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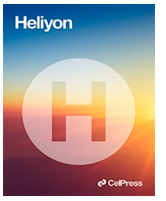

Research article

Peptidomic profiling of endogenous peptides in the spleens of mouse models of Graves' disease

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A R T I C L E I N F O

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A B S T R A C T

Background: Graves' disease (GD) is a common autoimmune thyroid disorder. The pathogenesis of GD involves an autoimmune response to the A subunit of the human thyrotropin receptor (hTSHR), although the specific mechanisms are not fully elucidated.
Methods: This study established a GD model by immunizing BALB/c mice with a recombinant adenovirus expressing the hTSHR A subunit. Spleen tissues were collected and analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to identify differentially expressed peptides (DEPs). Gene Ontology (GO) analysis and KEGG pathway analysis were further utilized to annotate the functions of these DEPs. Additionally, peptide bioactivity prediction and molecular docking studies were conducted using Alphafold and Pymol software, respectively, to assess the binding affinity of specific peptides to the hTSHR A subunit.
Results: The GD mouse model was successfully established, and 1,428 DEPs were identified in the spleen, with 368 upregulated and 1,060 downregulated. Functional analysis indicated that these DEPs are mainly involved in cellular endocytosis, regulation of gene expression, and nucleocytoplasmic transport. Notably, molecular docking studies revealed that the abnormally highly expressed peptide HG2A-24aa demonstrated potential bioactivity and strong binding affinity with hTSHR-289aa.
Conclusion: The specific bioactive peptides may play key roles in the pathogenesis of GD, particularly HG2A-24aa, which may have a significant role in the MHC II antigen presentation pathway mediated by the hTSHR A subunit.

1. Introduction

Graves' disease (GD) is a prevalent organ-specific autoimmune disorder primarily presenting with hyperthyroidism and diffuse goiter. The incidence of GD ranges from 20 to 50 cases per 100,000 individuals annually [1]. Despite advances in diagnostic and therapeutic strategies leading to milder disease phenotypes, thyroid storms—acute and life-threatening exacerbations—can still occur. Current treatment modalities for GD, such as antithyroid medications, radioiodine therapy, and thyroidectomy, are effective but often associated with significant side effects [2]. Given these challenges, it is imperative to delve deeper into the molecular mechanisms that drive GD pathogenesis. Understanding these mechanisms can facilitate the development of more precise diagnostic tools and tailored therapeutic approaches [2]. Molecular research can uncover specific biomarkers for early detection and identify novel targets for

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intervention, potentially minimizing adverse effects and improving patient outcomes.

Central to the pathogenesis of GD is the production of autoantibodies targeting the A subunit (amino acids 1–289) of the human thyrotropin receptor (hTSHR), a principal immunogenic element of hTSHR [3]. Upon release into circulation, this subunit is internalized and processed into peptides by antigen-presenting cells (APCs). These peptides then form complexes with MHC class II (MHC II) molecules and are presented to $CD4^+$ T cells. This interaction stimulates $CD4^+$ T cells to secrete a spectrum of cytokines and chemokines, driving B cells to produce thyrotropin receptor antibodies (TRAb), ultimately leading to hyperthyroidism [4]. Despite these insights, the precise molecular mechanisms by which the A subunit incites autoimmunity in GD remain incompletely understood, highlighting the need for further investigation to identify novel therapeutic targets.

Unlike in humans, GD does not occur spontaneously in animals. Immunization of mice with soluble TSHR protein and various adjuvants can induce TSHR antibodies and sometimes thyroiditis, but it does not result in overt hyperthyroidism. However, TSHR antibodies and hyperthyroidism can be induced through unconventional methods, such as in vivo expression of TSHR. These methods include injecting TSHR-transfected MHCII-positive fibroblasts into I-Ak mice or B cells into Balb/c mice, and performing naked DNA vaccination with plasmids containing TSHR cDNA in certain mouse strains. The most widely used GD model involves multiple immunizations of Balb/c female mice with an adenovirus vector carrying hTSHR, which induces TRAb and hyperthyroidism in approximately 70 % of the mice [5]. The TSHR undergoes cleavage on the cell surface into two subunits: an extracellular A subunit and a predominantly transmembrane B subunit, which remain linked by disulfide bonds. After cleavage, some A subunits shed from the cell surface. Evidence suggests that the free A subunit is a more effective antigen for inducing thyroid-stimulating autoantibodies compared to the intact receptor, as thyroid-stimulating antibodies are more likely to recognize the free A subunit than the intact receptor [6]. Additionally, adenoviral expression of the TSHR A subunit, compared to an adenovirus encoding a TSHR modified to prevent cleavage and shedding, more effectively induces hyperthyroidism in Balb/c mice [7]. The hTSHR A subunit immunization mouse model allows for a detailed study of the molecular mechanisms through which the TSHR A subunit triggers GD. For example, Ye et al. utilized a GD animal model induced by TSHR A subunit immunization to elucidate the immune-related molecular profile associated with GD pathogenesis [8]. Other studies have explored the dynamics of interactions between TSHR epitopes and MHC class II molecules and the impact of TSHR glycosylation on disease induction [9]. Despite these advances, research on the antigen presentation mechanisms and the resulting immune cascade induced by the hTSHR A subunit remains limited, necessitating further investigation.

Recent investigations have highlighted the role of endogenous peptides as intrinsic bioactive molecules, extensively distributed across tissues and possessing potent biological functionalities. These peptides serve as crucial immunomodulatory agents, activating both $CD4^+$ and $CD8^+$ T cells, facilitating their proliferation and effector functions, and playing a pivotal role in the pathogenesis of autoimmune diseases [10]. In this context, our study aims to identify and characterize differentially expressed peptides (DEPs) in the spleen tissues of GD and control mice using peptidomic approaches. We anticipate that this investigation will uncover functional peptides involved in the antigen presentation pathway mediated by the TSHR A subunit, offering novel insights and potential targets for the diagnosis and management of GD.

2. Methods

2.1. Construction and validation of the GD mouse model

To establish a GD mouse model, 10 female BALB/c mice aged 6–8 weeks were selected and maintained under specific pathogen-free conditions. The mice were divided into an experimental group (n = 5) and a control group (n = 5). Both groups received intramuscular injections of adenovirus (Ad) every three weeks, for a total of three injections, each delivering 50 μl at a concentration of 1 × 10ˆ10 PFU/ml. The experimental group was inoculated with adenovirus containing the TSHR sequence (Ad-TSHR289), while the control group received control adenovirus (Ad-NC), both provided by Hanheng Biotech Co., Ltd., China. One week after the final injection, mice were anesthetized, euthanized, and their serum, thyroid, and spleen tissues were collected.

Serological assays for FT3, FT4, and TRAb levels were conducted using an automated chemiluminescence immunoassay analyzer and reagents from Siemens Healthcare. The normal range was established by calculating the mean and three standard deviations (SD) of FT3, FT4, and TRAb in the control group (Ad-NC), with the upper limit serving as the criterion for successful GD model establishment. Three mice meeting the serological criteria were included in the GD group, and three Ad-NC mice were randomly selected for the control group (CON). Thyroid tissues were stained with H&E, sectioned, and analyzed under a microscope for image acquisition and analysis.

The study was approved by the Ethics Committee (Approval No. 2023-488).

2.2. Sample preparation

Mouse spleen samples were prepared as follows: Tris-HCl was added to the samples at a 1:3 vol ratio, boiled for 10 min, then rapidly cooled in an ice bath. Ultrasonication was performed at a frequency of 100 Hz for 2 min with 5-s intervals. Ice-cold acetic acid was added to a final concentration of 1 M, followed by 2 min of vortexing. The supernatant was collected and stored at −20 °C. An 80 % acetone solution was added, mixed by vortexing, and heated in a water bath, followed by ultrasonication at 20,000 rpm for 2 min at 4 °C, and then centrifuged at high speed for 30 min. The supernatant was transferred to new tubes, lyophilized, redissolved in 200 μl of 0.1 % TFA solution, and loaded onto a C18 column for desalting with a capacity of up to 80 μg. Samples were then lyophilized again in preparation for LC-MS analysis.

2.3. LC-MS/MS analysis

For LC-MS/MS analysis, samples were analyzed using a Q Exactive mass spectrometer equipped with a C18 column (3 μm, 150 mm × 5 μm). The mass spectrometer was set to positive ion mode, with a primary resolution of 70,000, AGC target of 3e6, and a scan range of 300–1,400 *m/z*. The top 20 ions were selected for MS/MS analysis at a secondary resolution of 17,500, AGC target of 5e4, and an isolation window of 3 *m/z*. The liquid chromatography used a C18 column (3 μm, 250 mm × 75 μm), with mobile phase A being 0.1 % formic acid in water, and mobile phase B being 0.1 % formic acid in acetonitrile. The flow rate was set at 300 nl/min, and the injection volume was 6 μl. The gradient for the mobile phase was as follows: from 6 % to 10 % B in 0–8 min, increasing to 30 % B over 8–60 min, rising to 42 % B between 60 and 79 min, quickly ascending to 95 % B at 79–80 min, held until 85 min, then rapidly dropped to 6 % B at 85–86 min, and maintained at 6 % B for the remaining time until 90 min. The total run time was 90 min.

2.4. Peptide spectrum analysis

Peptide analysis was performed using Mascot (version 2.5.1) and the SwissProt *Homo sapiens* database, with non-enzymatic digestion, methionine oxidation, and protein N-terminal acetylation as variable modifications; other settings were default. Data were processed using MAXQUANT (Bruker Daltonics). Peak intensity values were normalized by summing peaks across each sample. Differences in peptide expression between groups were estimated using the Mann-Whitney *U* test. Volcano plots and heatmaps were generated using the R package. The experiment included GD and CON groups.

2.5. Functional analysis

Functional analysis of the precursor proteins of differentially expressed peptides (DEPs) was conducted using the Gene Ontology (GO) website (http://geneontology.org/) for biological processes (BPs), molecular functions (MFs), and cellular components (CCs). The most significant signaling and metabolic pathways related to precursor proteins were identified using the KEGG pathway database (http://www.kegg.jp/).

2.6. Identification of differentially expressed peptides

Peptide precursors were initially identified through the UniProt database (http://beta.uniprot.org). Subsequently, the functional domains of proteins were analyzed using the protein function domain analysis tool at the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/). Additionally, the bioactivity of DEPs was predicted using the PeptideRanker

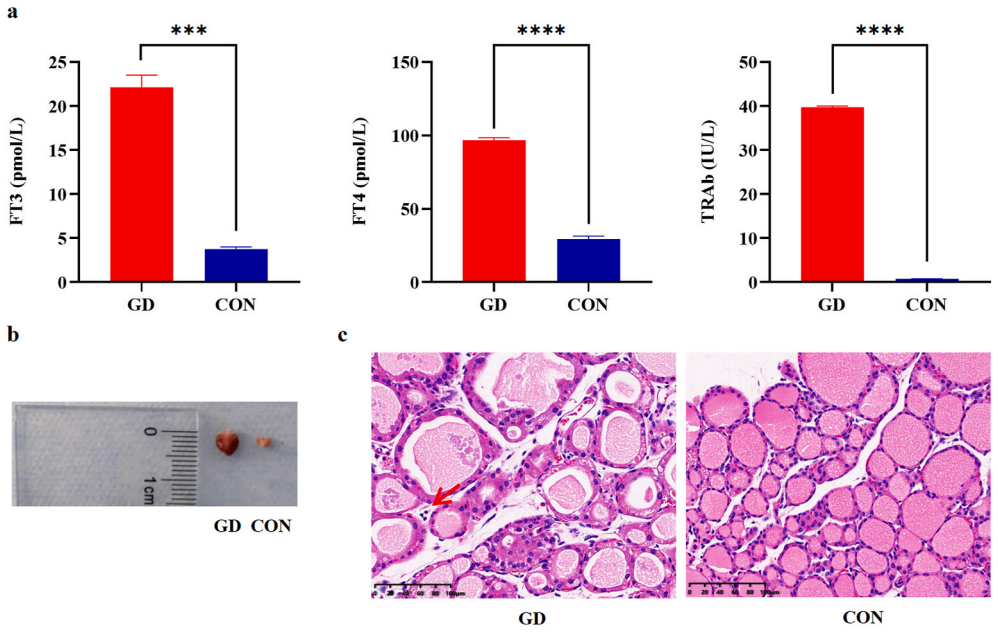

Fig. 1. Evaluation of the Graves' disease mouse model: a. Compared to the control group, significant increases in serum FT3, FT4, and TRAb levels were observed in the GD mice. b. Compared to the control group, GD mouse thyroid tissues showed noticeable enlargement and congestion. c. The thyroid follicular epithelial cells in GD mice exhibited marked hyperplasia, with a multilayered arrangement of epithelial cells, rough and irregular follicular margins, and reduced colloid in the follicular lumen. Additionally, there was sparse lymphocytic infiltration in the interstitium, a feature not observed in the control group. FT3: Free T3, FT4: Free T4, TRAb: Thyroid Stimulating Hormone Receptor Antibody, ***P < 0.001, ****P < 0.001.

platform (http://distilldeep.ucd.ie/PeptideRanker/).

2.7. Molecular docking

Multimer models in Alphafold (version 2.3.2) were used to predict the structures of peptide-protein multimers, with default parameters and database versions. Structures were analyzed using Pymol (version 2.5.0) to identify amino acid pairs forming hydrogen bonds between peptides and proteins. The binding free energy of the predicted multimer structures was analyzed using PRODIGY.

3. Results

3.1. GD mouse model

Following the construction of the GD mouse model, serum levels of FT3, FT4, and TRAb were assessed. Compared to the CON group, the GD group exhibited significantly elevated levels of FT3, FT4, and TRAb (Fig. 1a). Within the model group, two mice died of unknown causes, resulting in a success rate of 66 % for establishing the GD model, consistent with previous reports. GD mice displayed noticeable thyroid enlargement compared to the CON group (Fig. 1b). Histological analysis of H&E-stained thyroid tissues revealed follicular hyperplasia and proliferation of follicular epithelial cells in the GD group, accompanied by limited colloid depletion and interstitial lymphocyte infiltration, changes not observed in the CON group (Fig. 1c).

3.2. Identification of differentially expressed peptides in GD mouse spleens

A total of 1,428 differentially expressed peptides (DEPs) were identified in the spleens of GD mice, with 368 peptides significantly upregulated and 1,060 downregulated (FC $\geq$ 2, $p < 0.05$) compared to the CON group. These DEPs are presented in volcano plots and heatmaps (Fig. 2a–b).

3.3. Functional analysis

Endogenous peptides, typically derived from protein precursors, were sourced from 535 precursor proteins in GD mouse spleens. Functional enrichment analysis of these proteins was conducted to explore the potential functions of DEPs. GO analysis identified enrichments in 235 biological processes (BPs), 126 cellular components (CCs), and 105 molecular functions (MFs) ($p < 0.05$). DEPs were primarily involved in cellular endocytosis and the regulation of gene expression. CC enrichment suggested roles primarily in the cytoplasm and cell-cell junctions, while MF analysis revealed associations with binding to macromolecular complexes and specific

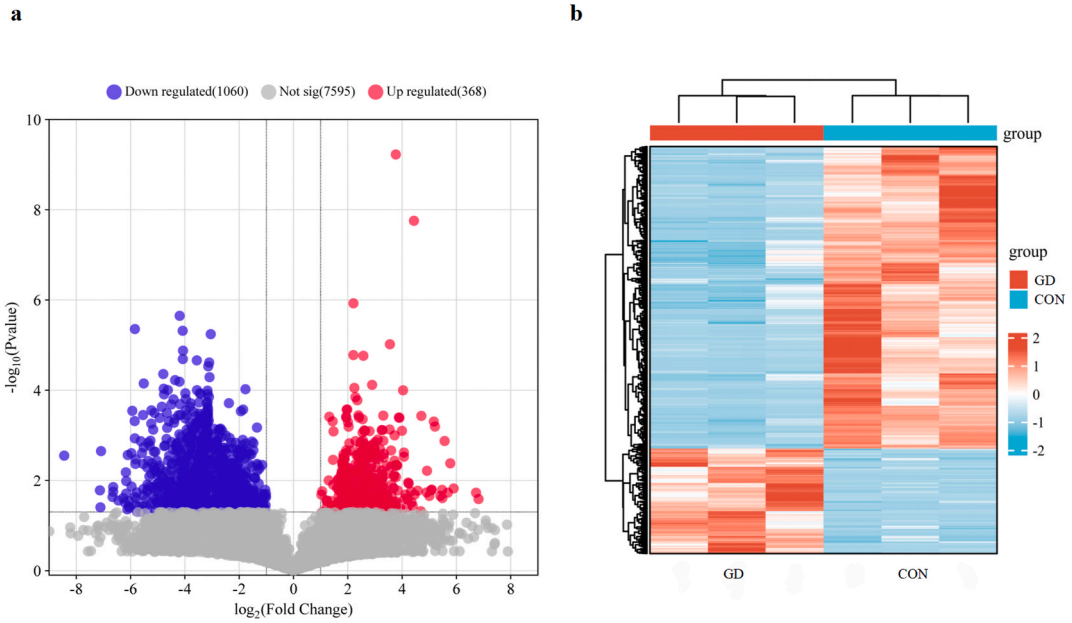

Fig. 2. Differential peptide expression spectrum in the spleens of Graves' disease mice: a. Volcano plot showing peptide expression data between the GD and CON groups. The x-axis represents log 2 (fold change) values, and the y-axis -log 10 P-values indicate the significance of the differences. Red or blue dots represent abnormally expressed peptides. b. A cluster diagram illustrates the differences in peptide expression between the GD and CON groups. Cluster analysis shows similarity clustering relationships in peptide expression. Blue indicates downregulated expression levels, and red indicates upregulated expression levels.

structural domains. The top 10 enriched terms for BP, CC, and MF are listed in Fig. 3a. KEGG pathway analysis indicated that DEPs in GD mouse spleens are predominantly involved in nucleocytoplasmic transport regulation, Fcγ receptor-mediated phagocytosis, antigen processing and presentation, and intercellular signaling pathways (Fig. 3b). The key pathway, particularly antigen processing and presentation, involved 18 DEPs derived from 9 protein precursors related to this pathway, as shown in Table S1.

3.4. Differentially expressed peptides in GD mouse spleens

Focusing on the MHC II antigen presentation pathway, which plays a critical role in autoimmune thyroid diseases, we identified 8 DEPs related to this pathway, primarily derived from the HG2A protein precursor. Five of these peptides were significantly upregulated in GD mouse spleens (Fig. 4a). Specifically, the peptide sequence MDDQRDLISNHEQLPILGNRPRE, named HG2A-24aa, derived from the MHC2-interact domain of the mouse HG2A protein (amino acids 1–24), showed a 79 % similarity to the corresponding segment in human HG2A protein, with a predicted bioactivity of 0.57 (Table 1). Fig. 4b displays the secondary mass spectrum of HG2A-24aa under natural conditions. The amount of HG2A-24aa between different groups is shown in Fig. 4c.

3.5. Molecular docking

To elucidate the role of HG2A-24aa in regulating the MHC II antigen presentation pathway mediated by the hTSHR subunit, homology modeling and molecular docking techniques were employed to analyze the interaction between hTSHR-289aa and HG2A-24aa. Predictive analysis demonstrated effective binding between HG2A-24aa and hTSHR-289aa, with a docking score of −10.9 kcal/mol. Specifically, amino acid residues R38, R80, R109, E107, and K183 in hTSHR-289aa interacted with the peptide through hydrogen bonding (Fig. 5). These findings suggest that hTSHR-289aa may regulate the MHC II antigen presentation pathway through binding with HG2A-24aa, thereby facilitating the presentation of self-antigens and participating in the pathogenesis of GD.

4. Discussion

In this study, we successfully DEPs in the spleens of GD mice induced by immunization with the hTSHR A subunit. Through GO and KEGG pathway analyses, we explored the potential functions of these DEPs in the pathogenesis of GD, particularly focusing on DEPs related to the antigen presentation pathway. This analysis revealed their possible mechanistic roles in disease progression, thereby enhancing our understanding of the molecular mechanisms of GD and providing new potential targets for diagnosis and treatment.

Animal disease models offer invaluable opportunities to investigate the underlying pathophysiological mechanisms of Graves' disease (GD), as they allow experimental manipulations that are not feasible in humans. Currently, the most commonly used method for constructing animal models of GD is the immunization of mice with adenovirus encoding human TSHR [11]. This approach facilitates the exploration of the role of the TSHR A subunit in the pathogenesis of GD. In the present study, we successfully established a mouse model of GD characterized by goiter, lymphocytic infiltration of thyroid tissue, and typical serum functional alterations indicative of thyrotoxicosis, achieving a high success rate. This model provides a robust foundation for subsequent studies on the pathogenicity of the TSHR A subunit.

With the advancement of high-throughput sequencing technologies, these methods have been widely applied in studies on the

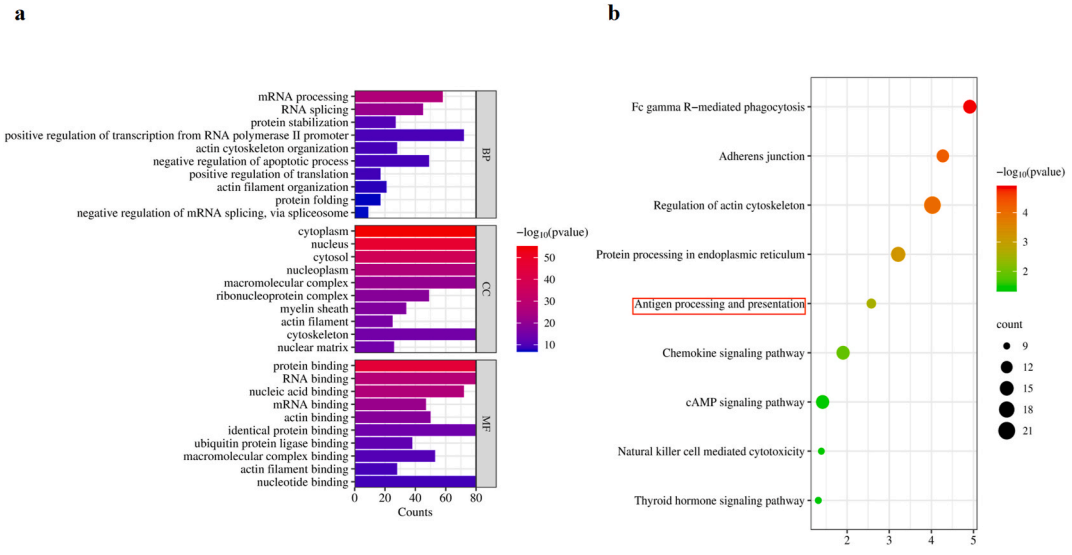

Fig. 3. Functional enrichment analysis of precursor proteins of differentially expressed peptides in the spleens of Graves' disease mice: a. GO analysis. b. KEGG analysis. Red boxes highlight the antigen presentation pathway.

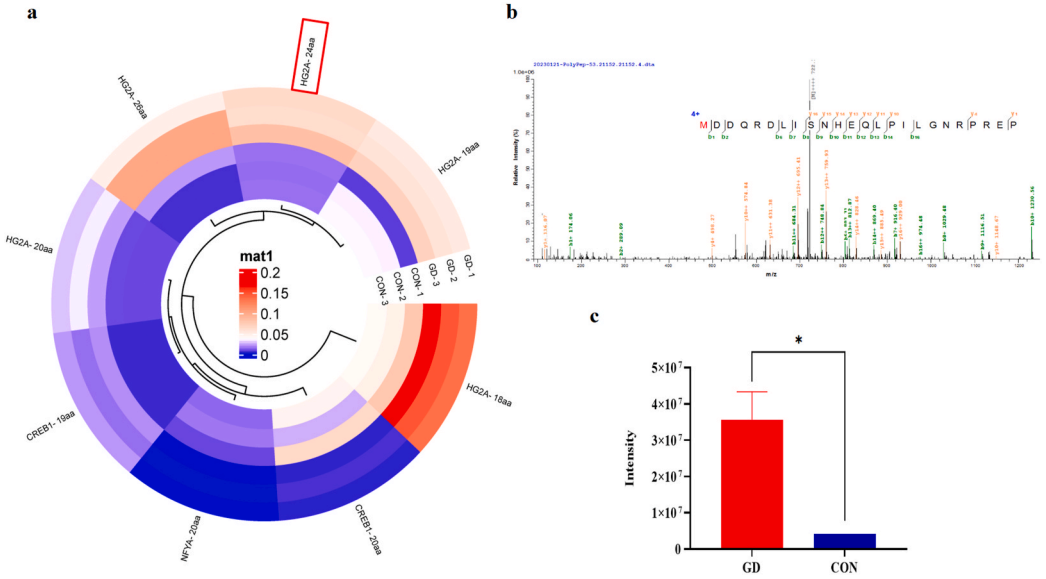

Fig. 4. MHC II antigen presentation pathway-related differentially expressed peptides in the spleens of GD: a. Eight differentially expressed peptides related to the MHC II antigen presentation pathway are shown, named after their precursor proteins and peptide length, with HG2A-24aa highlighted in a red box. b. Secondary mass spectrum of HG2A-24aa under natural conditions. c. Comparison of expression abundance between GD and CON groups, *P $<$ 0.05.

Table 1
Differential peptides derived from HG2A protein precursor.

Peptide	Location	Functional domain	FC	identity	Biological activity
MDDQRDLISNHEQLPILGNRPREP	1–24	MHC II-interact	4.87	79.17 %	0.57
RSGPLEYPQLKGTFPENLK	131–149	MHC assoc_trimer	2.34	68.42 %	0.51
SGPLEYPQLKGTFPENLK	132–149	MHC assoc_trimer	2.74	66.67 %	0.67
SGPLEYPQLKGTFPENLKH	132–150	MHC assoc_trimer	3.55	68.42 %	0.65
SGPLEYPQLKGTFPENLKHLKNSMDG	132–157	MHC assoc_trimer	8.73	69.23 %	0.33

pathogenesis of GD. Researchers have used transcriptomics (including miRNA, lncRNA, and circRNA) and proteomics to systematically analyze blood samples from patients with GD. For example, Zhang et al. identified 178 proteins abnormally expressed in GD by analyzing plasma and serum proteomes in both GD and healthy controls [12]. Furthermore, peptides, as products of protein degradation or limited proteolysis, play significant roles in disease mechanism studies and the development of diagnostic biomarkers, providing critical information about physiological status, behavioral changes, and disease severity. Peptidomics research has identified over 13,000 functional peptides in biological samples, playing vital roles in human physiological processes [13]. In this study, we analyzed the peptide spectrum in the spleens of GD mice induced by the hTSHR A subunit. Results showed 1,428 DEPs compared to the control group, with 368 significantly upregulated and 1,060 downregulated, originating from 535 protein precursors. This finding suggests that these DEPs could serve as novel diagnostic biomarkers for GD, though their functions and specific regulatory mechanisms require further exploration.

Based on the bioinformatics enrichment analysis of the precursor proteins of DEPs, we hypothesize that DEPs may be related to functions such as “endocytosis,” “regulation of gene expression,” and “protein binding to specific structural domains.” These DEPs might also be involved in Fc gamma R-mediated phagocytosis, antigen processing and presentation, and chemokine signaling pathways. Research indicates that GD is a process driven by the interplay of genetic and environmental factors, leading to the disruption of immune tolerance and immune homeostasis. This process involves the endocytosis of autoantigens by immune cells, the presentation of autoantigens by antigen-presenting cells, signaling between antigen-presenting cells and autoreactive T cells, B cell activation, and sustained stimulation of thyroid tissues by autoantibodies, thereby triggering a series of pathophysiological responses [14]. In this context, the presentation of autoantigens by immune cells is a crucial step in disease initiation, with the MHC II antigen presentation pathway playing a significant role in autoimmune diseases. Autoantigens are taken up by specialized presenting cells, such as Dendritic Cells (DCs), processed in early endosomes, and cleaved by proteases into peptides that bind to MHC II molecules and are expressed on the cell surface to induce an autoimmune response [15]. In GD, the endocytosis of the TSHR A subunit is the initiating step of antigen presentation. The antigen is internalized, forming endosomes, and in these endosomes, it binds to MHC II molecules, forming stable antigen-MHC complexes that are ultimately expressed on the cell surface, triggering an immune cascade. Additionally, chemokines, which are small cytokines, can induce chemotaxis of responsive cells, recruiting immune system cells such as DCs to sites of

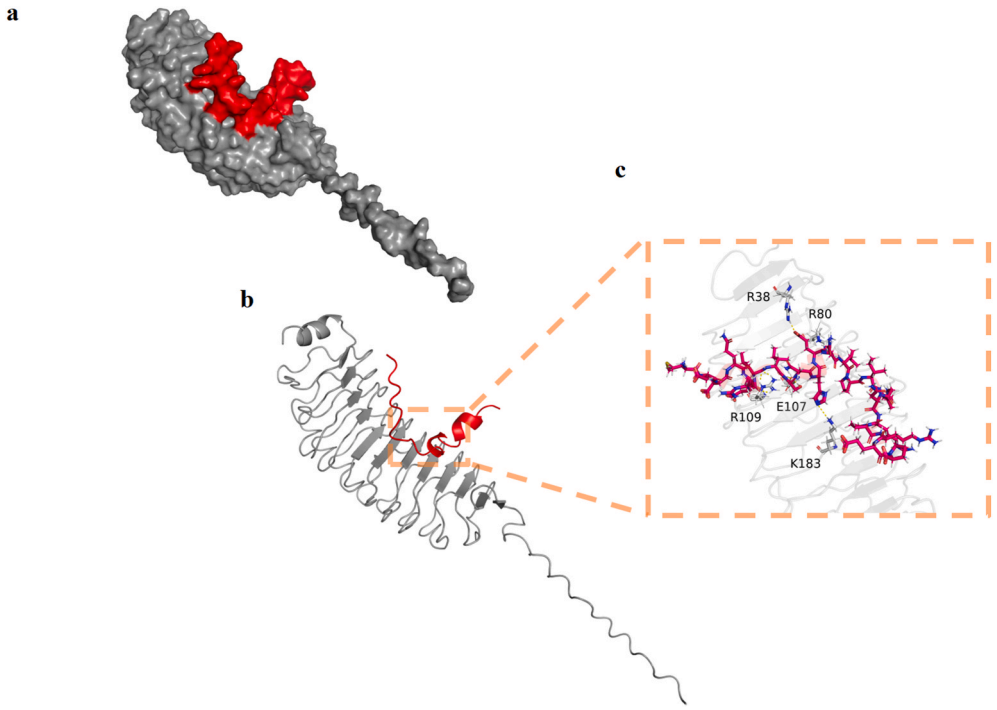

Fig. 5. Molecular docking model of the hTSHR A subunit with HG2A-24aa: a. 3D surface model of the hTSHR A subunit-HG2A-24aa interaction. b. Cartoon model of the hTSHR A subunit-HG2A-24aa interaction. c. Binding sites of the hTSHR A subunit with HG2A-24aa.

inflammation or infection, thereby promoting the presentation of autoantigens and lymphocyte infiltration [16]. Therefore, we speculate that the abnormally expressed peptides in GD may be involved in the MHC II-mediated antigen presentation of the TSHR A subunit, although the precise regulatory mechanisms remain unclear.

Most notably, the eight peptides that are abnormally upregulated in the spleens of GD mice and related to the MHC II antigen presentation pathway predominantly originate from HG2A_MOUSE, also known as the invariant chain (Ii). This chaperone protein for MHC II molecules is widely present on the surface of MHC II-positive cells, such as dendritic cells (DCs), monocytes, macrophages, and B cells [17]. It influences antigen processing, endocytosis maturation, and cell migration, and includes structural domains such as MHC-II-interact, MHCassoc_trimer, and TY [18]. The MHC-II-interact domain, also known as the Class II-associated invariant chain peptide (CLIP), binds to the groove of MHC II molecules, facilitating their transport through the endoplasmic reticulum and Golgi to the lysosomes or late endosomes containing antigen peptides and playing a crucial role in the antigen processing pathway [14,15]. Our study found that the abnormally upregulated peptide HG2A-24aa in the spleens of GD mice originates from this domain. This 24-amino acid peptide is highly homologous to the human HG2A protein, with a predicted bioactivity greater than 0.5, suggesting it may function as a bioactive peptide. The findings suggest that HG2A-24aa may be functionally related to CLIP and might participate in the presentation of the hTSHR A subunit by MHC II molecules. To further explore the regulatory role of this peptide, we performed homology modeling and molecular docking analysis, discovering that HG2A-24aa has multiple binding sites with the hTSHR A subunit. This indicates that the hTSHR A subunit may promote its presentation by MHC II molecules through interaction with HG2A-24aa, mediating the immune cascade reaction. However, further functional and molecular interaction studies are required to confirm this.

In summary, this study reveals the differentially expressed peptide profiles in the spleen of Graves' disease (GD) mice and explores their potential functions. We focused particularly on peptides closely related to the antigen presentation pathway and investigated the potential role of HG2A-24aa in presenting the hTSHR A subunit through bioinformatics analyses, including molecular docking. However, there are some limitations to our study. Primarily, our bioinformatics analysis was conducted on spleen peptides from an animal model. To validate the clinical relevance and biological functions of these differentially expressed peptides, future research should include clinical samples and both in vivo and in vitro disease models. Additionally, this study was based on the analysis of differential peptides at the initial stage of GD onset in mice. Future studies should cover different stages of disease progression following multiple immunizations to comprehensively analyze potential differential peptides and their functions. Despite these limitations, our study not only expands the understanding of GD pathogenesis but also provides new perspectives and methodologies for future research. Specifically, it lays the theoretical foundation for exploring critical functional peptides in the MHC II antigen presentation pathway and supports the development of therapeutic strategies targeting this pathway.

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Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author.

Declarations

The research protocols were approved by the Medical Ethics Committee (Approval No. 2023-488). All participants were informed about the research and were requested to provide written consent.

Consent for publication

Not applicable.

CRediT authorship contribution statement

Zhengrong Jiang: Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Lijun Chen:** Investigation, Funding acquisition, Data curation. **Huibin Huang:** Methodology, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36661.

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