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# The study of CTLA-4 and vitamin D receptor polymorphisms in the Romanian type 1 diabetes population

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## Abstract

Several studies suggested that part of the genetic susceptibility for Type 1 diabetes (T1DM) is encoded by some polymorphisms of CTLA-4 gene (2q33) and of Vitamin D Receptor gene (VDR; 12q12-14). Our aim was to assess their contribution to T1DM genetic susceptibility in the Romanian population. We typed CTLA-4 49 A/G and VDR *Fok*I (F/f), *Apa*I (A/a) and *Taq*I (T/t) polymorphisms by Sequence Specific Primer PCR (SSP-PCR) in 204 Romanian diabetic families (756 individuals: 212 T1DM probands and 544 unaffected parents and siblings). We studied alleles transmission using the Transmission Disequilibrium Test (TDT). We found an increased transmission of CTLA-4 49G allele to diabetics (54.8%, p=0.11). The transmission of F (56.1%, p=0.063), a (55.7%, p=0.061) and T (51.8%, p=0.37) alleles of VDR gene to diabetics was increased but did not reach statistical significance. In conclusion we found the same increased transmission of CTLA-4 49 G allele to diabetics as previously reported. VDR *Foq*I F allele seems to be predisposing while *Taq*I T allele seems to be protective.

**Keywords**: type I diabetes - genetic - CTLA-4 - vitamin D receptor - Romania

## Introduction

Type 1 diabetes mellitus (T1DM) or primary insulin dependent diabetes mellitus (IDDM) is a common chronic disease characterized by the autoimmune destruction of insulin secreting pancreatic cells. The destruction is complete and results in absolute insulin dependency. T1DM is a multifactorial disease the etiology of which involves both genetic and environmental factors [1, 2, 3].

Genetically, T1DM is a complex, polygenic disease, with multiple susceptibility and protective alleles interacting with each other. The study of candidate genes identified the two major susceptibility genes for T1DM: *IDDM1* encoded in the HLA region of the Major Histocompatibility

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Complex (MHC) on chromosome 6p21 [1, 4, 5] and mapped to the *DRB1, DQB1* and *DQA1* loci [6, 7] and *IDDM2* encoded by the insulin gene region mapped to the Variable Number of Tandem Repeats (*VNTR*) region 5' of the insulin gene on chromosome 11p15 [8]. This approach also raveled the association of a third locus with T1DM, at least in some populations: *IDDM*12 in the *CTLA-4* gene region on chromosome 2q [9, 10]. Using linkage analysis strategies by whole genome study, at least 20 other regions of the human genome were linked with T1DM [11, 12, 13] but none of these genes has been identified yet. All these "minor" genes have smaller contributions to T1DM genetic susceptibility. One of the two main T1DM susceptibility genes (*IDDM1* and *IDDM2*) is present in up to 90% of T1DM patients. This indicates that these main susceptibility genes are necessary for diabetogenesis. However they are not sufficient since around 8% of non-diabetic subjects carry the most diabetogenic haplotype (*IDDM1* and *IDDM2* positive). This suggests that in order to become diabetics, either some environmental factors must act on carriers of *IDDM1/IDDM2* or some of the "minor" genes should be involved.

We previously reported the *IDDM1* and *IDDM2* status for the Romanian population, characterized by one of the lowest T1DM incidences in Europe [14]. In this study on the Romanian population we aimed to study the role of two "minor genes: *CTLA-4* on chromosome 2q33 and *Vitamin D Receptor* (VDR) gene on chromosome 12q12-14. We tested for association with T1DM one *CTLA-4* gene and three VDR gene polymorphisms. For this, we typed 204 Romanian type 1 diabetic families using a unified method of genotyping multiple HLA and non-HLA polymorphisms under identical conditions employing the Sequence Specific Primer - SSP-PCR technique [15, 16, 17].

## Materials and methods

### *Families selection*

204 Romanian Type 1 diabetic families were genotyped in this study. We chose the Romanian population because of its very low reported incidence of Type 1 diabetes [18, 19]. Informed consent was obtained prior to blood collection for DNA extraction from all subjects involved and the local ethics committee approved the study. The ascertainment of families was made according to EURODIAB Study protocol as previously described [14]. Most were simplex families (196) but there were 3 multiplex families and 5 families with one parent also affected. Most were 4 member families (144) and the other 60 were 3 member families. Overall the study group comprised 756 individuals; 371 M (49.07%)/385 F (50.93%) of which 212 type 1 diabetic patients (106 M/106 F) with the onset of disease between 9 months and 43 years. The median age at the onset of disease was 12.1±6.7 years. Most patients (166 - 78.3%) had diabetes onset under 17 years age. There were 544 unaffected individuals (parents and siblings), 265 M (48.71%)/279 F (51.29%).

### *Selection of genes*

We selected for typing on the Romanian families the 49 A/G polymorphism of CTLA-4 gene (chromosome 2q33) and *Fok*I (F/f), *Apa*I (A/a) and *Taq*I (T/t) polymorphisms of Vitamin D Receptor gene (chromosome 12q12-14). All these polymorphisms were previously described [16, 17].

### *Primer design*

The primers used for typing of CTLA-4 and VDR gene polymorphisms were as previously reported [16, 17]. For all alleles we used an internal control for validation of amplification: *HLA-DRB1* intron 3 amplicon of 796 bp, forward primer TgCCAAgTggAgCACCCAA with annealing position exon 3 amino acid 173-179; reverse primer gCATCTTgCTCTgTgCAgAT with annealing position in exon 4 amino acid 193-200, both 12 ng/PCR amplification.

### *DNA extraction, PCR amplification and electrophoresis*

DNA extraction was done as previously described [14]. PCR amplification was done in 13 µl per reaction using the SSP-PCR technique as previously described [15, 17, 20]. Subsequently, PCR products were electrophoresed through 1% agarose gels containing 0.5 g/ml ethidium bromide in 0.5xTBE buffer (89 mmol Tris base, 89 mmol boric acid, 2 mmol EDTA, pH 8.0) for 20 - 30 min at 15 V/cm. Following electrophoresis, the products were visualised with UV illumination and the gel photographed with a Polaroid camera. Gel interpretation was simple, requiring the scoring of the presence or absence of an amplicon. For validation of PCR amplification, all reactions included an internal control, a 796 bp product depending on the HLA amplicon size, as detailed previously [15, 17, 20]. An example is given in Fig. 1.

### *Statistical analysis*

Data was initially analyzed using the Transmission Disequilibrium Test (TDT) [21]. The TDT tests the possibility that transmission of alleles from heterozygous parents to affected siblings is not the expected 50%. The TDT relative risk (RR) was calculated by dividing the number of times an allele was transmitted by the number not transmitted. After analysis we found a trend of association with some polymorphisms of Vitamin D receptor and CTLA-4 [22].

## Results

The results of the TDT analysis for CTLA-4 49 A/G polymorphism are shown in Table 1 while those for VDR polymorphisms are shown in Table 2. Our data show an increased transmission of 49G allele of CTLA-4 to diabetics compared to the unaffected siblings (54.84%, p=0.11). The percentage is similar to those reported for other Caucasian populations [9] but with p=0.11 the result does not reach statistical significance on this data set. We found an increased transmission of *Fok*I F allele to diabetics (56.14%, p=0.063) compared to the unaffected siblings (48.84% transmission) suggesting a possible predisposing effect. We also found an increased transmission of *Taq*I T allele to unaffected siblings (58.27%, p=0.038) compared to diabetics (51.82% transmission). This sustains that this allele could be protective. The transmission of *Apa*I alleles to diabetics was not different compared to the transmission to the unaffected siblings.

## Discussion

In this study we used a candidate gene approach to ascertain the genetic component of T1DM susceptibility on the Romanian population, using 204 Romanian Type 1 diabetic families. For this we used an SSP-PCR technique, which enabled us to test HLA and non-HLA polymorphisms under identical PCR amplification conditions. Previously we tested the Romanian families for *IDDM1* and *IDDM2* susceptibility genes in order to validate genetically for Type 1 diabetes this population. The results confirmed the major role of DR/DQ alleles and of Ins-VNTR alleles in T1DM susceptibility for the Romanian population [14].

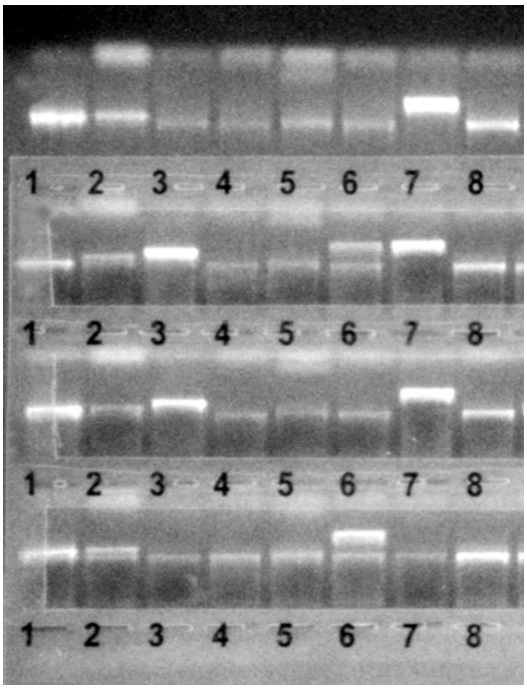


**Fig. 1** The polaroid photograph of an electrophoresis gel visualised with UV illumination. PCR products for VDR polymorphisms on each row in positions 1, 2, 3, 4, 5, 6 and of CTLA4 polymorphisms in positions 7 and 8.

Using the genome wide scan technique, a region of linkage was described on the long arm of chromosome 2 (2q33), which was later assigned as *IDDM12*. In fact, in a 23 cM region of 2q31-2q35 at least three loci linked with T1DM were described: *IDDM7, IDDM12* and *IDDM13* [9, 23, 24]. CTLA-4 (Citotoxic T Lymphocyte associated Antigen 4) gene on 2q33 is an ideal candidate gene for T1DM as an autoimmune disease. CTLA4 molecule is a member of the immunoglobulins superfamily and has multiple immunomodulatory actions. It mediates T cell apoptosis and negatively regulates T cell activation. It also downregulates expression of interleukin 2, an autocrine and paracrine growth factor responsible for continued T-cell proliferation.

An A/G transition in position 49 of exon 1 results in a Threonine to Alanine substitution in the CTLA-4 peptide. Initial studies showed that 49G allele is associated with T1DM in Italian and Spanish families but not for UK or USA [9]. Subsequently, the association was confirmed on a larger family collection from France, Spain and USA (Caucasian and Mexican-American) [25].

**Table 1** TDT for CTLA 4 alleles on Romanian families.

| | Polymorphism | T | NT | %T | *p* |
|---|---|---|---|---|---|
| Affected | CTLA-4 49 A | 84 | 102 | 45.16% | 0.89 |
| Unafected | CTLA-4 49 A | 56 | 63 | 47.06% | 0.71 |
| Affected | CTLA-4 49 G | 102 | 84 | 54.84% | 0.11 |
| Unafected | CTLA-4 49 G | 63 | 56 | 52.94% | 0.29 |

T - Transmitted, NT - Not-transmitted, %T - Transmission percentage.

Another polymorphism in the 3' untranslated region of CTLA-4 (which shows linkage disequilibrium with 49 A/G polymorphism) showed a tendency of association with T1DM on UK and USA families [26]. An association of CTLA-4 polymorphisms with Hashimoto and Graves diseases was also shown [27, 28], supporting the contribution of this gene in the pathogeny of autoimmune diseases.

In our study on 204 Romanian diabetic families, we found the same increased transmission of CTLA-4 49 G allele to diabetics (55%) as previous reported in other populations. This result, if replicated on a larger data set, will reach statistical significance and will confirm the role of CTLA-4 (*IDDM 12*) in T1DM genetic susceptibility for the Romanian population.

Vitamin D (1, 25 dihydroxy D3) has important immunoregulatory functions, including monocyte activation, inhibition of monocyte immunoglobulin receptor expression, inhibition of lymphocyte

**Table 2** TDT for Vitamin D receptor alleles on Romanian families.

| | Polymorphism | T | NT | %T | *p* |
|---|---|---|---|---|---|
| Affected | VDR ex2 f | 75 | 96 | 43.86% | 0.94 |
| Unafected | VDR ex2 f | 66 | 63 | 51.16% | 0.43 |
| Affected | VDR ex2 F | 96 | 75 | 56.14% | 0.063 |
| Unafected | VDR ex2 F | 63 | 66 | 48.84% | 0.57 |
| Affected | VDR intr8 a | 113 | 90 | 55.67% | 0.061 |
| Unnafected | VDR intr8 a | 79 | 62 | 56.03% | 0.089 |
| Affected | VDR intr8 A | 90 | 113 | 44.33% | 0.94 |
| Unafected | VDR intr8 A | 62 | 79 | 43.97% | 0.91 |
| Affected | VDR ex9 t | 66 | 71 | 48.18% | 0.63 |
| Unafected | VDR ex9 t | 53 | 74 | 41.73% | 0.96 |
| Affected | VDR ex9 T | 71 | 66 | 51.82% | 0.37 |
| Unafected | VDR ex9 T | 74 | 53 | 58.27% | 0.038 |

T - Transmitted, NT - Not-transmitted, %T - Transmission percentage.

activation and proliferation, suppression of immunoglobulin production, cytokine synthesis, MHC class II and CD4 expression on different cells [29]. There are several arguments for Vitamin D involvement in the pathogenesis of both murine and human T1DM. Thus, in NOD mice the development of diabetes can be prevented by administration of 1, 25(OH)2D3 which inhibits lymphocyte activation and restores the altered ratio of CD4/CD8 cells [30]. The data of EURODIAB epidemiologic multicentric study showed that the administration of Vitamin D supplements in new-borns and young children is associated with a reduction of T1DM incidence [31].

Vitamin D exerts its metabolic and immunomodulatory actions via the nuclear Vitamin D Receptor (VDR). VDR belongs to the steroid receptor super-family and is widely expressed in many cell types, including lymphocytes, macrophages and pancreatic cells. So VDR is a natural candidate gene for T1DM. VDR gene is located on chromosome 12q12-14, it was completely sequenced and several polymorphisms were described. A C/T transition at the junction of intron 1 and exon 2 creates an initiation codon (ATG) three codons proximal to downstream start site, leading to a protein variant with three additional amino-acids [32]. This transition introduces a site recognized by *Fok*I restriction enzyme. The two alleles of this polymorphism are F and f where f is recognized by *Fok*I. *In vitro* studies demonstrated an increased transcription rate (1.7 fold) of the VDR gene in cells with the FF haplotype [32]. Other polymorphisms induced by restriction enzymes *Bsm*I (alleles b/B) and *Apa*I (alleles a/A) in intron 8 and a „silent" T/C substitution in exon 9 which creates a *Taq*I restriction site (alleles t/T).

There are several studies regarding the association of VDR *Fok*I, *Bsm*I, *Apa*I and *Taq*1 polymorphisms with T1DM. In 1997, McDermott reports an association of "bAT" haplotype with T1DM on a collection of Indian ancestry families living in UK. „BAT" haplotype was protective [33]. These results were reconfirmed on a family collection from Germany [34], but in this population „bAT" haplotype seems to be also protective rather than predisposing. However, the association was not confirmed on some US families [35].

In our study on 204 Romanian T1DM families, *Foq*I F allele seems to be predisposing (56.14% transmission, p=0.063) while *Taq*I T allele seems to be protective (58.27% transmission, p=0.038). However these results must be replicated on a second family set ascertained in the same conditions as that examined in the present study.

In conclusion, our data sustain the role of CTLA-4 (*IDDM 12*) and Vitamin D Receptor in Type 1 diabetes genetic susceptibility for the Romanian population.

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