

Original Article

Lack of humoral response against *Helicobacter pylori* peptides homologous to human ZnT8 in Hashimoto's thyroiditis patients

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Abstract

Introduction: The *Helicobacter pylori* (HP) reinfection rate seems to be higher in developing countries than in developed ones. An increased seroprevalence of HP has also been reported in patients with type 1 diabetes (T1D) and Hashimoto's thyroiditis (HT). *Mycobacterium avium* subsp. *paratuberculosis* (MAP) has been linked to both T1D and HT. Quite a few lines of evidence indicate that autoantibodies against several epitopes belonging to human zinc transporter 8 (ZnT8) cross-recognize the homologous MAP3865c epitopes in both T1D and HT patients. HP may play a role in HT disease, most likely acting through a molecular mimicry mechanism that targets ZnT8 as reported for MAP and the two autoimmune diseases.

Methodology: An enzyme-linked immunosorbent assay (ELISA) has been developed for the detection of antibodies against several epitopes deriving from HP proteins, which are highly homologous to the immunodominant ZnT8 peptides previously identified: ZnT8₁₇₈₋₁₈₆ and ZnT8₁₈₆₋₁₉₄.

Results: None of the HP peptides tested were significantly recognized when the humoral responses of 92 HT patients and 91 healthy volunteers were analyzed.

Conclusions: These findings do not support a triggering role for HP (through ZnT8 mimicking) in HT. If a molecular mimicry phenomenon is taking place, it involves a different self-antigen. Moreover, the negative outcome of the experiments performed stresses the fact that sharing stretches of sequence homology is relevant, but not enough to trigger an antibody-mediated cross-recognition.

Key words: zinc transporter 8; Hashimoto's thyroiditis; *Helicobacter pylori*.

J Infect Dev Ctries 2015; 9(6):631-634. doi:10.3855/jidc.6284

(Received 19 November 2014 – Accepted 21 April 2015)

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Introduction

Autoimmune diseases, such as Hashimoto's thyroiditis (HT) and type 1 diabetes (T1D), stem from the interaction of genetic susceptibility and environmental exposures [1-4]. A distinctive marker of HT is the presence of autoantibodies (aAbs) against thyroglobulin (Tg), thyroperoxidase (TPO), and thyrotropin receptor (TR) [5]. Infectious agents such as bacteria and viruses may trigger autoimmunity [6,7] through a molecular mimicry mechanism, being able to mimic the antigenic profile of the epithelial cell lining the thyroid gland. Among the environmental factors activated is *Helicobacter pylori* (HP), a chronic pathogen living in the human stomach, which typically colonizes and infects the gastric mucosa. This Gram-negative bacterium is responsible for gastritis, which can progress to gastric/duodenal ulcers and carcinomas.

The overall prevalence of HP infection is higher in developing countries compared to developed nations [8].

Contradictory conclusions have been reported for HP infection and HT disease, both confirming and denying the existence of an association [9,10].

What is more, the existence of a remarkable correlation between *Mycobacterium avium* subsp. *paratuberculosis* (MAP) infection/seroprevalence and autoimmune diseases such as T1D and HT was recently demonstrated [1]. Researchers found a very strong humoral response mounted against epitopes belonging to one MAP membrane protein (MAP3865c), and against the homologous region present in human ZnT8, which is a relevant T1D autoantigen specifically expressed in the pancreatic β -cell and in the cubical epithelium that lines thyroid follicles [1-3].

The reported increased prevalence of anti-MAP3865c and anti-ZnT8 antibodies (Abs) in

Sardinian T1D and HT subjects indicates that MAP is a possible environmental trigger of both autoimmune diseases. MAP and HP may express proteins capable of mimicking self-antigens through a common mechanism, leading to the production of aAbs, which in turn may cause self-aggression.

To determine if this was true, and to identify in human ZnT8 the most likely common target for molecular mimicry in both HT and T1D, we investigated the prevalence anti-HP Abs developed against four HP peptides highly homologous to ZnT8 immunodominant (ZnT8₁₇₈₋₁₈₆ and ZnT8₁₈₆₋₁₉₄) epitopes in a Sardinian population cohort of 92 HT patient and 91 healthy volunteers.

Methodology

Subjects

A group of 92 HT patients (female : male ratio, 10.75 : 1; mean age, 47 ± 13 years) followed at the University Hospital of Sassari, Department of Endocrinology, and a control group consisting of 91 age and sex-matched healthy individuals were enrolled in the study. All the subjects were inhabitants of Sardinia, Italy. All healthy controls (HCs) had not undergone therapy for at least six months and had no history of autoimmune diseases or clinically inflammatory diseases. All study subjects provided signed informed consent prior to the sample and data collection, and the study protocol was approved by the University Hospital of Sassari, Italy.

Peptides

Four HP peptides highly homologous to human ZnT8₁₇₈₋₁₈₆ and ZnT8₁₈₆₋₁₉₄ were assayed in this study: J0I929_HELPX₁₋₁₁ [MIIGGGVSGCA] derived from HP quinone oxidoreductase J0I929_HELPX protein (UniProtKB accession number: J0I929); J0T7E7_HELPX₄₀₋₄₇ [IIINSCTV] derived from HP Radical SAM methylthiotransferase, MiaB/RimO family protein J0T7E7_HELPX protein (UniProtKB: accession number J0T7E7); T2S9C8_HELPX₇₆₋₈₂ [VAQNIVL] derived from HP T2S9C8_HELPX hypothetical protein (UniProtKB: accession number T2S9C8); T2T4W3_HELPX₉₉₋₁₀₅ [AGIVLTV] derived from HP Preprotein translocase subunit T2T4W3_HELPX (UniProtKB: accession number T2T4W3). All peptides were synthesized at > 90% purity commercially (LifeTein, South Plainfield, USA). Purified peptides were stocked as 10 mM solutions and stored in single-use aliquots at -80°C.

Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISAs were performed in order to verify the humoral response mounted against the selected peptides as previously described [3]. Briefly, ELISA 96-well Nunc immunoplates were coated with 10 µg/mL of peptides diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.5 (Sigma, St. Louis, USA) overnight at 4°C. The remaining protein-free sites in the plate were blocked by 200 µL/well of 5% phosphate-buffered saline (PBS)-non-fat dried milk (Sigma, St. Louis, USA) solution upon incubation for two hours at room temperature. After being washed twice with PBS containing 0.05% Tween-20 (PBS-T), serum samples at 1:100 dilution in 0.05% PBS-T were added and incubated for two hours at room temperature. Extensive washing of the plate was followed by addition of 100 µL of goat anti-human immunoglobulin G polyclonal Ab (1:1000; Sigma, St. Louis, USA) and incubation for one hour at room temperature. Finally, after five washes in PBS-T, the reaction was developed by adding 100 µL/well of para nitrophenylphosphate (Sigma, St. Louis, USA) as substrate for alkaline phosphatase. Plates were incubated in the dark for about 10 minutes, and optical density (OD) was measured at 405 nm using a VERSATunable Max microplate reader (Molecular Devices, Orleans Drive Sunnyvale, USA). Negative and positive control wells were included in each plate tested, and results are expressed as means of three different experiments performed.

Statistical analysis

Statistical analyses were performed using GraphPad 6 Software. Data was presented as mean ± standard error or median (25th and 75th percentiles) as clinical and laboratory data for each parameter analyzed. The results were analyzed by Fisher's exact test, and $p \leq 0.05$ was considered to be significant. The cut-off values were determined by receiver operating characteristic (ROC) curve analyses using values normalized to a strongly positive control included in each assay performed. The specificity was set at > 93.4% (*i.e.*, Ab+ HCs < 7%), and the sensitivity was calculated accordingly.

Results

BLAST analysis showed that J0T7E7_HELPX₄₀₋₄₇ and J0I929_HELPX₁₋₁₁ were homologous to human ZnT8₁₇₈₋₁₈₆ peptide, whereas T2S9C8_HELPX₇₆₋₈₂ and T2T4W3_HELPX₉₉₋₁₀₅ were homologous to human ZnT8₁₈₆₋₁₉₄ peptides. Both human peptides, which

derive from zinc transporter 8 protein, are known to be linked to HT and T1D [1-3].

J0T7E7_HELPX₄₀₋₄₇ was recognized by 7.6% of the HT patients and by 6.6% of HCs (area under the curve [AUC] = 0.5; *p* = 0.5), and J0I929_HELPX₁₋₁₁ was recognized by 15.2% of the HT patients and by 6.6% of HCs (AUC = 0.54; *p* = 0.16).

Regarding the peptides homologous to ZnT8₁₈₆₋₁₉₄, T2S9C8_HELPX₇₆₋₈₂ was recognized by 16.4% of the HT patients and by 6.6% of HCs (AUC = 0.54; *p* = 0.07), while T2T4W3_HELPX₉₉₋₁₀₅ was recognized by 12% of the HT patients and by 6.6% of HCs (AUC = 0.58, *p* = 0.46).

As shown in Figure 1, all selected peptides displayed the same Ab reactivity in the HCs group and very similar percent fraction of Ab-positivity in the HT one; in any case, there was no statistically significant difference between patients and controls.

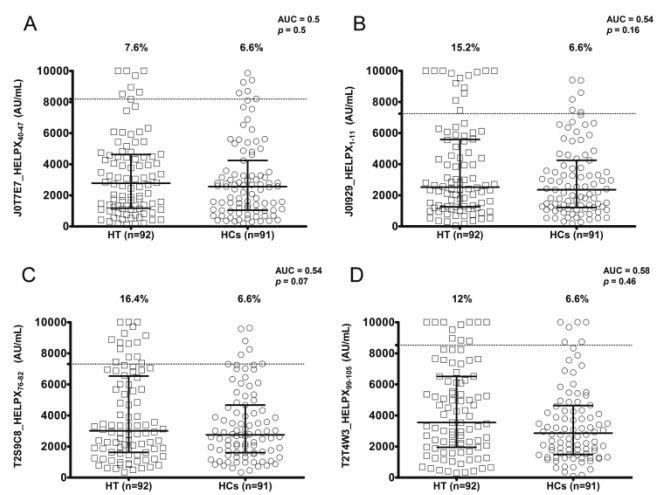
Discussion

We investigated whether HP might play a role in HT disease, probably acting through a molecular mimicry mechanism which targets human ZnT8 protein as it was reported for MAP [1-3]. We aimed to find an explanation accounting for the interconnection found between HP infection and two autoimmune diseases, HT and T1D [11], which have been proven to be associated with MAP infection [1-3].

To achieve this goal, we searched for Abs against four HP peptides (Table 1), which are highly homologous to two human ZnT8 immunodominant epitopes, ZnT8₁₇₈₋₁₈₆ and ZnT8₁₈₆₋₁₉₄, the latter being highly recognized by the sera of HT and T1D subjects [1-3].

Our group recently demonstrated that Abs recognizing epitopes belonging to MAP3865c protein (MAP3865c₁₂₅₋₁₃₃ and MAP3865c₁₃₃₋₁₄₁) cross-react with ZnT8 homologous ones (ZnT8₁₇₈₋₁₈₆ and ZnT8₁₈₆₋

Figure 1. Dot plot showing the distribution of IgG for the four *Helicobacter pylori* (HP) peptides measured by indirect ELISA



A total of 91 HT patients and 91 healthy controls were screened for IgG antibodies against J0T7E7_HELPX₄₀₋₄₇ (A) and J0I929_HELPX₁₋₁₁ (B) homologous to ZnT8₁₇₈₋₁₈₆, and against T2S9C8_HELPX₇₆₋₈₂ (C) and T2T4W3_HELPX₉₉₋₁₀₅ (D) homologous to ZnT8₁₈₆₋₁₉₄. The black bars represent the median with interquartile range. The dotted lines indicate the respective cut-off values calculated by ROC analysis and used as threshold in discriminating between positive and negative sera. Area under roc curve (AUC) and p values are displayed in the right corner. Each experiment was independently repeated at least three times.

194), mounting a molecular mimicry mechanism which may be in turn responsible for self-aggression [1-3].

Therefore, we postulated that MAP and HP may express proteins capable of mimicking the same self-antigens ZnT8. Nevertheless, this study failed to find a direct association between HP seropositivity and HT. Indeed, there was no statistically significant difference in the seroprevalence of HP infection in Sardinian HT patients compared to control participants, which were matched for age, sex, and socioeconomic status.

Accordingly, we concluded that these HP epitopes are not correlated with HT. If a molecular mimicry phenomenon is taking place, it certainly targets

Table 1. Alignment of the amino acid sequences of the identified peptides by ClustalW2.

Peptide name	Sequence alignment	Protein
ZnT8 ₁₇₈₋₁₈₆	MII--VSSCAV	Human zinc transporter 8
HPM5_0328 ₁₋₁₁	MIIGGGVSGCA-	<i>Helicobacter pylori</i> HPM5_0328
ZnT8 ₁₇₈₋₁₈₆	MIIVSSCAV	Human zinc transporter 8
HPM3_0496 ₄₀₋₄₇	-IIINSCTV	<i>Helicobacter pylori</i> HPHPM3_0496
ZnT8 ₁₈₆₋₁₉₄	VAANIVLTV	Human zinc transporter 8
HPSA50_1635 ₇₆₋₈₂	VAQNIVL--	<i>Helicobacter pylori</i> HPSA50_1635
ZnT8 ₁₈₆₋₁₉₄	VAANIVLTV	Human zinc transporter 8
SecD ₉₉₋₁₀₅	--AGIVLTV	<i>Helicobacter pylori</i> SecD

Result shows fully conserved residue (*), strongly similar properties (:), weakly similar properties (.) and missing regions (-).

different self-antigens. What is more, the negative outcome of the experiments performed stresses the fact that sharing stretches of sequence homology is relevant, but not enough to trigger an antibody-mediated cross-recognition.

Conclusions

These findings do not support a triggering role for HP (through ZnT8 mimicking) in HT disease. Indeed, we cannot discount a direct involvement of HP in HT, which might act as an environmental trigger, targeting different self-antigens via molecular mimicry. To verify this hypothesis, further studies should seek and identify new possible sources of cross-reactive Abs; moreover, the genetic polymorphism of the host needs to be investigated.

Acknowledgements

This article is dedicated to Professor Piero Cappuccinelli, a great man of science on the occasion of his retirement. This study was partially supported by the Sardinian Region, L.R. 7, progetti di ricerca di base 2010 and Fondazione Banco di Sardegna, to LA Sechi 2014.

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Conflict of interests: No conflict of interests is declared.