LETTER

Redundant enhancers and causal variants in the *TCF7L2* gene

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The *TCF7L2* gene is the locus with the strongest association with risk of type 2 diabetes (T2D) in different populations.^{1,2} The rs7903146 single-nucleotide polymorphism (SNP), located in intron 3 and the one with the strongest association to T2D risk, has been the most widely studied *TCF7L2* variant. Current evidence suggests that rs7903146 may affect risk of T2D through its effects on enhancer activity^{3,4} resulting in higher *TCF7L2* gene expression.^{3–5} The rs7903146 SNP has been found located inside islet-selective open chromatin sites using either formaldehyde-assisted isolation of regulatory elements coupled with high throughput sequencing (FAIRE-seq),³ or histone H3 lysine methylation modifications as marks of regulatory elements.⁴ In addition, the T-allele of rs7903146 (ie, the allele associated with higher risk of T2D) has been found associated with higher *TCF7L2* gene expression in human pancreatic islets,⁵ and in luciferase reporter assays in the mouse pancreatic MIN6 cell line,^{3,4} and the rat pancreatic 832/13 cell line.³ More recently Savic *et al*^{6,7} reported that the *TCF7L2* gene harbors several highly conserved *cis*-regulatory elements that show both *in vitro* and *in vivo* enhancer activity. These enhancer elements are located in a 92-kb interval that harbors all the *TCF7L2* SNPs, not just rs7903146, associated with T2D risk. The physical overlap between multiple enhancer elements and *TCF7L2* variants suggests that this gene may carry more than one causal variant related to the development of T2D. The present letter discusses the implications of these recent findings and presents possible mechanisms in how genetic variants in the *TCF7L2* gene may affect the development of T2D.

Causal variants in *TCF7L2*

Although the 92-kb enhancer-rich region in *TCF7L2* harbors several SNPs that have been found associated with risk of T2D, it is commonly assumed the presence of a single causal variant in the region.^{8–10} However, a critical assessment of published results does not rule out the presence of an independent secondary signal. Helgason *et al*⁸ carried out haplotype analysis of SNPs, rs7903146 and rs12255372, and the microsatellite DG10S478 (ie, the three markers that were initially found with the strongest association with T2D¹¹) in Danish and Icelandic populations, and concluded that rs7903146 is the most likely causal variant. However, this same study also did single SNP analysis in West African groups, and although rs7903146 was indeed the marker with the strongest association with T2D (odds ratio (OR) = 1.45, 95% confidence interval (CI) = 1.19–1.77), the rs12255372 showed a weaker but still significant association with T2D (OR = 1.31, 95% CI = 1.01–1.69). Given the nonexistent linkage disequilibrium (LD) between both SNPs in West African

populations (YRI $r^2 = 0.003$), these results are consistent with the presence of at least two different causal variants in the *TCF7L2* gene; a stronger signal tagged by rs7903146 and a weaker signal tagged by rs12255372. In contrast, the high LD between these two SNPs in European ancestry populations (CEU $r^2 = 0.70$) would mask the presence of the secondary signal. Palmer *et al*⁹ in a recent re-sequencing study of a small region of 4.3 kb region in the *TCF7L2* gene in African Americans proposed rs7903146 as the causal T2D polymorphism. However, the re-sequenced region did not cover rs12255372 that is 50.5 kb away from rs7903146. It is worth noting that the same research group in an earlier study found both rs7903146 (OR = 1.51, 95% CI = 1.27–1.80) and rs12255372 (OR = 1.20, 95% CI = 1.00–1.43) being associated with risk of T2D in African Americans.¹² Because these two SNPs are not correlated in African Americans (ASW $r^2 = 0.01$), these results also suggest the presence of at least two causal variants rather than a single causal variant. Maller *et al*¹⁰ used Bayesian-based fine mapping in the Wellcome Trust Case Control Consortium (WTCCC) GWAS and estimated that 75% of the posterior weight of association in *TCF7L2* rests on rs7903146. However, WTCCC data are inadequate to identify a secondary signal even if there is one present, given the high LD in the *TCF7L2* gene in European populations. In point of fact, in the only three genes where the WTCCC study found evidence of additional independent signals for T2D, the secondary SNP was in low LD with the index SNP; *FTO* (index rs17817449 and secondary rs8063946, CEU $r^2 = 0.051$), *CDKN2A* (index rs12555274 and secondary rs10965250, CEU $r^2 = 0.067$), and *CDKAL1* (index rs7756992 and secondary rs6456360, CEU $r^2 = 0.006$). Additional evidence of the relevance of rs12255372 independent from rs7903146 was reported by Parra *et al*¹³ who found that rs12255372 (OR = 1.78, 95% CI = 1.11–2.88) shows a stronger association with T2D in Hispanics relative to rs7903146 (OR = 1.39, 95% CI = 0.89–2.17). Because the minor allele frequency (MAF) of rs12255372 was lower (11.6%) than the MAF of rs7903146 (16.1%) in the studied Hispanic population, the stronger association of rs12255372 cannot be attributed to higher statistical power compared with rs7903146. At last, a recent study found that rs12255372 but not rs7903146 interacts with dietary fat intake in relation to changes in body fat composition.¹⁴ In summary, these results do not rule out the role of rs12255372 as an independent secondary signal in the *TCF7L2* gene.

Alternative use of redundant enhancers

In addition to the high LD that may be masking additional signals in *TCF7L2* another possibility should be considered, namely that *TCF7L2* enhancers may be redundant and they may show person-to-person variation on their use. Although the actual number of enhancer elements in the *TCF7L2* gene is still unclear, multiple enhancers that show overlapping activity in several cell lines and tissues are present in this region.^{6,7} These observations raise the question whether some of those enhancer elements could be showing functional redundancy, as it has been shown for other genes with multiple enhancers.^{15,16} The Encyclopedia of DNA Elements (ENCODE) project has recently depicted a complex picture of chromatin interactions, where on average 3.9 enhancer elements physically interact with 1 transcription start site (TSS) and 2.5 TSSs physically interact with 1 enhancer element.¹⁷ The occurrence of a multitude of enhancer–TSS interactions raises the possibility that some of those interactions are interchangeable (ie, redundant) and that enhancer functional redundancy may be a general feature of gene

expression regulation. Whether this is the case or all these enhancer-TSS interactions are strictly necessary for normal gene expression is an open question that requires an answer.

Person-to-person variation in the use of redundant enhancers may add a layer of complexity to gene expression regulation and risk of disease. Recent evidence suggests the presence of inter-individual variation in the use of alternative promoters^{18,19} and even usage differences between ethnic groups.²⁰ The author recently proposed the hypothesis that person-to-person variation in the use of alternative regulatory elements may extend to both gene promoters and enhancers.²¹ This hypothesis predicts that, in presence of inter-individual variation in the use of redundant regulatory elements (eg, gene promoters and enhancers) and if functional SNPs are located inside those elements, the observed effects of these functional variants would be attenuated by a factor equal to the proportion of chromosomes in the population using their respective regulatory element.²² Kasowski *et al*²³ have

recently reported the presence of considerable inter-individual variation in chromatin states as assessed by histone modifications, and binding of cohesin and CTCF. In particular, they found person-to-person variation in enhancer activity without major changes in gene expression,²³ confirming the hypothesis of inter-individual variation in the use of redundant enhancers. It is noteworthy that rare variants (allele frequency <1%) seems to explain in part this variation on enhancer use.²³ Whether *TCF7L2* enhancers also show this type of variability is still unknown.

Table 1 shows the 28 elements tested for enhancer activity by Savic *et al*^{6,7} in the *TCF7L2* gene. Positions of the different constructs were obtained by blasting construct primer sequences to the reference human genome sequence version 19. It is worth noting that (a) SNPs associated with T2D risk are either inside or surrounded by elements with enhancer activity, (b) some elements with enhancer activity do not harbor any reported SNP associated with risk of T2D, even

Table 1 Position of the 28 conserved elements in the *TCF7L2* gene (chromosome 10) assessed by Savic *et al.* for enhancer activity

Element ^a	Start_hg19	End_hg19	Enhancer activity		SNPs with MAF ≥ 1% ^b	T2D SNPs
			<i>In vivo</i>	<i>In vitro</i>		
1*	114,732,126	114,738,118	χ ^c		24	
2*	114,738,098	114,743,939			20	
3*	114,743,762	114,749,675	χ ^d		22	
4	114,749,526	114,751,619		χ ^e	9	
5*	114,751,369	114,752,970	χ ^f	χ ^{e,g,h,i}	7	
6	114,752,830	114,757,031			15	rs7901695, rs4506565
7*	114,756,892	114,761,317	χ ^j	χ ^h	14	rs7903146
8	114,761,177	114,763,507			7	
9	114,762,605	114,766,935			10	
10*	114,766,763	114,772,697	χ ^k		19	
11	114,772,333	114,774,613			9	
12*	114,774,599	114,775,647			1	
13	114,775,077	114,777,888			7	
14	114,777,595	114,782,410		χ ^{e,g,h,i,l}	21	
15	114,781,831	114,783,202		χ ^l	9	
16*	114,783,097	114,788,917	χ ^m		24	
17	114,788,643	114,793,365			12	
18	114,793,343	114,796,279		χ ^{h,l}	9	
19*	114,796,072	114,797,988	χ ⁿ		7	
20	114,797,572	114,800,251			7	
21*	114,799,764	114,803,427		χ ^e	6	rs7895340
22*	114,803,305	114,804,588	χ ^j	χ ^{e,g,h,i}	3	
23	114,804,162	114,809,085			16	rs12255372, rs11196205
24	114,808,760	114,813,606			12	
25	114,813,428	114,818,344		χ ^{e,g,h,i}	17	
26*	114,818,226	114,821,745			12	
27	114,821,559	114,824,769			14	
28*	114,823,386	114,825,149	χ ^f		6	

^aThe *in vivo* scan analyzed only 13 elements, those marked by an *; the *in vitro* scan assessed all the twenty eight conserved elements.

^bNumber of SNPs with minor allele frequency ≥ 1% in the combined populations from 1000 genomes project.

^cStomach.

^dSpinal neurons.

^eNeuro-2a cell line.

^fBone.

^gHCT-116 cell line.

^hC2C12 cell line.

ⁱU2OS cell line.

^jBrain.

^kWalking pads.

^lMIN6 cell line.

^mLimb vasculature.

ⁿBrain vasculature.

though they contain polymorphic variants, and (c) elements with enhancer activity harboring T2D risk variants are located on both ends of the 92-kb region. Although the rs12255372 SNP (ie, the one the present letter proposes as an secondary signal) is not located inside any of the elements with enhancer activity reported by Savic *et al.*,^{6,7} chromatin segmentation analysis using the HaploReg software version 2²⁴ with data from the NIH Roadmap Epigenomics Mapping Consortium (<http://www.roadmapepigenomics.org/>) shows that rs12255372 is located inside elements with chromatin mark characteristics of active enhancers in 10 different cell types and weak enhancers in 20 different cell types (Table 2). It is noteworthy that some of the tissues represented by these cell lines (eg, the adipose, pancreas, gastric, and brain) are involved in glucose metabolism. The rs7903146 is located inside elements with chromatin mark characteristics of promoter activity in

Table 2 Regulatory chromatin states in different cell lines from the NIH Roadmap Epigenomics Mapping Consortium around SNPs rs7903146 and rs12255372 in the *TCF7L2* gene

Chromatin states in different cell lines				
SNP	Promoter activity	Weak promoter activity	Enhancer activity	Weak enhancer activity
rs7903146	ADI.MSC	PANC.I	ADI.NUC	BN.HM150
			HD.CD184EC	BR.MYO
			HUES48	COL.SMUS
				LNG.FE
rs12255372				ST.MUC
			ADI.NUC	ADI.MSC
			BN.AG	BM.MSC
			BN.HM150	BN.AC
			BN.SN	BN.FE2
			HD.CD56MESC	BR.MYO
			H1.DMSC	DUO.SMUS
			MSC.ADIPC	ES.I3
			NCC.COR2	GAS
			PFF.1	HUES6
			PFF.2	HUES64
				H1.BMP4DT
				H1.DNP
				H9
				IPS.DF6
				IPS.DF19
				IPS.15
				IPS.18
				IPS.20
				NCC.GED2
				PANC

Abbreviations: ADI.MSC, adipose-derived mesenchymal stem cell; ADI.NUC, adipose nuclei; BM.MSC, bone marrow-derived mesenchymal stem cell; BN.AC, brain anterior caudate; BN.AG, brain angular gyrus; BN.FE2, fetal brain donor HuFNSC02; BN.HM150, brain hippocampus middle donor 150; BN.SN, brain substantia nigra; BR.MYO, breast myoepithelial cells; COL.SMUS, colon smooth muscle; DUO.SMUS, duodenum smooth muscle; ES.I3, embryonic stem cell-I3; GAS, gastric; HD.CD184EC, hESC-derived CD184 + endoderm cultured cells; HD.CD56MESC, hESC-derived CD56 + mesoderm-cultured cells; HUES6, human embryonic stem cells-6; HUES48, human embryonic stem cells-48; HUES64, human embryonic stem cells-64; H1.BMP4DT, H1 BMP4-derived trophoblast-cultured cells; H1.DMSC, H1-derived mesenchymal stem cells; H1.DNP, H1-derived neuronal progenitor-cultured cells; H9, H9 cell line; IPS.DF6, induced pluripotent stem cells DF 6.9; IPS.DF19, induced pluripotent stem cells DF 19.11; IPS.15, induced pluripotent stem cells 15b; IPS.18, induced pluripotent stem cells 18; IPS.20, induced pluripotent stem cells 20b; LNG.FE, fetal lung; MSC.ADIPC, mesenchymal stem cell-derived adipocyte cultured cells; NCC.COR2, neurosphere-cultured cells cortex-derived donor HuFNSC02; NCC.GED2, neurosphere-cultured cells ganglionic eminence-derived donor HuFNSC02; PANC, pancreas; PANC.I, pancreatic islets; PFF.1, penis foreskin fibroblast primary cells donor skin01; PFF.2, penis foreskin fibroblast primary cells donor skin02; ST.MUC, stomach mucosa.

one cell line, weak promoter activity in one cell line, enhancer activity in three cell lines, and weak enhancer activity in five cell lines (Table 2). These results suggest that both rs7903146 and rs12255372 may affect risk of T2D through enhancer-mediated mechanisms. On the basis of the proposed hypothesis of inter-individual variation in the use of redundant enhancers, rs7903146 would show the strongest association with T2D in most of the studied populations because its respective enhancer would be the most frequently used. However, it is possible that in populations with a different usage of redundant enhancers other *TCF7L2* variants may also affect risk of T2D. Presence of additional functional SNPs is suggested by the recent functional analysis of *TCF7L2* polymorphisms by Pang *et al.* who found that five SNPs including rs7903146 showed allele-specific binding patterns in electrophoretic mobility shift assays. These results need *in vivo* confirmation as *in vitro* assays do not give a complete picture in how the complex chromatin structure inside cells affects binding of transcription factors. For example, Chakrabarti *et al.*²⁶ reported that *in vitro* immunoprecipitation assays overestimate the number of binding sites of the transcription factor Pdx1 in both β -cells and non β -cells as measured by chromatin immunoprecipitation. Nevertheless three of the *TCF7L2* SNPs assessed by Pang *et al.*²⁵ had allele-specific luciferase activity in both Huh7 (hepatocarcinoma) and WiDr (colorectal adenocarcinoma) cell lines (rs4132670), or in Huh7 cell line only (rs4506565, and rs12255372),²⁵ suggesting that these SNPs may have *in vivo* functional relevance. Although results from these two nonpancreatic tumor-derived cell lines must be interpreted with caution, they stress the need to assess *TCF7L2* SNP function in a variety of organs other than pancreas, as it has been shown that *TCF7L2* regulates glucose homeostasis through its action in a variety of nonpancreatic tissues such as the liver,^{27,28} gut,²⁹ and brain.³⁰

Testing the hypothesis of alternative use redundant enhancers in the *TCF7L2* gene

Testing the hypothesis of person-to-person variation in the use of redundant enhancers in the *TCF7L2* gene requires the ability of distinguishing active enhancers from poised/inactive enhancers. Recent work has shown that active enhancers are bi-directionally transcribed resulting in short (<2 kb) noncoding RNAs (enhancer RNAs or eRNAs).³¹ Zhu *et al.*³² reported that expression levels of eRNAs, and therefore enhancer activity, can be reliably predicted by assessing H3K27ac, H3K27me3, H3K79m1, and H3K9ac histone modifications (area under the curve (AUC)=0.94, and Matthew's correlation coefficient (MCC)=0.71), and the predictive model seems to be cell-type independent. These observations offer an approach to measure enhancer activity and to test the hypothesis that active enhancers vary from person to person. Preliminary chromatin segmentation analysis of the 92-kb enhancer-rich region of the *TCF7L2* gene using available data from different cells/tissues with more than one donor from the NIH Roadmap Epigenomics Mapping Consortium (<http://www.roadmapepigenomics.org/>) shows variability on chromatin states from tissue-to-tissue and from person-to-person for the same tissue (Supplementary Figure 1). It is noteworthy that the rs12255372 SNP (ie, the one proposed as a secondary signal) is located inside regions with weak enhancer activity in some of the tissues. A crucial next step should be the measurement of enhancer activity by assessing histone modifications in the whole range of tissues where *TCF7L2* is expressed. This assessment must move from the traditional 2-dimension approach enhancer x tissue to a more comprehensive 3-dimension scenario subject x enhancer x tissue (ie, to allow for inter-individual variation in the use of redundant enhancers).

Summary

TCF7L2 is the gene with the strongest association with T2D, and it is generally accepted that the rs7903146 SNP is the single causal variant in this locus.^{8–10} The present letter discussed published data from European and nonEuropean populations and postulated that an independent secondary signal tagged by the rs12255372 SNP is also present in the *TCF7L2* gene. The author also discussed recent results that show the presence of multiple enhancers in the *TCF7L2* gene,^{6,7} and advanced the hypothesis that these enhancers are redundant and there is person-to-person variability in their use. This hypothesis may explain in part why the rs12255372 shows a weaker association with T2D compared with rs7903146; namely rs12255372 would be located inside elements with weak enhancer activity and with less frequency of use. Preliminary analysis of available data from the NIH Roadmap Epigenomics Mapping Consortium (<http://www.roadmapepigenomics.org/>) seems to bring support to this hypothesis. A comprehensive assessment of enhancer functional redundancy in all tissues where *TCF7L2* is expressed may clarify the interplay between genetic variants and chromatin modifications, and how they jointly affect the development of T2D.

CONFLICT OF INTEREST

The author declares no conflict of interest.

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DISCLAIMER

The content is solely the responsibility of the author and does not necessarily represent the official views of the National Institute on Minority Health and Health Disparities, the National Cancer Institute, the National Institutes of Health, or the American Heart Association.

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