Minor contribution of SMAD7 and KLF10 variants to genetic susceptibility of type 2 diabetes

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Abstract

Background. – Transgenic mice over-expressing SMAD7 in pancreatic beta-cells develop type 2 diabetes (T2D). The expression of SMAD7 is affected by KLF11, which contains gene variants that have previously been shown to be involved in genetic susceptibility to T2D, and by the highly homologous KLF10. This study aims to assess the genetic contribution of SMAD7 and KLF10 gene variants to T2D susceptibility in the French population.

Methods. – We screened both genes to identify rare and frequent variants by direct sequencing and then genotyped these variants. Six frequent variants of SMAD7 and six of KLF10 were analyzed in 349 T2D patients and 349 normoglycemic adult subjects. Variants with statistically significant differences in allele and/or genotype distribution were further analyzed in a population sample of 1,712 T2D patients and 1,072 normoglycemic subjects.

Results. – Two variants showed a significant association under a recessive model: The intronic SMAD7 IVS2–21 had an odds ratio of 0.62 (P = 0.007, 95% CI = 0.44–0.88; P = 0.034 when adjusting for age, sex and BMI by logistic regression), and the KLF10 3′UTR +1002 variant had an Odds Ratio of 0.81 (P = 0.009, 95% CI = 0.69–0.95; P = 0.042 when adjusting for age, sex and BMI).

Conclusion. – Although the observed association of SMAD7 and KLF10 gene variants with T2D is modest, they may weakly contribute to a particular genetic background that increases the susceptibility to development of T2D.

Résumé

Contribution mineure des variants SMAD7 et KLF10 à la prédisposition génétique au diabète de type 2

Contexte. – Des souris transgéniques surexprimant SMAD7 dans les cellules β-pancréatiques développent un diabète de type 2 (DT2). L’expression de SMAD7 est régulée par les facteurs de transcription KLF11 et son plus proche homologue KLF10. Nous avons précédemment montré que KLF11 était impliqué dans la susceptibilité génétique au DT2. Dans cette étude, nous analysons la contribution génétique des variants des gènes SMAD7 et KLF10 à la susceptibilité au DT2 dans la population française.

Méthodes. – Une recherche des variants rares et fréquents des deux gènes a été effectuée par séquençage direct. Les variants fréquents, six SNPs de SMAD7 et six SNPs de KLF10, ont été génotypés chez 349 patients diabétiques et 349 individus adultes normoglycémiens. Les variants montrant des différences de distribution allélique et/ou génotypique ont été analysés dans une deuxième population qui incluait 1712 patients atteints de DT2 et 1072 individus normoglycémiens.

Abbreviations: BMI: body mass index; C.I.: confidence interval; KLF: Krüppel-like factor; MAF: minor allele frequency; OR: odds-ratio; T2D: type 2 diabetes.

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The upregulation of i-Smad7 by TGF-β phosphorylation of r-Smads involved in TGF-β gene transcription. The inhibitor-Smad7 (i-Smad7) prevents plex is then translocated to the nucleus where it regulates target endocrine β-cells. The development of T2D in transgenic mice that over express i-Smad7 in pancreatic islet function, and that rare and frequent genetic variants of the KLF10 gene could also be associated with T2D. We have recently shown that TIEG2/KLF11 plays a role in endocrine β-cell function, and that rare and frequent genetic KLF11 variants cosegregate with early onset familial diabetes or are associated with late-onset T2D, respectively [3]. KLF11 is also known to regulate exocrine cell growth and behaves as a tumour suppressor in pancreatic cancers [4]. Interestingly, TGF-β induces KLF11, and KLF11 represses the SMAD7 gene [5]. Thus, up regulation of KLF11 may create a feed-forward loop to TGF-β signalling. It is possible that the contribution of aberrant KLF11 function to T2D susceptibility depends on its transcriptional regulation of SMAD7. Since i-smad7 over expression is diabeticogenic [1], we assessed whether the SMAD7 gene could also harbour genetic variants associated with T2D.

The TGF-β induced transcription factor TIEG/KLF10 is highly homologous to KLF11 and may have similar functions [6,7]. Members of the KLF transcription factor family have three homologous SP1-like DNA-binding domains. Moreover, both KLF10 and KLF11 have three similar, evolutionary conserved, repressor domains that are involved in co-factor binding [6]. In addition, KLF10 has also been shown to repress transcription of the SMAD7 gene [8]. However, both the knockout mouse model of KLF11 and of KLF10 showed no apparent pathological defects of pancreatic function under basal conditions [9,10]. If KLF10 and KLF11 affect transcriptional regulation of the same set of genes (including SMAD7), we may expect they contribute similarly to the development of T2D, and that genetic variants of the KLF10 gene could also be associated with T2D.

To test our hypothesis, we assessed the contribution of SMAD7 and KLF10 gene variants in genetic susceptibility to T2D. We screened the promoter regions, UTRs, coding and flanking intron sequences of these genes to identify frequent and rare variants. We then analyzed frequent variants in a two-stage case-control study and analyzed rare variants in their isolated families.

1. Introduction

SMAD7 has recently been shown to be associated with T2D in transgenic mice [1]. Smads are the principal signal transducers of TGF-β [2]. Upon phosphorylation by TGF-β-receptor-I, two receptor-associated Smads (r-Smad2 and r-Smad3) form a complex with the coactivator Smad4 (co-Smad4). This complex is then translocated to the nucleus where it regulates target gene transcription. The inhibitor-Smad7 (i-Smad7) prevents phosphorylation of r-Smads involved in TGF-β signalling. The upregulation of i-Smad7 by TGF-β serves as negative feedback loop to TGF-β signalling [2]. The development of T2D in transgenic mice that over express i-Smad7 in pancreatic cells suggests that disrupted islet TGF-β signalling may impair insulin production in adult β-cells.

We have recently shown that TIEG2/KLF11 plays a role in endocrine β-cell function, and that rare and frequent genetic KLF11 variants cosegregate with early onset familial diabetes or are associated with late-onset T2D, respectively [3]. KLF11 is also known to regulate exocrine cell growth and behaves as a tumour suppressor in pancreatic cancers [4]. Interestingly, TGF-β induces KLF11, and KLF11 represses the SMAD7 gene [5]. Thus, up regulation of KLF11 may create a feed-forward loop to TGF-β signalling. It is possible that the contribution of aberrant KLF11 function to T2D susceptibility depends on its transcriptional regulation of SMAD7. Since i-smad7 over expression is diabeticogenic [1], we assessed whether the SMAD7 gene could also harbour genetic variants associated with T2D.

The TGF-β induced transcription factor TIEG/KLF10 is highly homologous to KLF11 and may have similar functions [6,7]. Members of the KLF transcription factor family have three homologous SP1-like DNA-binding domains. Moreover, both KLF10 and KLF11 have three similar, evolutionary conserved, repressor domains that are involved in co-factor binding [6]. In addition, KLF10 has also been shown to repress transcription of the SMAD7 gene [8]. However, both the knockout mouse model of KLF11 and of KLF10 showed no apparent pathological defects of pancreatic function under basal conditions [9,10]. If KLF10 and KLF11 affect transcriptional regulation of the same set of genes (including SMAD7), we may expect they contribute similarly to the development of T2D, and that genetic variants of the KLF10 gene could also be associated with T2D.

To test our hypothesis, we assessed the contribution of SMAD7 and KLF10 gene variants in genetic susceptibility to T2D. We screened the promoter regions, UTRs, coding and flanking intron sequences of these genes to identify frequent and rare variants. We then analyzed frequent variants in a two-stage case-control study and analyzed rare variants in their isolated families.

2. Materials and methods

2.1. Study population

Frequent variants were analyzed in an initial case-control study from a French Caucasian cohort that included 349 unrelated T2D patients with at least one affected first-degree relative (185 men/163 women, age at T2D diagnosis 45 ± 11 years, BMI 26.8 ± 3.8 kg/m²) and 349 unrelated normoglycaemic spouses (128 men/221 women, age at examination 59 ± 13 years, BMI 26.1 ± 5.0 kg/m²) recruited by the “Centre National de la Recherche Scientifique” Unit 8090 in Lille. The second set consisted of 1.712 T2D patients (1049 men/663 women, age at T2D diagnosis 49 ± 10 years, BMI 30.0 ± 4.5 kg/m²) recruited at the Endocrinology-Diabetology Department of the Corbeil-Essonne Hospital (Paris region), and 1.072 normoglycaemic control subjects from the general population cohorts D.E.S.I.R. (West-Central France; [11]) and MONICA-Lille (Northern France; [12]) (433 men/639 women, age at examination 54 ± 6 years, BMI 23.9 ± 2.9 kg/m²).

2.2. SNP selection and genotyping

To identify genetic variants in SMAD7 and KLF10, we sequenced the promoter regions (~1.5 Kb), UTRs, coding and flanking intron sequences from both genes (ABI Prism 377 DNA sequencer) in 124 DNA samples from unrelated individuals (32 T2D subjects and 92 probands from early onset T2D families). The identification numbers of SNPs used in this study as well as the PCR primers used to amplify SNP-containing fragments are listed in Supplementary Table 1. Three KLF10 SNPs were genotyped in DNA pools using pyrosequencing™ technology as previously described by Neve et al. [13] (Pyrosequencing AB, Uppsala, Sweden), one SNP by TaqMan® SNP Genotyping Assays (gift of TJ Woodage, Foster City, CA USA) and four SNPs by SNPllex assays (Applied Biosystems, Foster City, CA, USA). The others variants were...
genotyped using Lightcycler technology, which measures differences in melting curves based on fluorescence resonance energy transfer (Roche, Meylan, France). Genotypes were validated by a second independent reading and by 1% duplicate genotyping. There were no discrepancies in the genotype scores. With one exception (Table 2), allele distributions for all frequent variants conformed to Hardy-Weinberg equilibrium (HWE) \((P > 0.05)\); according to the Finetti program at http://linkage.rockefeller.edu/soft). For the variant in HWE-disequilibrium, the genotypes were further validated by automated DNA sequencing (DNA Analyser, Applied Biosystems, Courtaboeuf, France).

2.3. Statistical analysis

Common variants were analyzed in an initial case-control study of 698 subjects, which has a power of 76% to detect association with an Odds Ratio (OR) of 1.4 and a minor allele frequency (MAF) of 0.20 at a significance level of \(P = 0.05\) (calculated with the PAWE program (Power for Association With Errors; [14])). For variants with a MAF below 0.05 the statistical analyses were omitted as the power of this study would then drop to 33%. Variant allele frequencies of normoglycaemic and T2D subjects were compared by a Chi-2 test using the Finetti program (http://linkage.rockefeller.edu/soft). SPSS software was used for general statistical analysis (Chicago, IL, USA). We analyzed variants showing a significant T2D association under an allelic, dominant or recessive model \((P \leq 0.05)\) in the second set of 2.784 subjects, which has a power of 99% to detect an OR of 1.4 with a significant level of \(P = 0.01\) and a MAF of 0.20. The OR and the 95% confidence interval (CI) reported were calculated using the Mantel-Haenszel Test [15].

2.4. Reporter assays

The KLF10 variant Ala316Thr construct was generated by subcloning a PCR-amplified DNA fragment from the proband.

### Table 1

<table>
<thead>
<tr>
<th>Identified SNPs</th>
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<th>T2D</th>
<th>Chi-test P-value</th>
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Per SNP is represented: number of subjects carrying two major (NN), heterozygous (NM) or two minor alleles (MM); total number of subjects genotyped (for rare variants an additional 92 early onset T2D subjects were included); MAF, minor allele frequency; Chi-test \(P\)-value for differences between control and T2D subjects of allele frequencies, and *of genotype frequencies under a recessive model; §For variants with a MAF below 0.05 the statistical analysis was omitted; pool, MAF measured in a pool of DNA samples from 211 T2D and 309 normoglycaemic subjects by pyrosequencing.
with the variant sequence (BamH1-site forward primer 5′-AAAGGATCCATGGTCCAACCTTGGGTGC and XbaI-site reverse primer 5′-AATCTAGATGTGACTCCTTATGC) in the pcDNA-constructs previously described [16]. All constructs were sequenced to confirm the correct sequence of the inserts (automated sequencing, Applied Biosystems, Foster city, CA). Then, MIN6 cells were transfected with the constructs using TransFast™ Transfection (Promega, Charbonnieres, France). The KLF10 wild type or variant construct was cotransfected with the human insulin promoter (gift of Dr. D. Melloul, Hadassah University Hospital, Jerusalem, Israel) as previously described [3] and after 48 h luciferase activity was measured using the luciferase assay system (Promega) according to the manufacturer’s instructions.

3. Results

We first analyzed the genetic variants of the inhibitor SMAD7 gene by sequencing the DNA of 124 subjects. We identified ten exonic and 3′-UTR SMAD7 variants with a MAF below 0.05, but the SMAD7 exons harbored no frequent coding variants (Table 1). Three frequent variants identified at proximal intron sequences and three frequent variants located in linkage disequilibrium blocks of SMAD7 (SNP browser 3.0, Applied Biosystems) were analyzed in our first set of case control subjects. Of these six variants, IVS2-21C>T showed a significant association with T2D under a recessive model (P = 0.004, OR = 0.25, 95% C.I. = 0.09–0.70). In the second set, the number of subjects carrying two minor alleles of this variant also tended to be lower in the T2D group than in the normoglycemic control group (Table 2; P = 0.08; TT genotype frequency = 0.03 vs. 0.05, respectively). Analysis of the combined set 1 and 2 showed a significant association with T2D (Table 2; P = 0.007, combined OR = 0.62, 95% C.I. = 0.44–0.88). We analyzed 12 frequent SNPs in our study (including six of SMAD7) and the P-value after Bonferroni adjustment is 0.082. After adjusting for age, sex and BMI in a logistic regression model SMAD7 IVS2-21C>T contributes to the association with P = 0.034.

The ten rare exonic and 3′-UTR SMAD7 variants that we identified were further analyzed for cosegregation with T2D in the pedigrees of the proband families. However, none of these variants showed an apparent segregation with T2D (data not shown). In addition, we analyzed all of the less frequent variants in the SMAD7 gene by creating a binary parameter coding for presence or absence of one of these variants. We did not observe a significant difference between the normoglycemic and T2D subjects (frequencies of 0.056 vs. 0.064, respectively).

Next, we screened for genetic variants of the KLF10 gene. We identified six frequent variants, which we genotyped in the first set of T2D patients and normoglycemic subjects (Table 1). The variant KLF10 3′-UTR +1002 A>C (rs6935) showed a significant association with T2D under an allelic model (P = 0.01, OR = 0.75, 95% C.I. = 0.60–0.94) and a trend for association under a recessive model (P = 0.07, OR = 0.71, 95% C.I. = 0.48–1.04). In the second set, this variant also trends for association under a recessive model; the number of subjects carrying two minor alleles of this variant tended to be lower in the T2D group than in the normoglycemic control group (Table 2, P = 0.04, MAF = 0.26 vs. 0.22, respectively). In the combined sets, we observed T2D association also under a recessive model (P = 0.009, P = 0.11 with Bonferroni correction, combined OR = 0.81, 95% CI = 0.69–0.95). After adjusting for age, sex and BMI in a logistic regression model KLF10 3′-UTR + 1002 A>C contributed to the association with P = 0.042. The combined analysis of CC-allele carriers of the KLF10 variant with TT-allele carriers of SMAD7 IVS2-21C>T did not strengthen the evidence for T2D association, suggesting these gene variants do not interact.

The KLF10 screen also identified five rare variants (Table 1). Two of them were observed exclusively in diabetic subjects and were further analyzed in the family pedigrees. The non-synonymous variant His129Leu (+386 A>T) was present in two probands but did not cosegregate in the one informative family we analyzed (data not shown). The second rare non-synonymous variant Ala316Thr (+946 G>A) was found in two unrelated families with early onset T2D (Fig. 1a). In the most informative family, this variant, located in the conserved repressor domain 3, partly co-segregates with T2D. Indeed, Ala316Thr was observed in five T2D, one glucose-intolerant and three normoglycemic subjects in this family. However,
one T2D subject did not carry the variant allele. Similar to the effects of KLF11 [3], in vitro transfection of pancreatic β-cells (MIN6) with KLF10 pcDNA3.1-constructs evokes a two-fold activation of the preproinsulin promoter construct (Fig. 1b). However, 316Thr-KLF10 had an impaired capacity to activate this promoter, suggesting the Ala316Thr variant of KLF10 may indeed have functional implications.

In order to better cover the two loci we studied, we also analyzed the data we previously generated in our recently published genome wide association study [17]. Nineteen frequent intronic/intragenic database SNPs located in the SMAD7 and KLF10 regions have been genotyped in this study. None of these SNPs showed a significant association with T2D in this French population (data not shown), suggesting there are no variants that significantly contribute to T2D risk outside the regions we sequenced.

4. Discussion

We assessed the contribution of SMAD7 and KLF10 gene variants to the T2D genetic susceptibility in a two-stage design case control study. The modest size of the first set obviously could have limited our ability to detect and select variants with a weak but true association with T2D, for further analysis in set 2. However, offset 1 allowed us to study T2D patients with affected first degree relatives and controls sharing the same environment, which probably increased our power to detect an association with T2D. We may suppose that the second set has the same genetic background as it came from the same topographic region, although the T2D patients were not selected from T2D families.

The combined analysis of the case/control studies showed two variants with modest T2D association. One intronic variant amongst the SMAD7 gene variants, IVS2-21 C>T, showed an association with T2D under a recessive model. The overall genetic effect size is small because of the rarity of the TT genotype; however, the OR of 0.62 suggests that the biological effect of the genotype, for those few people who carry it, may be strong. This variant may affect the binding of the splicing factor SRp40 according to the predictions of the ESEfinder program (at CSH: http://rulai.cshl.edu/cgi-bin/tools/ESE/esefinder.cgi), however, no alternative splicing has previously been reported for the SMAD7 gene.
Furthermore, none of the rare variants cosegregated with T2D suggesting that the SMAD7 gene variants do not play a major role in the genetic susceptibility to early onset T2D. SMAD7 inhibits TGF/β-Smad-signaling, but may also inhibit Wnt/β-catenin signaling as reported for the skin differentiation program [18]. The transcription factor TCF7L2 of the Wnt/β-catenin signaling pathway is a major contributor to genetic susceptibility for the development of T2D [17,19] and therefore it could be interesting to assess the effect of SMAD7 on TCF7L2 signaling.

Our genetic analyses of the KLF10 gene identified a frequent variant in the 3′-UTR + 1002 A>C with a modest association with T2D. According to the DIANAmet program [20], the folding structure of the mRNA under minimal free energy may be different in the presence of the variant allele, but further functional studies are needed to determine if this variant affects stability of KLF10 mRNA levels. In addition, the causal association may be based on linkage disequilibrium with other intronic variants not analyzed by us, or by the HapMap consortium. The rare non-synonymous variant 316Thr-KLF10 that partly cosegregated with T2D, seemed to affect KLF10 capacity to stimulate the insulin promoter in vivo. It is unclear if KLF10 could also regulate this promoter in vivo. Interestingly, we have previously shown that the KLF11 variant, Ala347Ser, also located in the conserved repressor domain, segregated with early onset T2D. Interactions of these repressor domain 3 regions with specific cofactors are currently unknown, but it is likely that KLF10 and KLF11 bind the same factors, suggesting that they might compete for binding. It will be interesting to analyze these cofactors, once identified.

We observed no evidence for an interaction between the two T2D-associated SMAD7 and KLF10 gene variants. Effects of TGF-β signaling mediated either by KLF11, KLF10 and SMAD7 may largely depend on the relative status quo of several signaling pathways involved in β-cell function. In the transgenic mice over-expressing SMAD7 in pancreatic cells (Pdx1-tTA/SMAD7) and developing T2D, the level of KLF11 mRNA expression might be increased compared to control mice (3.3 ± 0.2 vs. 2.2 ± 0.3 relative units; 1), which might suggest KLF11 is implicated. The T2D association of the KLF10 and SMAD7 variants was modest compared to the association previously observed for non-synonymous KLF11 variants [3]. To which extent the frequent Gln62Arg KLF11 variant contributes to diabetes risk remains to be assessed as recently the association with T2D was not confirmed in a European-derived population [21]. Thus, it is unclear whether the potential effects of SMAD7, KLF10 and KLF11 gene variants on TGF-β signalling are pathogenic mechanisms that lead to T2D. Since KLF11 may directly interfere with insulin promoter activity [3], we believe that direct target gene regulation by KLF11 also interferes with the pathogenic mechanisms involved in T2D. Therefore, characterization of KLF11 and KLF10 specific target genes is required.

In conclusion, the observed potential T2D association for SMAD7 and KLF10 gene variants suggests they might weakly contribute to a particular genetic background that increases the susceptibility for the development of T2D.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi10.1016/j.diabet.2007.06.002.

References


