

CLINICAL STUDY

Vitamin D 1 α -hydroxylase (CYP1 α) polymorphism in Graves' disease, Hashimoto's thyroiditis and type 1 diabetes mellitus

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Abstract

Objective: The vitamin D endocrine system plays a role in the regulation of (auto)immunity and cell proliferation. Vitamin D 1 α -hydroxylase (CYP1 α) is one of the key enzymes regulating both systemic and tissue levels of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃). Administration of 1,25(OH)₂D₃, whose serum levels were found to be reduced in type 1 diabetes and thyroid autoimmunity, prevents these diseases in animal models. We therefore investigated a recently reported CYP1 α polymorphism for an association with type 1 diabetes mellitus, Graves' disease and Hashimoto's thyroiditis.

Design and methods: Four hundred and seven Caucasian pedigrees with one offspring affected by either type 1 diabetes (209 families), Graves' disease (92 families) or Hashimoto's thyroiditis (106 families) were genotyped for a C/T polymorphism in intron 6 of the CYP1 α gene on chromosome 12q13.1–13.3 and transmission disequilibrium testing (TDT) was performed. Subsets of affected offspring stratified for HLA-DQ haplotype were compared using χ^2 testing.

Results: There was no deviation from the expected transmission frequency in either type 1 diabetes mellitus ($P = 0.825$), Graves' disease ($P = 0.909$) or Hashimoto's thyroiditis ($P = 0.204$). However, in Hashimoto's thyroiditis the CYP1 α C allele was significantly more often transmitted to HLA-DQ2[−] patients (27 transmitted vs 14 not transmitted; TDT: $P = 0.042$) than expected. The C allele was less often transmitted to HLA-DQ2⁺ patients (9 transmitted vs 12 not transmitted; TDT: $P = 0.513$), although the difference was not significant (χ^2 test: $P = 0.143$). A similar difference was observed in type 1 diabetes between offspring with high and low risk HLA-DQ haplotypes (χ^2 test: $P = 0.095$).

Conclusions: The CYP1 α intron 6 polymorphism appears not to be associated with type 1 diabetes mellitus, Graves' disease and Hashimoto's thyroiditis. A potential association in subsets of patients with type 1 diabetes and Hashimoto's thyroiditis should be further investigated as well as its functional implications.

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Introduction

The vitamin D endocrine system is not only a major regulator of calcium homeostasis, but also has important immunomodulatory properties. 1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃), the most active natural vitamin D metabolite, effectively prevents the development of autoimmune diabetes mellitus (1) and autoimmune thyroiditis (2) in animal models. Vitamin D 1 α -hydroxylase (CYP1 α) is the key enzyme for both systemic and tissue levels of 1,25(OH)₂D₃ (reviewed in 3 and 4). 1,25(OH)₂D₃ exerts its immunomodulatory actions by inhibiting HLA class II expression on endocrine cells (5, 6), T cell proliferation and secretion of inflammatory cytokines (7). In addition, other

autoimmune disorders such as experimentally induced autoimmune encephalitis (8) can be favourably influenced by administering 1,25(OH)₂D₃.

Numerous extrarenal tissues express CYP1 α suggesting that this enzyme has endocrine as well as para- and autocrine functions (9). Whereas inflammatory cytokines induce the expression of CYP1 α in murine macrophages, its induction in macrophages of diabetic NOD mice was shown to be defective (10). Mutations of the CYP1 α gene which severely impair 1 α -hydroxylase activity and cause vitamin D-dependent rickets were recently identified (reviewed in 4). In the search for mutations, a C/T polymorphism was reported in intron 6 of the CYP1 α gene (11). A CYP1 α polymorphism could affect systemic or

tissue-specific $1,25(\text{OH})_2\text{D}_3$ levels. The *CYP1 α* gene is located on chromosome 12q13.1–13.3, ten megabases centromeric of the vitamin D receptor (VDR) locus. Genetic VDR polymorphisms have been implicated in mediating susceptibility to autoimmunity (12–14).

We therefore investigated this *CYP1 α* intron 6 polymorphism for an association with type 1 diabetes mellitus, Graves' disease and Hashimoto's thyroiditis.

Subjects and methods

Subjects

Two hundred and nine subjects with type 1 diabetes mellitus, 106 patients with Graves' disease and 92 individuals with Hashimoto's thyroiditis were recruited from the endocrine outpatient clinics at the University Hospital Frankfurt am Main (Germany), at the University Hospital Göttingen (Germany) and the paediatric endocrine outpatient clinic, University 'La Sapienza', Rome (Italy). All families with type 1 diabetes were recruited at Frankfurt. Type 1 diabetes mellitus was diagnosed according to WHO criteria. The male:female ratio of patients with type 1 diabetes was 1:1.1 and the mean age of disease onset (\pm S.D.) was 8.9 ± 5.6 years. Patients with thyroid autoimmunity were recruited from Frankfurt and Göttingen (all postpubertal disease onset) and Rome (all prepubertal disease onset). Five patients with Hashimoto's thyroiditis (all females) and 82 with Graves' disease (15 males and 67 females) were from Frankfurt and Göttingen. The diagnosis of Graves' disease rested on autoimmune hyperthyroidism with thyrotrophin (TSH) receptor antibodies and/or ophthalmopathy. Hashimoto's thyroiditis was diagnosed by positive thyroglobulin and/or thyroid peroxidase antibodies, reduced echogenicity on thyroid ultrasound and normal or elevated TSH levels. Whole blood samples were drawn from patients and parents, a total of 1221 subjects, after having obtained informed consent from each individual.

The male:female ratios for Graves' disease and Hashimoto's thyroiditis were 1:5.5 and 1:4.6 respectively. All individuals had previously been typed for HLA-DQ. Patients were assigned to subsets of HLA-DQ haplotypes mediating either a high, medium or low disease risk (DQ2: DQA*0501-DQB*0201; DQ8: DQA*0301-DQB*0302; DQn: neither DQ2 nor DQ8). Type 1 diabetes: 74 patients had high risk (DQ2/DQ8), 100 (DQ2/DQ2; DQ2/DQn; DQ8/DQ8; DQ8/DQn) had medium risk and 35 patients had low risk (DQn/DQn) HLA-DQ haplotypes. Graves' disease: 40 patients had high risk (DQ2⁺) and 52 patients had low risk (DQ2⁻) HLA-DQ haplotypes. Hashimoto's thyroiditis: 48 patients had high risk (DQ2⁺) and 58 patients had low risk (DQ2⁻) HLA-DQ haplotypes. Furthermore, transmission frequencies of maternal and paternal alleles were compared. The comparison of transmission to male vs female offspring was only

performed for type 1 diabetes as the number of male patients with Graves' disease and Hashimoto's thyroiditis was too small for such an analysis.

Genotyping and haplotyping

Following DNA extraction according to standard protocols, individuals were genotyped for the *CYP1 α* intron 6 polymorphism (Genbank accession no. AF072470) using polymerase chain reaction followed by single strand conformation polymorphism analysis as described previously (11). HLA-DQ typing was performed using standard allele-specific primers. Additionally, all individuals had been genotyped for the VDR polymorphisms *FokI* and *BsmI* as previously described (13).

Statistical analyses

Transmission disequilibrium testing (TDT) (15) was applied to detect an association in the presence of linkage. Only heterozygous parents are considered in TDT, which measures the likelihood that the difference between observed and expected (50%) transmission frequency is solely due to chance. Transmission differences among the subsets were compared using χ^2 testing TDT and χ^2 testing was performed with the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) software, version 9.0. Linkage disequilibrium \pm S.D. was calculated (16). Probabilities were calculated by applying the appropriate degree of freedom of the respective analysis, but were not corrected for the number of subsets analyzed. TDT power calculations were carried out using the template provided by David Curtis (<http://www.mds.qmw.ac.uk/statgen/dcurtis/software.html>) based on Risch & Merikangas (17). PASS2000 (NCSS, Kaysville, UT, USA) was used to calculate the power of the χ^2 comparisons and powers are given as $1 - \beta$ (β : type 2 error).

Results

No preferential transmission was observed for the *CYP1 α* intron 6 polymorphism in type 1 diabetes mellitus (see Table 1). The C allele was 91 times transmitted compared with 94 times not transmitted by heterozygous parents to affected offspring (TDT: $P = 0.825$; $1 - \beta = 0.85$). Similarly, we could not detect a significant transmission disequilibrium in families with Graves' disease (C allele: 39 times transmitted vs 38 times not transmitted; TDT: $P = 0.909$; $1 - \beta = 0.55$) and with Hashimoto's thyroiditis (C allele: 36 times transmitted vs 26 times not transmitted; TDT: $P = 0.204$; $1 - \beta = 0.60$).

The *CYP1 α* allele frequencies in all 814 non-related parents were 25.4% C and 74.6% T respectively. The genotype frequencies in the investigated population were in Hardy–Weinberg equilibrium (data not shown).

Table 1 Transmission frequencies of the CYP1 α intron 6 polymorphism in type 1 diabetes mellitus, Graves' disease and Hashimoto's thyroiditis.

Disease	No. of families	Allele	Transmitted	Not transmitted	χ^2	P
Type 1 diabetes mellitus	209	C T	91 (49.2%) 94 (50.8%)	94 (50.8%) 91 (49.2%)	0.049	0.825
Graves' disease	92	C T	39 (50.6%) 38 (49.4%)	38 (49.4%) 39 (50.6%)	0.013	0.909
Hashimoto's thyroiditis	106	C T	36 (58.1%) 26 (41.9%)	26 (41.9%) 36 (58.1%)	1.613	0.204

Subsequently, affected offspring were stratified according to their HLA-DQ haplotypes and transmission frequencies of the CYP1 α polymorphism were compared. The results are shown in Table 2. Whereas the CYP1 α C allele was less often transmitted to type 1 diabetes patients with high risk HLA-DQ haplotypes (25 transmitted vs 38 not transmitted; TDT: $P = 0.101$), it was more often transmitted to affected offspring with low risk HLA-DQ haplotypes (15 transmitted vs 9 not transmitted; TDT: $P = 0.221$; χ^2 : $P = 0.095$; $1 - \beta = 0.76$). Also, in Hashimoto's thyroiditis, the CYP1 α C allele was significantly more often transmitted to HLA-DQ2⁻ patients (27 transmitted vs 14 not transmitted; TDT: $P = 0.042$; $1 - \beta = 0.75$) than expected, whereas it was less often transmitted to HLA-DQ2⁺ patients (9 transmitted vs 15 transmitted; TDT: $P = 0.513$). In contrast, no transmission differences were observed in Graves' disease between high (16 transmitted vs 16 transmitted; TDT: $P = 1$) and low risk (22 transmitted vs 22 not transmitted; TDT: $P = 1$) HLA-DQ haplotypes (χ^2 : $P = 0.816$).

Maternal and paternal transmission frequencies did not differ for any of the diseases investigated (data not shown). Also, transmission frequencies in pedigrees with type 1 diabetes were very similar between male and female affected offspring (χ^2 : $P = 0.786$). No

linkage disequilibrium was observed with polymorphisms within the VDR gene polymorphisms located ten megabases centromeric of the CYP1 α gene (CYP1 α T vs VDR FokI 'f': linkage disequilibrium = -0.0104 ± 0.0128) and (CYP1 α T vs VDR BsmI 'b': linkage disequilibrium = 0.0043 ± 0.0185).

Discussion

In this study of CYP1 α variants in endocrine autoimmunity we did not observe an association of the intron 6 C/T polymorphism with type 1 diabetes, Graves' disease and Hashimoto's thyroiditis in the Caucasian population investigated. However, the C allele was significantly more often transmitted to HLA-DQ2⁻ offspring affected by Hashimoto's thyroiditis. Also, a trend was observed for a differential transmission of the CYP1 α intron 6 polymorphism among HLA-DQ subsets in both type 1 diabetes and Hashimoto's thyroiditis. However, correction for multiple testing in the HLA-DQ subset analyses would reduce these differences.

We found the nearby VDR gene to be associated with type 1 diabetes (13), Graves' disease and Hashimoto's thyroiditis (authors unpublished data). Due to an

Table 2 Transmission of the CYP1 α C allele in subsets stratified for HLA-DQ haplotype.

Disease	No. of families	HLA-DQ risk subset	Transmitted	Not transmitted	TDT P	χ^2 test (high vs low HLA-DQ risk) P
Type 1 diabetes mellitus	74 100 35	High Medium Low	25 (39.7%) 48 (51.1%) 15 (62.5%)	38 (60.3%) 46 (48.9%) 9 (37.5%)	0.101 0.837 0.221	0.095*
Graves' disease	40 52	High Low	16 (50.0%) 22 (50.0%)	16 (50.0%) 22 (50.0%)	1 1	0.816
Hashimoto's thyroiditis	48 58	High Low	9 (42.9%) 27 (65.9%)	12 (67.1%) 14 (34.1%)	0.513 0.042	0.143

TDT was applied to detect preferential transmission within a subset. χ^2 testing was used to compare transmission patterns of two subsets with each other. * Comparison of the combined HLA-DQ subsets resulted in no significant differences in either high vs medium + low (χ^2 : $P = 0.109$) or high + medium vs low (χ^2 : $P = 0.214$).

absent linkage disequilibrium between *CYP1 α* and *VDR* polymorphisms it is rather unlikely that *VDR* alleles might act as confounding factors.

The vitamin D endocrine system has important immunomodulatory properties and 1,25(OH)₂D₃ can prevent autoimmune diabetes (1) and autoimmune thyroiditis (2) in animal models. Also, 1,25(OH)₂D₃ serum levels were reported to be reduced in type 1 diabetes – even at disease onset (18, 19) – and in autoimmune hyperthyroidism compared with non-autoimmune hyperthyroidism (20). A recent study investigating various parameters of vitamin D metabolism in mono- and dizygotic twins found 65% of the 1,25(OH)₂D₃ serum levels to be genetically determined (21). A number of tissues, including pancreas, adrenal gland and macrophages, express vitamin D 1 α -hydroxylase (9). The expression of *CYP1 α* and *CYP24* (vitamin D 24-hydroxylase) is normally inversely regulated, permitting the concentration of 1,25(OH)₂D₃ to be locally fine tuned. Interestingly, induction of *CYP1 α* expression was found to be defective in macrophages of diabetic NOD mice, a model for type 1 diabetes (10). *CYP1 α* knockout mice displayed enlarged lymph nodes in the vicinity of the thyroid gland and a reduction in peripheral CD4⁺ and CD8⁺ T lymphocytes (22).

Vitamin D 1 α -hydroxylase plays an important role in the immunomodulatory properties of vitamin D, but also in mediating its antiproliferative effects as shown for prostate and colorectal cancer (23–25). Genetic *CYP1 α* variants may therefore affect both autoimmunity as well as malignancy.

The observed allele frequencies were in accordance with those reported previously (11). Our results suggest that the *CYP1 α* intron 6 polymorphism is not associated with type 1 diabetes and Graves' disease. There is only very weak evidence for an association with Hashimoto's thyroiditis in the subset of HLA-DQ2⁺ patients. However, since our study has limited power to detect a potential association of the *CYP1 α* intron 6 polymorphism with Graves' disease and Hashimoto's thyroiditis, additional genetic as well as functional studies are warranted to detect and characterise further polymorphisms within and adjacent to the *CYP1 α* gene. About 180 families per disease would be required to detect a transmission difference ($\gamma = 1.5$) with a power of 0.8. The recently reported *CYP1 α* splice variants (26) may also correlate with differences at the genomic level.

Since susceptibility to endocrine autoimmune disease is polygenic, conditioning of patients for other genetic variants allows us to address the issue of differential predisposition and genomic interaction. Although it has been reported that intronic sequences can alter protein function (27, 28), the *CYP1 α* intron 6 polymorphism has not yet been studied functionally. Thus the *CYP1 α* variant studied is not excluded as a candidate susceptibility gene in thyroid autoimmunity,

but may predispose in individuals with low HLA-DQ risk.

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