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DISSECTING THE ROLE OF THE FOXP3 GENE IN THE JOINT GENETIC SUSCEPTIBILITY TO AUTOIMMUNE THYROIDITIS AND DIABETES: A GENETIC AND FUNCTIONAL ANALYSIS

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Abstract

We have previously shown that a (TC)_n microsatellite in intron 5 of the Forkhead Box Protein 3 (FOXP3) gene was associated with a variant of the autoimmune polyglandular syndrome type 3 (APS3v), that is defined as the co-occurrence of type 1 diabetes (T1D) and autoimmune thyroiditis (AITD). Allele 10, containing 25 repeats of the microsatellite (long repeats), is preferentially transmitted to offspring with APS3v, while allele 2, containing 14 repeats of the microsatellite (short repeats), is protective. We hypothesized that the long repeats of the intron 5 microsatellite decrease FOXP3 splicing and function, thereby reducing regulatory T cell activity and promoting the development of APS3v.

We cloned genomic DNA from two males hemizygous for the long and short repeats of the microsatellite on their X-chromosomes and transfected them into human embryonic kidney 293 (HEK 293) cells to perform direct splicing analysis.

We identified a novel splice variant of FOXP3 lacking exon 6, and showed that it is expressed in human thymus and lymph node. However, the length of the repeats in the microsatellite did not significantly influence the expression of this FOXP3 splice variant *in vitro*. Interestingly, this splice variant was expressed in human regulatory T cells, suggesting it may play a role in their function.

In conclusion, we identified a novel splice variant FOXP3_{Δ6}. The role of its expression in regulatory T cells in the development of autoimmunity remains to be determined.

Keywords

autoimmune thyroiditis; type 1 diabetes; FOXP3; gene splicing; transcription

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1. Introduction

Autoimmune endocrine disorders frequently aggregate within families (McCanlies et al., 1998) and may co-occur in the same individual (Huber et al., 2008). Autoimmune endocrine disorders occurring in the same individual constitute the autoimmune polyglandular syndrome (APS) (Eisenbarth and Gottlieb, 2004). There are several types of APS (Betterle and Zanchetta, 2003). APS type 1 (APS1) is caused by AIRE gene mutations and is characterized by chronic candidiasis, hypoparathyroidism and Addison's disease. APS type 2 (APS2) is diagnosed by the co-occurrence of Addison's disease, plus autoimmune thyroiditis (AITD) and/or type 1 diabetes (T1D). APS type 3 (APS3) refers to autoimmune thyroiditis associated with other autoimmune diseases, excluding Addison's disease. The co-occurrence of T1D and AITD is a common variant of APS3 (APS3v) (Jenkins and Weetman, 2002). Indeed, the percentage of T1D patients developing thyroid-specific antibodies (TAb), including anti-thyroid peroxidase and anti-thyroglobulin, varies from 8% to 44% among different populations, and it is significantly higher than the frequency of TAb in healthy individuals (Riley et al., 1981; Betterle et al., 1984; Kontiainen et al., 1990; McKenna et al., 1990; Darendeliler et al., 1994; Lorini et al., 1996; Lindberg et al., 1997). Conversely, 2.3% of AITD children were reported to have islet cell antibodies (ICA) compared to 0% of control children (Bright et al., 1982). It was also found that higher percentage of first-degree relatives of T1D patients have ICA or TAb than controls (Jaeger et al., 2001; Sougioultzoglou et al., 2005; Anaya et al., 2006), and there is increased frequency of TAb in siblings of T1D patients (Burek et al., 1990). These observations suggest a strong genetic association between T1D and AITD.

Several genetic (Jacobson and Tomer, 2007; Huber et al., 2008), epigenetic (Stefan et al., 2011; Stefan et al., 2014), and environmental factors (Hasham and Tomer, 2011; Dabelea et al., 2014) have been reported to play a role in the etiology of AITD and T1D. Moreover, a growing body of evidence points to shared susceptibility genes for T1D and AITD (Huber et al., 2008). Three genes were confirmed to confer joint susceptibility to T1D and AITD: HLA class II genes (Torfs et al., 1986; Payami et al., 1989; Santamaria et al., 1994; Dorman et al., 1997; Levin et al., 2004; Golden et al., 2005), cytotoxic T-lymphocyte antigen (CTLA-4) (Golden et al., 2005; Ikegami et al., 2006; Teft et al., 2006; Howson et al., 2007), and the protein tyrosine phosphatase non-receptor type 22 gene (PTPN22) (Cloutier and Veillette, 1999; Saccucci et al., 2008). Besides HLA class II, CTLA-4 and PTPN22, other genes might play a role in the joint susceptibility to T1D and AITD. Our group used whole genome linkage analysis to identify shared susceptibility genes for T1D and AITD (Villano et al., 2009). One of the genes, located within the Xp locus, that was linked and associated with APS3v is Forkhead Box Protein 3 (FOXP3). Significant association was found between the (TC)_n microsatellite in intron 5 of FOXP3 and APS3v. Allele 10, containing 25 repeats, was preferentially transmitted to offspring with APS3v, while allele 2, containing 14 repeats, was protective (Villano et al., 2009). We hypothesized that the length of the (TC)_n microsatellite could influence FOXP3 expression and/or splicing as intronic microsatellites have been shown to be regulators of genes splicing (Pagani et al., 2000; Gabellini, 2001). Changes in FOXP3 splicing pattern could alter the function of T regulatory cell activity which could predispose to autoimmunity. In the current study we used the minigene system

(Cooper, 2005) to determine directly whether the (TC)_n microsatellite alters FOXP3 splicing.

2. Material and methods

2.1 Cloning of genomic DNA containing the FOXP3 intron 5 (TC)_n microsatellite

The project was approved by the Icahn School of Medicine at Mount Sinai Institutional Review Board. We cloned a genomic segment of the FOXP3 gene containing exon 5 through exon 7 including the microsatellite of interest (TC)_n in intron 5 from the genomic DNA of 2 males: One hemizygous for allele 2 (14 repeats), and another hemizygous for allele 10 (25 repeats) on their X-chromosomes. Forward primer (FOXP3_f8) 5' GTGGATCCCACCCCTTTCGGCTGTGCCCCAGAG 3' and reverse primer (FOXP3_r8) 5' ACGCGGCCGCCACAGATGAAGCCTTGGTCAGTGC 3' were used. We introduced a BamHI site in the forward primer and a NotI site in the reverse primer (underlined). All primers were synthesized by Integrated DNA Technologies (Coralville, IA). The PCR reaction included 10x buffer, 22.5 μM dNTPs, 1.125 mM MgCl₂, 0.225 μM of each primer and 0.9 U of Hotstar polymerase (Qiagen, Valencia, CA). The PCR conditions were as follows: 95 °C for 15 min, followed by 35 cycles of 95 °C for 1 min, 62 °C for 1 min, 72 °C for 1 min, then a final extension step of 6 min. The plasmid pcDNA3.1(+) (Life Technologies, Carlsbad, CA) and the PCR products were digested with BamHI and NotI (New England Biolabs, Ipswich, MA). The digested plasmid and the PCR products were gel-purified using QIAquick gel extraction kit (Qiagen, Valencia, CA), and ligation was performed using T4 DNA ligase (Promega, Madison, WI). Double digestion with BamHI and NotI were carried out to check for the presence of inserts. The constructs were sequenced using forward primer (FOXP3_f6) 5' TGAGTGACAGAGCTGGGATT 3' and reverse primer (FOXP3_r6) 5' GTTCCCTTAACACATGCCCC 3' to verify the sequence of the cloned segments and the number of TC repeats in the microsatellite. Sequencing was performed using the Big Dye DNA Sequencing kit (Life Technologies, Carlsbad, CA) and the sequencing products were separated on an ABI-3130 automated sequencer (Life Technologies, Carlsbad, CA).

2.2 Cell culture

Human embryonic kidney 293 (HEK 293) cells (American Type Culture Collection [ATCC], Manassas, VA) were grown in Dulbecco's Modified Eagle's Medium (ATCC) supplied with 10% FBS (Sigma-Aldrich, St. Louis, MO) using T75 flasks (Corning, NY) and passaged twice a week.

2.3 Transfection

The two FOXP3 constructs containing the (TC)_n intron 5 microsatellite (alleles 2 and 10) were amplified using the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA). The empty vector pcDNA3.1(+) was also prepared as a control construct. On the day before transfection, the HEK 293 cells were seeded at 4 × 10⁵ cells/ml in 24-well plates (BD Biosciences, Franklin Lakes, NJ). On the next day when the cells reached 90% confluence, the cells were transfected with the 3 constructs: empty vector, allele 2 and allele 10. Two controls were included: cells only or cells with Lipofectamine only. After 24 h incubation at

37 °C, the medium was removed from wells and the cells were collected for RNA extraction.

2.4 Regulatory T cells purification

We obtained four buffy coats from anonymous donors through the New York Blood Center. Since these were anonymous donors, no clinical or demographic information is available. The buffy coats were rinsed with RPMI1640 (Corning, NY). Ficoll (GE Healthcare, Cleveland, OH) was added and centrifuged for 30 min at room temperature with no brake. The mononuclear cell layer which contained the peripheral blood mononuclear cells (PBMC's) was collected in new tubes. The cells were centrifuged at 1200 rpm for 7 min and the pellets were resuspended and pooled. On average, 300×10^6 PBMC's were used for regulatory T cell isolation. The cells were centrifuged at 1200 rpm for 7 min at room temperature, and the pellet was resuspended in buffer (PBS pH 7.2, 0.5% BSA, 2 mM EDTA, Miltenyi Biotech, Auburn, CA). The regulatory T cells were isolated using the CD4⁺CD25⁺CD127^{dim/-} Regulatory T Cell Isolation Kit II, human (Miltenyi Biotech, Auburn, CA) according to the manufacturer's instructions. All the isolated regulatory T cells were put in 24-well plate and stimulated with the Human T-Activator CD3/CD28 Dynabeads (Life Technologies, Carlsbad, CA) at 25 μ l/ 10^6 cells for 48 h at 37 °C. The cells were collected in Trizol reagent for RNA extraction followed by reverse transcription and PCR.

2.5 Polymerase chain reaction

RNA was extracted using Trizol reagent and reverse transcribed. The following primers were used for PCR amplification: for the transfected HEK 293 cells, forward primer (FOXP3_f10) 5' AAATGGTGTCTGCAAGTGGCCCGG 3' and reverse primer (FOXP3_r10) 5' ATTTTCCCAGCCAGGTGGGCCTGC 3' were used to check for the presence of the splice variant. For the human tissues (lymph node, kidney, spleen, pancreas, muscle, skin, thyroid, thymus) (Agilent Technologies, Santa Clara, CA) and regulatory T cells cDNA samples, forward primer (FOXP3_f13) 5' CACCCTTTCGGCTGTGCCCC 3' and reverse primer (FOXP3_r14) 5' CCAGCACCAGTTGAGGAAGT 3' were used for amplification. The forward primer FOXP3_f13 spans a sequence within exon 5. The reverse primer FOXP3_r14 spans a sequence that is part in exon 5 and part in exon 7. Therefore, the splice variant would be amplified only if exon 6 is spliced out. The PCR mix contained 10x buffer, 22.5 μ M dNTPs, 1.125 mM MgCl₂, 0.225 μ M of each primer and 0.9 U of Hotstar polymerase (Qiagen, Valencia, CA). The PCR conditions were as follows: 95 °C for 12 min, followed by 34 cycles (for transfected HEK 293 cells) or 40 cycles (for human tissues or regulatory T cells) of 94 °C for 30 sec, 60 °C for 30 sec and 72 °C for 40 sec, and then a final extension step of 72 °C at 10 min. PCR was performed using Bio-rad's (Hercules, CA) PCR thermal cycler and the PCR products were run on a 1.5% agarose gel for visualization.

2.6 Real-time quantitative PCR

Real-time quantitative PCR was used for quantification of the splice variants detected in the transfected HEK 293 cells. Forward primer (FOXP3_f13) 5' CACCCTTTCGGCTGTGCCCC 3' and reverse primer (FOXP3_r14) 5' CCAGCACCAGTTGAGGAAGT 3' were used for amplification. The forward primer

FOXP3_f13 spans a sequence within exon 5. The reverse primer FOXP3_r14 spans a sequence that is part in exon 5 and part in exon 7. Therefore, the splice variant would be amplified only if exon 6 is spliced out. In a 20 µl reaction, 0.5 µM of each primer and 2x SYBR green PCR Master Mix (Life Technologies, Carlsbad, CA) were added. The reaction was carried out using the following program: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min, using the Applied Biosystems 7300 Real-time PCR system (Life Technologies, Carlsbad, CA). GAPDH was amplified as the control housekeeping gene, with forward primer 5'ACAGTCAGCCGCATCTTCT 3' and reverse primer 5' ACGACCAAATCCGTTGACTC 3'.

3. Results

3.1 Confirmation of the number of TC repeats in the two constructs expressing long/short repeats of the FOXP3 intron 5 microsatellite

The two minigene constructs expressing different number of repeats in the intron 5 (TC)_n microsatellite were sequenced. Our sequencing results showed that allele 2 had 14 TC repeats, while allele 10 had 25 TC repeats (data not shown).

3.2 Expression of FOXP3 splice variant in HEK 293 cells after transfection

The primers FOXP3_f10 and FOXP3_r10 were used to amplify exon 5 through 7 from cDNA. With all three exons present in the mRNA, the expected size of the PCR product is 209 bp. If exon 6 is spliced out, the expected size of the PCR product would be 121 bp. Figure 1 shows the size of each exon and intron. HEK 293 cells were transfected with the two minigene constructs with short (allele 2, (TC)₁₄) and long (allele 10, (TC)₂₅) repeats of the intron 5 microsatellite respectively. The empty vector pcDNA3.1(+) was also transfected as a negative control. Negative controls with cells only or cells plus lipofectamine alone were also included. All 5 groups (cells only, cells with lipofectamine, empty vector, (TC)₁₄, (TC)₂₅) showed expression of the full-length non-spliced FOXP3 PCR product of size 209 bp, demonstrating endogenous expression of FOXP3 in HEK 293 cells. However, the splice variant (121 bp) was only detected in cells transfected with the constructs expressing the long/short repeats of the intron 5 (TC)_n microsatellite (Fig. 2). We sequenced the PCR products at the junction of the exons. Our results showed that the upper band of (TC)₁₄ had exon 5 directly followed by exon 6, while the lower band of (TC)₁₄ showed exon 5 directly followed by exon 7, with exon 6 spliced out. Similar results were found for (TC)₂₅ (Figure 1).

The other set of primers FOXP3_f13 and FOXP3_r14 is a set of primers that amplify a product of size 117 bp only if exon 6 is spliced out. FOXP3_f13 is on exon 5, while FOXP3_r14 spans part of exon 5 and part of exon 7, as depicted in supplemental figure 1. This splice variant was only seen in cells transfected by the two constructs (lanes 4-6, short repeats; lanes 7-9, long repeats), but not in control cells (lanes 1-3) (Supplemental figure 2). This set of primers further confirmed the expression of the splice variant in the transfected cells.

3.3 Quantification of the FOXP3 6 splice variant expression levels

To determine whether the long and short repeats of the (TC)_n microsatellite influence the expression levels of the splice variant, we transfected the HEK 293 cells with the two constructs for 24 h. The transfection experiment was done with each group (long and short repeats) having 10 replicates. Real-time quantitative PCR was performed to measure the quantity of splice variant produced after transfection of the two constructs. The experiment was repeated four times. No significant differences in the levels of splice variant were found when transfecting with the two constructs. Figure 3 shows the result of a representative experiment. The relative expression levels of the splice variant after transfecting with the two alleles did not differ significantly.

3.4 Expression of the FOXP3 6 splice variant in human tissues

We investigated the expression of the splice variant in several human tissues (lymph node, kidney, spleen, pancreas, muscle, skin, thyroid, thymus). Expression of the FOXP3 splice variant was detected in human thymus (lane 8) and lymph node (lane 1), but not in other tissues including kidney, spleen, pancreas, muscle, skin and thyroid (lanes 2-7) (Figure 4A). This suggests that this splice variant may play a role in lymphocyte function. Testing Graves' thyroid tissues we found no expression of the splice variant (data not shown).

3.5 Expression of the FOXP3 6 splice variant in regulatory T cells

We investigated the expression of the splice variant in human regulatory T cells. Expression of this splice variant was detected in the regulatory T cells from four different donors (lanes 1-4) (Figure 4B), suggesting that this splice variant may play a role in the development and/or function of regulatory T cells.

4. Discussion

FOXP3 is an X-linked gene located on chromosome Xp11.23 (Ban et al., 2007). It is a protein consisting of 431 amino acids that are encoded by 11 exons (Chatila et al., 2000; Wildin et al., 2001), and is a member of forkhead/winged-helix family of DNA-binding transcription factors (Kaufmann and Knochel, 1996; Brunkow et al., 2001). FOXP3 contains a forkhead domain at the C-terminus, which mediates DNA binding and nuclear localization (Lopes et al., 2006). It also has a C2H2 zinc finger domain which is necessary for dimerization and repressive activity, and a leucine zipper motif that is essential for FOXP3 homo- and hetero-dimerization (Lopes et al., 2006). FOXP3 can act both as transcriptional repressor (Schubert et al., 2001) or activator (Wu et al., 2006; Marson et al., 2007; Zheng et al., 2007) mostly for immune response genes. For example, FOXP3 binds to the Runt-related transcription factors to inhibit the expression of IL-2 and IFN- γ (Ono et al., 2007), while it can also activate CD25 expression by co-operatively binding with NF- κ B to the CD25 promoter upon activation of CD28 (Camperio et al., 2012). The role of FOXP3 in autoimmunity was unveiled when the mouse mutant, *scurfy*, was found to be defective in FOXP3. *Scurfy* mutant was lethal in hemizygous males, characterized by massive hyperproliferation of CD4⁺ T cells and multi-organ infiltration (Brunkow et al., 2001). In humans, mutations in FOXP3 lead to an X-linked syndrome characterized by immune

dysregulation, polyendocrinopathy and enteropathy (IPEX) (Chatila et al., 2000; Bennett et al., 2001; Wildin et al., 2001; Wildin and Freitas, 2005; Katoh et al., 2013).

In order to maintain immunological self-tolerance, regulatory T cells suppress peripheral self-reactive lymphocytes that have escaped central tolerance in the thymus (Maloy and Powrie, 2001; Sakaguchi et al., 2001). FOXP3 is a unique marker of CD4⁺CD25⁺ regulatory T cells and studies have shown that FOXP3 is essential for development and function of CD4⁺CD25⁺ regulatory T cells (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Ectopic expression of FOXP3 commits naïve T cells to become regulatory T cells (Hori et al., 2003). The mutations in FOXP3 resulting in *scurfy* mice (Brunkow et al., 2001) and IPEX in humans (Chatila et al., 2000; Bennett et al., 2001; Wildin et al., 2001) suggest that abnormal FOXP3 function may be associated with autoimmunity.

Interestingly, FOXP3 is expressed as a single isoform in mouse T-cells, while several splice variants of FOXP3 are found in human T-cells (Walker et al., 2003; Manavalan et al., 2004; Yagi et al., 2004; Allan et al., 2005; Smith et al., 2006; Mailer et al., 2009; Kaur et al., 2010). FOXP3 splice variants lacking exon 2 (FOXP3^{Δ2}), exon 7 (FOXP3^{Δ7}), or both (FOXP3^{Δ2-7}), have been reported. FOXP3 exon 2 is important for repressor function (Du et al., 2008; Ichiyama et al., 2008), while exon 7 encodes for a leucine zipper motif that is important for dimerization (Landschulz et al., 1988; Chae et al., 2006). FOXP3^{Δ2} was found to be expressed in human peripheral blood mononuclear cells (PBMC's) (Smith et al., 2006), CD4⁺CD25⁺ regulatory T cells (Yagi et al., 2004; Allan et al., 2005) and CD8⁺CD28⁻ T-cells (Manavalan et al., 2004). FOXP3^{Δ7} was found in CD4⁺CD25⁺ and CD8⁺ regulatory T cells (Kaur et al., 2010). FOXP3^{Δ2-7}, lacking both exon 2 and exon 7, was also found to be expressed in human PBMC's (Smith et al., 2006) and CD4⁺CD25⁺ human regulatory T cells (Mailer et al., 2009). FOXP3^{Δ2-7} was overexpressed by malignant T-cells in Sezary syndrome (Krejsgaard et al., 2008), which was the first description of FOXP3 splice forms in human disease.

Previously, we identified a (TC)_n microsatellite in intron 5 of the FOXP3 gene that was associated with a variant of autoimmune polyglandular syndrome type 3 (APS3v) (Villano et al., 2009). Therefore, we hypothesized that this microsatellite may alter FOXP3 splicing, thereby changing its activity and contributing to the development of APS3v. In the current study, using direct splicing analysis, we identified a novel splice variant of FOXP3 in which exon 6 was skipped. This new splice variant (FOXP3^{Δ6}) was expressed in human thymus, lymph nodes and in human CD4⁺CD25⁺ regulatory T cells. Whether this new FOXP3 splice variant has a role in development and function of regulatory T cells needs to be determined in further studies. Due to its location and size, the (TC)_n microsatellite could potentially influence the splicing efficiency of exon 6, altering the levels of FOXP3^{Δ6}. However, we did not observe a significant difference in the levels of FOXP3^{Δ6} mRNA *in vitro* when the HEK 293 cells were transfected with the long or short alleles of the (TC)_n microsatellite. It should be noted that this is an artificial system and it may not correlate directly with the levels of splice variant present in human regulatory T cells. To evaluate this possibility, further studies correlating FOXP3^{Δ6} splice variant levels in regulatory T cells with the (TC)_n genotypes are needed. One potential weakness of our study is that we tested splicing in HEK 293 cells, since the splicing machinery in HEK293 cells may be different than in T-

cells. However, the fact that we were able to identify the splice variant in regulatory T cells and in the thymus and lymph nodes suggests that the splicing we saw in the HEK 293 cells also happens in lymphocytes. Although we did not detect the expression of this splice variant in thyroid samples of GD patients or controls, the possibility that it might exist in untreated Graves' patients' thyroid samples cannot be eliminated. The role of this splice variant in thyroid autoimmunity warrants further investigation.

When we amplified the FOXP3 gene using primers spanning exon 6 in different tissues we could not visualize the splice variant by Ethidium Bromide staining and only the full-length FOXP3 (which includes exon 6, 209 bp) was visualized (data not shown). However, when we amplified using primers unique for the splice variant, the FOXP3 splice variant with exon 6 spliced out could be clearly detected in lymph node and thymus tissues (Figure 4). We think that this is because of the dominant expression of the full-length FOXP3 gene over the splice variant which probably has low levels of expression compared to the full-length FOXP3.

Our results showed that the full-length FOXP3 gene is expressed endogenously in non-immune HEK 293 cells, which might seem surprising. In fact, FOXP3 expression in normal human tissues has been tested (www.genecards.org). Many tissues from the immune, nervous, muscle, internal, secretory and reproductive systems were tested for their expression for FOXP3 gene. Microarray data from BioGPS (<http://biogps.org>) and RNAseq data from Illumina Body Map both showed expression of FOXP3 in the kidney. Our data showed that the full-length FOXP3 gene was expressed in lymph node, kidney, spleen, pancreas, muscle, skin, thyroid and thymus. Therefore, our findings showing expression of the full-length FOXP3 gene in HEK 293 cells (human embryonic kidney cells) agreed with the expression profiles published.

Several groups reported associations between FOXP3 variants and autoimmune diseases. We found an association between the (TC)_n microsatellite in intron 5 and autoimmune thyroid diseases (AITD) in Caucasian males, but not in a Japanese cohort (Ban et al., 2007). We also found association between the DXS573 microsatellite, which is in LD with FOXP3, and AITD in Caucasian female AITD patients (Ban et al., 2007). Owen et al. reported a FOXP3 haplotype that showed association with Graves' disease (GD), although it was not significant after correction for multiple testing (Owen et al., 2006). Moreover, Inoue et al. reported a FOXP3 polymorphism (-3279A/C) that was associated with development and intractability of GD, while another FOXP3 polymorphism (-2383 CC genotype) was associated with the severity of Hashimoto's thyroiditis (Inoue et al., 2010). Taken together, these data suggest that the FOXP3 gene plays a role in the development of thyroid autoimmunity. Similarly, several reports showed association of FOXP3 with type 1 diabetes (T1D). One study showed that a polymorphism in the promoter/enhancer region of FOXP3 was associated with T1D (Bassuny et al., 2003). A (GT)_n microsatellite in intron zero was also found to be associated with T1D in the Japanese, although the (TC)_n microsatellite in intron 5 did not show an association in this study. In contrast, another study from Italy found no association between FOXP3 and T1D (Zavattari et al., 2004). Polymorphisms in FOXP3 were also found to be associated with other autoimmune diseases such as systemic sclerosis (D'Amico et al., 2013), primary biliary cirrhosis (PBC) (Park et al., 2005), psoriasis (Gao et

al., 2010), systemic lupus erythematosus (Lin et al., 2011) and generalized vitiligo (Birlea et al., 2011). FOXP3 variants and their associations with autoimmune diseases are summarized in Table 1.

In summary, FOXP3 is emerging as an important susceptibility gene for autoimmune endocrine disorders, as well as other autoimmune conditions. A (TC)_n microsatellite in intron 5 is associated with autoimmune polyglandular syndrome 3 variant manifested by the co-occurrence of AITD and T1D. We have identified a new splice variant in the FOXP3 gene, FOXP3₆, but the levels of FOXP3₆ were not associated with the (TC)_n polymorphisms. Further studies are needed to elucidate potential role of FOXP3₆ in regulatory T cells function and endocrine autoimmunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

APS3v	autoimmune polyglandular syndrome type 3
AIRE	autoimmune regulator
AITD	autoimmune thyroiditis
bp	base pair(s)
CTLA-4	cytotoxic T-lymphocyte antigen deletion
dNTP	deoxyribonucleoside triphosphate
FOXP3	Forkhead Box Protein 3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GD	Graves' Disease
HEK	human embryonic kidney
HLA	human leukocyte antigen
IPEX	immune dysregulation, polyendocrinopathy and enteropathy
IFNγ	interferon gamma
IL-2	interleukin 2
ICA	islet cell antibodies
LD	linkage disequilibrium

NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
PBMC	peripheral blood mononuclear cells
PBC	primary biliary cirrhosis
PTPN22	protein tyrosine phosphatase non-receptor type 22 gene
TAbs	thyroid-specific antibodies
T1D	type 1 diabetes

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Highlights

1. A (TC)_n repeat in intron 5 of FOXP3 was associated with T1D+AITD.
2. We dissected the mechanisms by which the (TC)_n repeat may trigger autoimmunity.
3. Using direct splicing analysis we identified a novel splice variant FOXP3₆.
4. The (TC)_n microsatellite alleles did not influence the splicing of FOXP3.
5. Expression of FOXP3₆ in Tregs has potential role in development of autoimmunity.

Figure 1.

Schematic diagram of FOXP3 exon 5 through 7. The (TC)_n microsatellite is located in intron 5. Primers FOXP3_f10 and FOXP3_r10 were used for amplification. The expected size of the PCR product with all 3 exons included is 209 bp. If exon 6 is skipped, the product size is 121 bp. The bottom part shows the sequencing results of the PCR products at the exon junctions.

Figure 2.

PCR results showing expression of FOXP3 in transfected HEK 293 cells using primers FOXP3_f10 and FOXP3_r10. HEK 293 cells were incubated with medium only (lanes 1-3), lipofectamine only (lanes 4-6), or transfected with empty pcDNA 3.1 (+) (lanes 7-9), constructs with microsatellites (TC)₁₄ from individual hemizygous for allele 2 (lanes 10-12) or (TC)₂₅ from individual hemizygous for allele 10 (lanes 13-15). Lane 16 is the blank control and lane 17 is the marker. The size of the upper band is 209 bp, and the lower band is 121 bp.

Figure 3.

Real-time quantitative PCR results showing the relative expression of the FOXP3 splice variant when HEK 293 cells were transfected with the constructs containing (TC)₁₄ or (TC)₂₅ microsatellite in intron 5. No significant difference in the amount of splice variant was detected.

Figure 4A.

PCR results showing expression of the FOXP3 splice variant in human tissues. From left: lymph node, kidney, spleen, pancreas, muscle, skin, thyroid, thymus (lanes 1-8). Product size is 117 bp.

Figure 4B.

PCR results showing the expression of the FOXP3 splice variant in regulatory T cells. From left: donor 1 to 4 (lanes 1-4), followed by the HEK 293 cells transfected with (TC)₁₄ microsatellite (lane 5) or (TC)₂₅ microsatellite (lane 6) as positive controls and the blank control (lane 7). The product size is 117 bp.

Table 1

FOXP3 variants and their associations with autoimmune diseases

FOXP3 variant	Reference	Association with autoimmune diseases
(TC) _n microsatellite in intron 5	Ban et al, 2007	AITD in Caucasian males
DXS573 microsatellite (in LD with FOXP3)	Ban et al, 2007	AITD in Caucasian females
-3279 A/C polymorphism	Inoue et al, 2010	Development and intractability with GD
-2383 CC genotype	Inoue et al, 2010	Severity of Hashimoto's thyroiditis
(GT) _n microsatellite in intron zero	Bassuny et al, 2003	T1D in Japanese
rs2280883 (IVS9+459T/C)	D'Amico et al, 2013	Females with systemic sclerosis in Italian Caucasians
rs2280883 (IVS9+459T/C)	Park et al, 2005	Primary biliary cirrhosis
-3279 AC genotype and combined AC+AA genotype	Gao et al, 2010	Increased risk of psoriasis
IVS9+459 GG genotype	Gao et al, 2010	Increased risk of psoriasis
-3279 AC+AA genotype	Gao et al, 2010	Severe psoriasis
IVS9+459 GA+GG	Gao et al, 2010	Severe psoriasis
-6054, del>ATA polymorphism	Lin et al, 2011	Renal disorder in female SLE patients
-3279 A carrier	Lin et al, 2011	Lower anti-dsDNA levels in SLE patients
rs3761547	Birlea et al, 2011	Generalized vitiligo