results showed that HLA class I positively correlated to TTF-1 ($r_s = 0.542$, $P < 0.05$). Lymphocytic infiltration and IFN-γ expression were found in all samples, however, the results did not show HLA class I positively correlated to IFN-γ ($r_s = 0.382$, $P > 0.05$) or the degree of lymphocytic infiltration ($r_s = 0.392$, $P > 0.05$); this may attribute to the heterogeneity including the difference of affected tissues and length of disease inside the group with Hashimoto’s disease.

Discussion

HLA class I molecules present autoantigen peptides to immune cells. Quantitative and qualitative variations in HLA class I and/or autoantigen have been demonstrated to be responsible for autoimmune diseases (23, 24). Several clinical studies have reported that HLA class I and thyroid-specific autoantigen are both upregulated in autoimmune diseases (4, 9–11). Methimazole and iodine, which are widely used to treat Graves’ disease, can synchronously decrease the expressions of HLA class I (19, 21, 23, 25) and thyroid autoantigens, such as the TSH receptor (TSHR), synchronously (9, 26). TSHR is one of the most important autoantigens in the thyroid and is responsible for the upregulation of other thyroid autoantigens including TG and TPO. Both autoantigens have been demonstrated to play an important role in the pathological process of Hashimoto’s disease. This phenomenon strongly indicates that some coordinate regulatory factors, which synchronously upregulate HLA class I and specific thyroid autoantigen, may play a key role in the pathological process of autoimmune reaction.

TTF-1, one of the coordinate regulatory factors for HLA class I and TSHR, can bind to cis-elements on the 5’-flanking regions of both genes and together promote the expressions of HLA class I and TSHR.

Table 4 Immunohistochemistry detecting TTF-1 in Hashimoto’s disease and control groups.

<table>
<thead>
<tr>
<th>Staining result</th>
<th>Cases</th>
<th>Negative</th>
<th>Positive</th>
<th>Strong positive</th>
<th>Positive rate (%)</th>
<th>Strong positive rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>$n$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hashimoto’s</td>
<td>22</td>
<td>0</td>
<td>7</td>
<td>15</td>
<td>100</td>
<td>68*</td>
</tr>
<tr>
<td>Control</td>
<td>30</td>
<td>0</td>
<td>24</td>
<td>6</td>
<td>100</td>
<td>20*</td>
</tr>
</tbody>
</table>

$^*P<0.01.$
Table 5 Immuno histochemistry detecting the expression of IFN-γ and the degree of lymphocytic infiltration in 22 Hashimoto’s disease samples.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Staining result</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative (case)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0</td>
</tr>
<tr>
<td>Lymphocytic infiltration</td>
<td>0</td>
</tr>
</tbody>
</table>

The 5′-flanking region of the HLA class I gene has a ‘hormone-sensitive region’ modulated by cytokines, growth factors, drugs, and the TSH (6, 19). Among these factors, iodine (27), TSH (19, 23), paired box gene 8 (PAX8), and other TTFs (11) inhibit the expression of HLA class I, whereas IFN-γ and TTF-1 promote the expression of HLA class I (19, 25). In particular, TTF-1 promotes the expressions of thyroid autoantigens including TSHR, TG, and TPO (28, 29), and it increases the expression of HLA class I. Therefore, it may play an important role in the development of autoimmune thyroid diseases (5, 23, 25).

Hashimoto’s disease is an organ-specific autoimmune thyroid disease characterized by the production of high-titer TG and TPO autoantibodies, local infiltration of lymphocytes, and a different degree of structural damage and dysfunction. The patients often undergo medical treatment instead of surgery, so obtaining thyroid tissues samples from Hashimoto’s disease is difficult. In this study, some cases were treated with methimazole, a drug that was thought to decrease HLA class I (19, 21, 23, 25), however, both HLA class I and TTF-1 in these cases were still found significantly increased. For TTF-1 positively regulated HLA class I and the thyroid-restrictive gene, the upregulation of TTF-1 leads to the synchronous increase of both HLA class I and thyroid autoantigens. This means that autoimmune reaction is triggered when more TG, TPO, or TSHr peptides were presented to immune cells, which might induce a clinically detectable autoimmune response. So, the findings provide a clinical evidence for possible triggering autoimmune reaction.

Although the expression of IFN-γ and lymphocytic infiltration was found in all samples with Hashimoto’s disease and both TTF-1 and IFN-γ were confirmed to enhance the expression of HLA class I (19, 25), only TTF-1 positively correlated to HLA class I. This means the overexpression of HLA class I may attribute to the upregulation of TTF-1 and may just trigger autoimmune reaction rather than involve in the degree of immune damage.

A recent study on nontoxic goiters found that TG stored in the follicular lumen strongly promotes the expression of HLA class I (11). However, the autoimmune reaction is mild because TG simultaneously suppresses TTF-1, which can efficiently decrease the expressions of thyroid autoantigens, namely, TG, TPO, and TSHR (30). On the contrary, the data from previous studies showed that under physiological condition, TSH and PAX8 promote thyroid physiological functions by upregulating the autoantigens TG, TPO, and TSHR (25), but simultaneously decreasing class I, so the autoantigens cannot be efficiently presented. Based on these findings, an assumption is made, in which the increase in the thyroid physiological functions results in the upregulation of special thyroid autoantigens. The thyroid protects itself from triggering autoimmune reaction by inhibiting the expressions of HLA class I under physiological condition. In addition, the upregulation of TTF-1 results in the synchronous upregulation of class I and thyroid autoantigens, which might represent a pathological status of the thyroid and finally lead to thyroid autoimmune diseases.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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29 Saji M, Moriarty J, Ban T, Singer DS & Kohn LD. Major histocompatibility complex class I gene expression in rat thyroid cells is regulated by hormones, methimazole, and iodide as well as interferon. Journal of Clinical Endocrinology and Metabolism 1992 75 871–878. (doi:10.1210/jc.75.3.871)

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