UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS: ENDOCRINOLOGIA

ALTERAÇÕES GENÉTICAS NO GENE DA IODOTIRONINA DESIODASE TIPO 2 E RESISTÊNCIA INSULÍNICA

Tese de Doutorado

Leonardo Barbosa Leiria

Orientadora: Prof[a]. Dr[a]. Ana Luiza Maia

Co-orientadora: Prof[a]. Dr[a]. Daisy Crispim Moreira

Porto Alegre, Novembro de 2012.

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2. D2 Thr92Ala and PPARγ2 Pro12Ala polymorphisms interact in the modulation of insulin resistance in type 2 diabetic patients. Obesity. 2011;19(4):825-832.
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Lista de abreviaturas e siglas

AP-1: Activator protein 1 – proteína ativadora 1

BAT: Brown adipose tissue – tecido adiposo marrom

BMI: Body mass index – índice de massa corporal (IMC)

BP: Blood pressure – pressão sanguínea

cAMP: Adenosina monofosfato cíclico

cDNA: DNA complementar

CREB: cAMP response element binding protein – proteina de ligação ao elemento de resposta ao cAMP

D1: Iodothyronine deiodinase type 1 - iodotironina desiodase tipo 1

D2: Iodothyronine deiodinase type 2 - iodotironina desiodase tipo 2

D3: Iodothyronine deiodinase type 3 - iodotironina desiodase tipo 3

Db/db mouse: Mouse model for diabetic dyslipidemia – modelo murino diabético

DIO2: Gene D2 em humanos

DM: Diabetes mellitus

DNA: Ácido desoxirribonucleico

ESECIS: Selenocysteine insertion sequence element – elemento de inserção de selenocisteína

GHb: Glycated hemoglobin – hemoglobina glicada

GLUT4: Glucose transporter 4 – transportador de glicose tipo 4

HCPA: Hospital de Clínicas de Porto Alegre

HOMA: Homeostasis model assessment – modelo de avaliação da homeostase

MCT: Monocarboxylate transporter – transportador monocarboxilado

mRNA: Messenger ribonucleic acid – ácido ribonucleico mensageiro

NF-kβ: nuclear factor kβ - fator de transcrição nuclear kappa beta

NTCP: Na^+ taurocholate cotransporting polypeptide – proteína cotransportadora de Na/tauroclorato

OATP: Organic anion transporting polypeptide – polipeptídeo transportador de ânions orgânicos independentes de Na

PAX8- Paired box 8 – homeobox 8

PPARγ: Peroxisome proliferator-activated receptor-γ – receptor ativado pelo proliferador de peroxissoma gama

PPRE: Peroxisome proliferator response element - elemento de resposta aos proliferadores de peroxissoma

RI: Insulin resistance - resistência à insulina

rT_3: T_3 reverso

RXR: Retinoid X receptor - receptor retinóide X

SBP2: SECIS binding protein 2 – Proteína 2 de ligação ao SECIS

SECIS: Selenocysteine insertion sequence – sequencia de inserção de selenocisteína

T_2: 3,5-diiodotironina

T_3: 3,5,3'-triiodotironina

T_4: Tiroxina

TDM2: Diabetes mellitus type 2 – diabetes mellitus tipo 2

TFF-1: Thyroid transcriptional factor 1 – fator de transcrição da tireoide

TNF: Fator de necrose tumoral

TR: Thyroid receptor - receptor do hormônio tireoidiano

TER: Elemento responsivo ao hormônio tireoidiano

TRβ: Thyroid hormone receptor β – receptor beta do hormônio tireoidiano

TRH: Hormônio liberador de tireotrofina

TSH: Thyroid stimulating hormone - Hormônio estimulador da tireoide humana (tireotrofina)

WHR: Waist-to-hip ratio – razão cintura-quadril

Resumo

Introdução. O hormônio tireoidiano na sua forma ativa (T_3) possui um importante papel no crescimento, desenvolvimento e no metabolismo dos organismos complexos. A iodotironina desiodase tipo 2 (D2) é uma selenoenzima responsável pela ativação do T_3. O polimorfismo de troca única (SNP) Tre92Ala (rs225014) no gene que codifica a D2 foi associado com resistência à insulina em algumas populações; porém, estudos funcionais *ex vivo* indicaram que esse polimorfismo não seria o fator causal para o desenvolvimento de resistência à insulina. Objetivos. Identificar outras alterações no gene da D2 que pudessem contribuir para o desenvolvimento de resistência à insulina em pacientes com diabetes mellitus tipo 2 (DM2) na presença ou não do polimorfismo rs225014 (Tre92Ala). Material e Métodos. A busca por SNPs que pudessem estar associadas com resistência à insulina foi realizada através do sequenciamento automático do gene *DIO2* em 12 pacientes com DM2 apresentando diferentes graus de resistência à insulina e 2 indivíduos não-diabéticos. Variantes potencialmente informativas foram estudadas em 1077 pacientes com DM2 e 516 indivíduos não-diabéticos. Além disso, foi verificado se os SNPs informativos encontravam-se em desequilíbrio de ligação e se a estrutura predita do mRNA estaria alterada nas variantes selvagens e polimórficas da D2. Resultados. Durante a varredura no gene *DIO2* foram identificados 5 polimorfismos candidatos: rs199598135, rs12885300, rs225014, rs225015 e rs225017. Entre estes, verificou-se que a frequência do genótipo TT do rs225017 era mais elevada naqueles pacientes com índices de HOMA-IR mais altos do que naqueles com índices de HOMA-IR baixo. A partir desses dados, foi realizado um estudo de associação entre a presença desse polimorfismo e o desenvolvimento de resistência à insulina e desenvolvimento de DM2. Pacientes com DM2 que eram

homozigotos para o alelo polimórfico (T/T) apresentaram um nível mais elevado de insulina plasmática em jejum (15,7 vs. 10,6; P=0.005) e índices de HOMA-IR (5,20 vs. 3,50; P=0,005) quando comparados com pacientes portadores do alelo A. O genótipo TT foi associado com aumento dos níveis de insulina e índice de HOMA-IR de forma independente e em combinação com o genótipo Ala92Ala (rs225014) (P=0,001 e P=0,010; respectivamente). No estudo de caso-controle, não foi verificada uma associação entre a presença do genótipo TT (rs225017) e o desenvolvimento de DM2 (OR ajustada = 1,15, IC 95% 0,86 – 1,55, P=0,354). No entanto, a presença combinada deste genótipo com o genótipo Ala92Ala (rs225014) foi associada a um maior risco de desenvolvimento de DM2 (OR ajustada = 1,70, IC 95% 1,11-2,61; P=0,015) do que somente na presença do genótipo Ala92Ala (OR ajustada = 1,59, IC 95% 1,10 – 2,31; P=0,010). Estudos adicionais demonstraram que os polimorfismos rs225017 e rs225014 estão em um forte desequilíbrio de ligação. A análise da estrutura secundária predita do mRNA da *DIO2* sugere uma interação entre os polimorfismos rs225017 e rs225014. Conclusões. Pacientes com DM2 homozigotos para o genótipo T/T do polimorfismo rs225017 apresentaram níveis mais severos de resistência à insulina. Essa associação foi mais acentuada na presença do polimorfismo rs225014, sugerindo uma interação entre estes polimorfismos na modulação da resistência à insulina.

Capítulo 1. O papel da iodotironina desiodase tipo 2 (D2) na resistência à insulina.

O papel da iodotironina desiodase tipo 2 (D2) na resistência à insulina.

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Resumo

Os hormônios tireoidianos são importantes na regulação do metabolismo. Alterações no status tireoidiano estão associadas com um aumento da resistência à insulina. A captação de glicose mediada pela insulina é realizada por transportadores tecido-específicos, sendo um dos principais fatores responsáveis pela homeostase glicêmica. O principal receptor expresso no músculo esquelético e tecido adiposo é o transportador de glicose do tipo 4 (GLUT-4). O gene GLUT-4 é induzido pela forma ativa do hormônio tireoidiano (T_3), em humanos e em modelos murinos, o que poderia explicar a associação entre o status tireoidiano e a resistência à insulina. Nos tecidos alvos o pro-hormônio tiroxina (T_4) é convertido em T_3, sobretudo pela iodotironina desiodase tipo 2 (D2). Em humanos, o polimorfismo rs225014 (Tre92Ala) no gene *DIO2* foi associado com aumento da resistência à insulina em diferentes populações, sugerindo que a D2 possa participar da regulação da homeostase glicêmica.

O metabolismo dos hormônios tireoidianos

Os hormônios tireoidianos são importantes na regulação do metabolismo, no crescimento e no desenvolvimento dos tecidos humanos e de outros organismos. A produção dos hormônios tireoidianos é regulada pelo eixo hipotálamo-hipófise-tireoide, onde os hormônios são secretados na corrente sanguínea e transportados aos tecidos periféricos sensíveis ao hormônio para realizar sua ação biológica nas células (Figura 1). A tiroxina (T_4) é o principal produto secretado pela tireoide e atua como um precursor inativo do hormônio tiroidiano, sendo necessária sua ativação biológica em triiodotironina (T_3), para que possa realizar sua ação genômica (1-3).

O principal regulador da função tireoidiana é o hormônio estimulador da tireoide (TSH), secretado pela hipófise em resposta ao hormônio liberador de tireotrofina (TRH) produzido pelo hipotálamo. O TSH estimula as células foliculares da tireoide a sintetizar e secretar os hormônios tireoidianos T3 e T4 (3, 4).

Os hormônios tireoidianos são regulados através de um mecanismo de retroalimentação (*feedback*) negativo via receptor do hormônio tireoidiano beta (TRβ) no hipotálamo e na pituitária, inibindo a expressão gênica do TRH, sem efeito sobre outros grupos neuronais hipotalâmicos, mantendo uma correlação fisiológica inversa entre a biossíntese hormonal tireoidiana e a secreção do TSH (3-5).

Após serem secretados na circulação sanguínea os hormônios tireoidianos são transportados para o interior celular através de transportadores tecido-específicos dependentes de energia, temperatura e gradiente de íons Na^+. No entanto, em sítios de baixa afinidade o transporte ocorre através da ligação do hormônio tireoidiano a proteínas carreadoras específicas de membrana, independente de gradiente eletroquímico ou energia. Os principais transportadores tecido-específicos do hormônio

tireoidiano são as proteínas cotransportadoras de Na/taurocolato (NTCP), polipeptídeos transportadores de ânions orgânicos independentes de Na (OATP) e os transportadores monocarboxilados (MCTs), dentre os quais o MCT8 é o transportador específico para o hormônio tireoidiano, com maior afinidade ao T_3 do que ao T_4 (6, 7). Dentro das células alvo os hormônios tireoidianos são metabolizados pelas iodotironina desiodases (8).

As iodotironina desiodases são selenoenzimas, proteínas que contêm em seu sítio ativo resíduos de selenocisteína, um aminoácido essencial para o funcionamento catalítico da enzima. As iodotironinas desiodases do tipo 1 (D1), tipo 2 (D2) e tipo 3 (D3) fazem parte da super família das oxirredutases e catalisam a remoção do iodo do anel externo (D1 e D2) ou interno (D1 e D3) dos hormônios tireoidianos. Tanto a D1 quanto a D2 convertem o pro-hormônio T_4 na sua forma biologicamente ativa T_3, enquanto que a D3, juntamente com a D1, inativa os hormônios T_4 e T_3, gerando T3 reverso (rT_3) e T_2, respectivamente (Figura 1) (2, 3, 8).

No núcleo, a forma ativa do hormônio (T_3) atua através da sua ligação a receptores nucleares (TRs) localizados em regiões específicas na região promotora dos genes alvos, denominados de elementos responsivos ao hormônio tireoidiano (TERs), modulando a expressão desses genes. Outros fatores de transcrição como o receptor retinóide X (RXR) e outros correguladores atuam juntamente com o T_3-TR nesse complexo mecanismo de regulação gênica (5, 8-10). Na ausência do T_3, os TRs ligam-se ao DNA na forma de monômeros, homodímeros e, preferencialmente, heterodímeros com o receptor RXR e a ligação de correpressores da expressão gênica. Na presença do T_3, este se liga aos TRs, o que acarreta em uma mudança conformacional que leva à remoção de proteínas repressoras e o recrutamento de proteínas coativadoras, juntamente com o RXR e proteínas acessórias induzindo, assim, a transcrição gênica (9, 10).

A iodotironina desiodase tipo 2

A D2 é a principal enzima responsável pela produção do T_3 circulante em indivíduos eutireoideos e no hipotireoidismo (1), tendo um importante papel na regulação do *feedback* negativo da produção do TSH hipofisário (8). Em humanos, cerca de 80% do T3 é produzido através da desiodação do anel externo da molécula de T_4 pela D2 (1, 2). A regulação da D2 é um complexo processo que envolve fatores hormonais, nutricionais, genéticos e ambientais, tendo no hormônio tireoidiano seu principal regulador (5, 11, 12).

A D2 possui um papel crítico na manutenção intracelular dos níveis de T_3 nos tecidos especializados tais como a hipófise, cérebro e tecido adiposo. Também é expressa na tireoide, nos músculos lisos da aorta e da artéria coronária, no coração, no músculo esquelético, na medula espinhal, na pele, na placenta, no fígado e no pâncreas, protegendo esses tecidos de um estado de hipotireoidismo (13-17). Além disso, a D2 também está associada com outros mecanismos que não os da produção de hormônios tireóideos, como a regulação da termogênese adaptativa, obesidade e o aumento da resistência à insulina (RI) (2, 13, 18-20).

Resistência à insulina (RI) e diabetes mellitus tipo 2 (DM2)

A resistência à insulina (RI) pode ser definida como um estado fisiológico onde é reduzido o efeito biológico de uma determinada concentração de insulina (21) ou como a incapacidade dos tecidos sensíveis à insulina responderem de forma eficiente à mesma (22). A insulina tem como principal função diminuir o nível de glicose no plasma mantendo a glicemia em valores normais. Na presença da RI, a normoglicemia é alcançada pelo aumento da secreção de insulina pelas células-beta pancreáticas, resultando em hiperinsulinemia. Entretanto, com o passar do tempo, ocorre uma "exaustão" na capacidade secretória das células-beta, fazendo com que a homeostase glicêmica no jejum não possa mais ser mantida. A forma clínica de DM2 é detectada quando os níveis de insulina não são mais suficientes para manter a glicemia normal no estado de jejum (22).

O mecanismo que desencadeia a RI não é bem conhecido, porém várias alterações em proteínas, lipídios, lipoproteínas e hormônios em diversos tecidos, e principalmente, no músculo esquelético, no tecido adiposo e fígado, parecem ter uma forte relação com o desenvolvimento da RI (21-23).

A RI apresenta uma etiologia multifatorial onde a interação de fatores ambientais e genéticos está relacionada com a origem e o agravamento da doença. Fatores ambientais como dieta, estresse e fumo são fatores de risco associados ao desenvolvimento da RI (24). A RI está associada a diversos fatores de risco cardiovasculares, hiperglicemia decorrente do DM2, obesidade, dislipidemias e hipertensão arterial, compondo assim um quadro chamado de síndrome metabólica (22, 24).

Estudos mostram que a prevalência da RI na população em geral vem aumentando progressivamente ao longo dos anos (22). Ela pode estar presente de forma transiente ou permanente durante a gravidez, estresse cirúrgico, traumas ou inanição, porém é mais prevalente em pacientes com DM2 (21, 22, 25). O espectro clínico associado à RI evolui progressivamente de hiperinsulinemia para intolerância à glicose e finalmente para DM2. Com a evolução da RI, outros problemas como a disfunção endotelial, a arteriosclerose e inflamações tendem a se agravar (26).

Diabetes mellitus (DM) é uma doença metabólica de etiologia múltipla caracterizada por hiperglicemia crônica devido a defeitos na secreção de insulina, na ação da insulina ou ambos (27-29). O diabetes mellitus tipo 2 (DM2) é observado em 90 a 95% dos casos de DM e representa uma questão de saúde pública mundial devido aos elevados custos, à crescente prevalência e à acentuada morbidade e mortalidade relacionadas as suas complicações (27-30). Esse tipo de DM se caracteriza inicialmente por um estado de hiperinsulinemia, em resposta à RI, sem alterações no controle glicêmico, e posteriormente por um estado de hipoinsulinemia gradual (27).

Em pacientes com DM2, a RI resulta na desinibição da gliconeogênese hepática e/ou na diminuição das taxas de glicose dentro das células. O transportador de glicose insulino-dependente (GLUT4) medeia o transporte de glicose no músculo esquelético e tecido adiposo. A expressão do GLUT4 é regulada positivamente através dos hormônios tireoidianos e a sua expressão aumentada em ratos db/db insulino-resistentes melhora o controle da glicemia em dietas hipercalóricas (31, 32), demonstrando o importante papel fisiológico dos hormônios tireoideanos na regulação da RI e no estado de DM2.

A desregulação lipídica e lipoprotéica está fortemente relacionada com o estado de RI. Defeitos no armazenamento e no metabolismo de ácidos graxos aumentam os níveis de insulina, modificando a expressão dos transportadores de glicose, o que resulta

em um estado de RI e lipólise. Juntamente com esse processo, moléculas inflamatórias são ativadas, como citocinas (interleucina-6) e adipocinas (fator de necrose tumoral alfa - TNF-α), diminuindo a sensibilidade à insulina através da modulação do metabolismo dos ácidos graxos e do GLUT4 (21, 22, 33). Além disso, o sistema neuroendócrino também contribui para o desenvolvimento da RI. Concentrações elevadas de cortisol, hormônio de crescimento e catecolaminas podem gerar um efeito antiinsulínico que resulta no aumento da glicose hepática (22).

Os hormônios tireoidianos e a homeostase do metabolismo glicêmico

Os hormônios tireoidianos possuem um papel importante na produção de glicose endógena, na gliconeogênese e na glicogenólise, na formação de lactato no músculo e no tecido adiposo; na produção do glucagon e na secreção de adrenalina no fígado, na concentração dos transportadores de glicose nos tecidos alvos e na concentração de ácidos graxos livres no plasma, alterando a homeostase celular da glicose (34, 35).

Pacientes com hipertireoidismo apresentam um aumento na eliminação hepática de glicose endógena e na captação de glicose mediada por insulina quando comparados com indivíduos eutireoideos (36-38). Por outro lado, pacientes com hipotireoidismo mostraram uma diminuição na absorção de insulina mediada por glicose, no tempo de eliminação de glicose no músculo e uma capacidade diminuída da insulina para estimular a eliminação glicose quando comparados com indivíduos com status tireoidiano normal (37).

Estudos em modelos animais demonstraram que na ausência dos hormônios tireoidianos ocorre uma diminuição na expressão do transportador de glicose insulino-dependente do tipo 4 (GLUT4) (39, 40). O GLUT4 é a principal proteína

transmembrana transportadora de glicose em diferentes tecidos e encontra-se associada a vesículas intracelulares nos tecidos musculares esqueléticos e cardíacos e no tecido adiposo (41). Em resposta à insulina estas vesículas são translocadas para a membrana plasmática e captam glicose intensamente nestes tecidos insulino-sensíveis (Figura 2).

O gene da iodotironina desiodase tipo 2 (*DIO2*) e a resistência à insulina (RI)

O gene que codifica a D2 (*DIO2*) está localizado no braço longo do cromossomo 14 na posição 14q24.3, sendo codificado por três éxons separados por dois íntrons de aproximadamente 7,4 kb. Ele é estruturalmente complexo e bem descrito, contendo regiões de interação com diversos fatores transcricionais e proteínas de ligação ao DNA (TFF-1, AP-1, NF-kβ), elementos de resposta ao cAMP (CRE) e sequências de inserção de selenocisteínas (SECIS). Além disso, possui uma complexa regulação pós-transcricional e pós-traducional, esse último via ciclo da ubiquitina (8).

Em humanos, foi demonstrado que polimorfismos nos genes das desiodases podem interferir na expressão destas enzimas (13, 14, 42, 43). Alguns estudos sugeriram que o polimorfismo rs225014 (A/G) no gene *DIO2*, que resulta na troca de uma treonina por uma alanina no códon 92 (Tre92Ala), está associado a uma redução de cerca de 20% na taxa de disponibilidade de glicose em mulheres e a uma elevada RI em pacientes com DM2 (13, 20, 44, 45); porém, outros estudos não encontraram tal associação (46, 47). É interessante observar que o polimorfismo rs225014 (Tre92Ala) tem sido associado com o aumento no risco de desenvolvimento de retardo mental, alterações no quociente de inteligência em regiões deficientes de iodo, DM2, Doença de Graves, osteoartrite, redução da massa óssea e maior *turnover* ósseo, hipertensão arterial e bem estar psicológico em resposta ao tratamento com T_3 ou T_4 (45, 47-52), sendo

essas associações independentes dos níveis séricos de hormônios tireoidianos. Adicionalmente, a frequência do genótipo polimórfico AlaAla (rs225014) é significativamente mais elevada em populações marcadamente com altos níveis de RI, como os índios Pima e mexicanos (20, 52).

O nosso grupo demonstrou que nos pacientes com o genótipo Ala/Ala do polimorfismo rs225014, a atividade da D2 (*ex vivo*) na tiroide e músculo esquelético é aproximadamente a metade daquela apresentada por indivíduos com os genótipos Tre/Ala ou Tre/Tre (13). A atividade reduzida da D2 poderia resultar na diminuição da geração de T3 no músculo esquelético, criando um estado de hipotiroidismo intracelular relativo, o que diminuiria a expressão de genes envolvidos no gasto energético, tais como o GLUT4, e consequentemente, levaria ao surgimento da RI (20) (Figura 2). No entanto, estudos funcionais *in vitro* de cinética e de atividade enzimática da D2 contendo o alelo mutado (92Ala) não demonstraram alterações significativas quando comparados com a cinética e atividades *in vitro* da enzima contendo o alelo selvagem (Tre92), sugerindo que embora o polimorfismo rs225014 possa ser um marcador para RI, ele não é o fator causal da RI (13).

Recentemente, demonstrou-se que o gene DIO2 apresenta na sua região promotora uma região de interação com o PPAR-γ, chamada de elemento de resposta aos proliferadores de peroxissoma (peroxisome proliferator response element – PRE), similar ao motivo de interação entre o PPAR-γ e o RXR (23, 44, 53). O PPAR-γ é membro de uma superfamília de receptores nucleares e fatores de transcrição ativados por ligantes e está envolvido na regulação transcricional de diversos genes relacionados ao metabolismo de lipídios e glicose, proliferação e diferenciação celular, inflamação, adipogênese e outros processos celulares também envolvidos na patogênese da RI e da DM2 (54).

Dois estudos demonstraram a associação entre a interação entre esse polimorfismo na D2 e o polimorfismo Pro12Ala (rs1801282) no PPAR-γ com o desenvolvimento da RI e síndrome metabólica (23, 44). Essa interação sugere que o PPAR-γ possa modular a expressão da D2 e que possa atuar conjuntamente nos processos biológicos e patológicos, indicando que alterações no gene da D2 possam ter um papel fundamental na etiologia da RI e na patogênese do DM2 (44).

Conclusão

Os hormônios tireoidianos possuem um papel fundamental no metabolismo glicêmico, regulando-o em diversos níveis. Alterações no status tireoidiano estão associadas com a RI a partir da captação deficiente de glicose mediada pela insulina dependente do GLUT-4. Visto que o gene do GLUT-4 é regulado pelas concentrações de T_3, a diminuição da atividade da enzima D2 poderia levar a um hipotireoidismo intracelular local, consequentemente, levando a uma alteração na expressão dos genes relacionados ao metabolismo, tais como o GLUT-4, o que poderia explicar em parte a relação entre o status tireoidiano e a RI. O melhor entendimento dos mecanismos moleculares que expliquem a associação entre a redução da atividade da D2, e consequentemente dos níveis de T_3, e o aumento da RI, sugerem um papel central do T_3 na fisiopatologia da RI e do DM2, e sinalizam a D2 e o T_3 como alvos terapêuticos potenciais para o desenvolvimento de novos fármacos.

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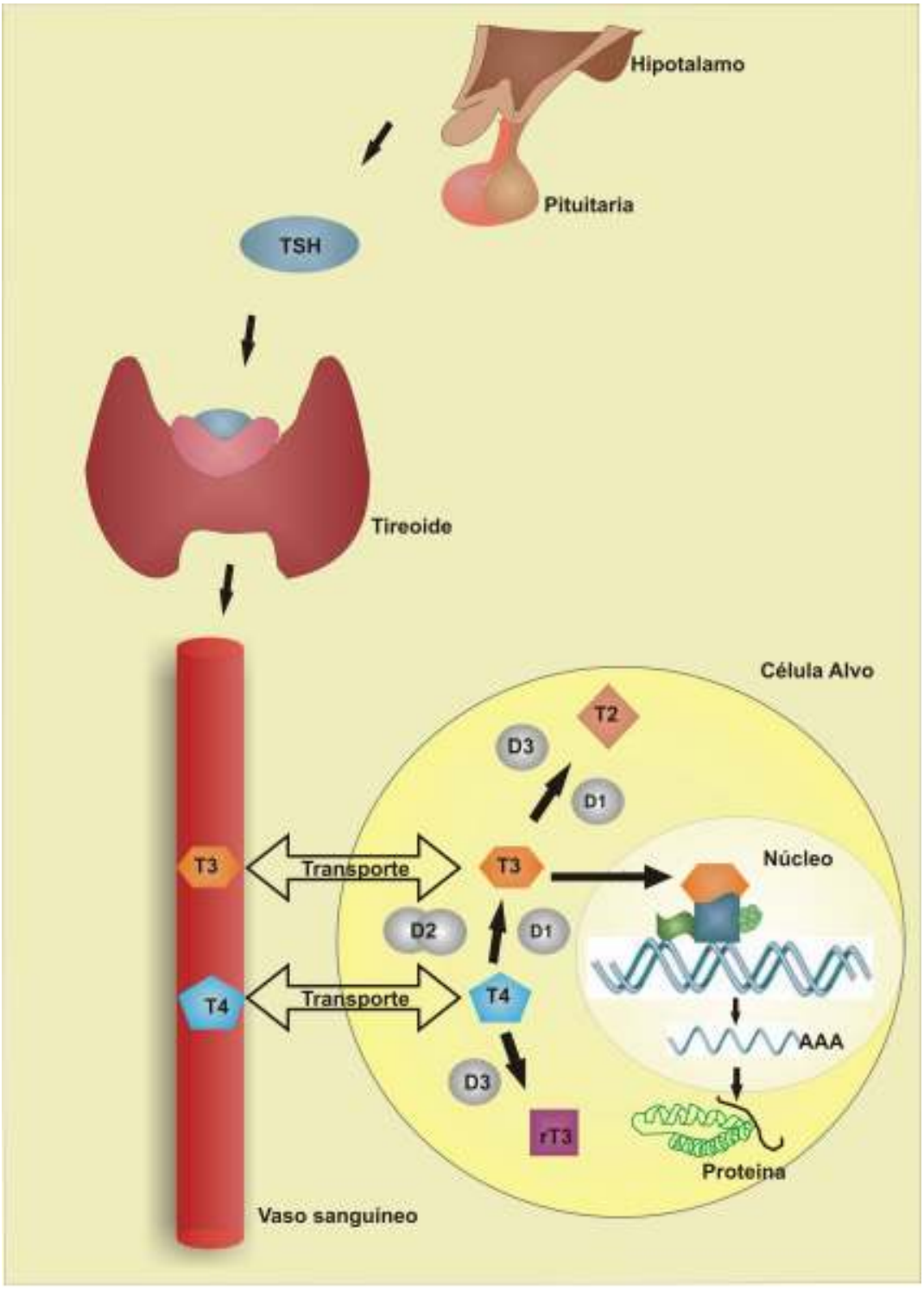

Figura 1. Metabolismo dos hormônios tireoidianos.

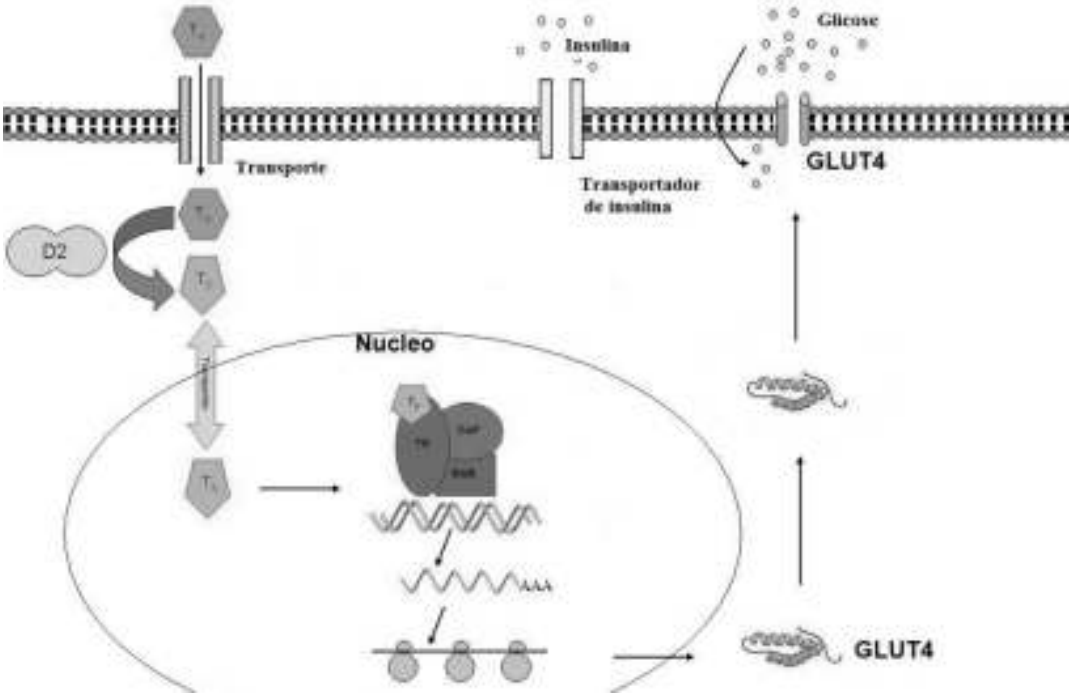

Figura 2. Mecanismo hipotético da regulação do transporte de glicose pelo hormonio tireoideano.

Capítulo 2. The rs225017 polymorphism in the 3'UTR of the human *DIO2* gene is associated with increased insulin resistance.

The rs225017 polymorphism in the 3'UTR of the human *DIO2* gene is associated with increased insulin resistance

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Running title: New polymorphism in *DIO2* is associated with insulin resistance

Key words: D2 polymorphism, insulin resistance, type 2 diabetes

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ABSTRACT

Background: The type 2 deiodinase (D2) enzyme has a key role in the activation of the prohormone thyroxine (T4) to the active hormone triiodotironine (T3). The Thr92Ala (rs225014) polymorphism in the gene codifying D2 (*DIO2*) has been associated with increased insulin resistance (IR) in both nondiabetic and type 2 diabetic (T2DM) subjects. Moreover, the Ala allele in homozygosis was associated with decreased enzyme activity in human tissues. Nevertheless, kinetic studies did not detect significant changes in the biochemical properties of the mutant enzyme, suggesting that this variant could only be a marker for abnormal *DIO2* expression. Objectives: To investigate whether other genetic polymorphisms in the *DIO2* gene may contribute to IR, individually or in combination with the Thr92Ala polymorphism. Methods: Initially, we sequenced the *DIO2* gene in 12 T2DM patients and 2 nondiabetic subjects. Potentially informative *DIO2* variants were evaluated in 1077 T2DM patients and 516 nondiabetic subjects. Haplotypes constructed from the combination of these variants with the Thr92Ala polymorphism were inferred using a Bayesian statistical method. Results: Screening of the *DIO2* gene did not show any mutations, although 5 already described polymorphisms were identified (rs199598135, rs12885300, rs225014, rs225015 and rs225017). Among them, the rs225017 (T/A) polymorphism was selected for further analysis. The frequency of rs225017 T/T genotype did not differ between T2DM patients and nondiabetic subjects (26.9% *vs*. 24.6%; P=0.452), even after adjusting for age and gender (OR=1.15, 95% CI=0.86-1.55; P=0.354). However, the frequency of patients carried T/T (rs225017) and Ala/Ala (rs225014) genotypes together were more elevated that frequency in nondiabetic patients (14% vs 9%; OR=1.70, 95% CI=1.11 –

2.61; P=0.015). The median fasting plasma insulin was significantly higher in T2DM patients with the rs225017 T/T genotype than in patients carrying the A allele (15.71 UI/ml *vs*. 10.57 UI/ml, adjusting P=0.013). Moreover, patients with the rs225017 T/T genotype displayed higher HOMA-IR index when compared with A allele carriers (5.20 *vs*. 3.50, adjusting P=0.002). The associations of the rs225017 T/T genotype with fasting plasma insulin and HOMA-IR were magnified in the presence of the Ala92Ala genotype (P=0.001 and P=0.01, respectively). Interestingly, further analysis for predicting the *DIO2* mRNA structure constituted by the rs205017 and Thr92Ala polymorphisms suggests an interaction between them. Conclusions: The rs225017 polymorphism is associated with greater IR in patients with T2DM, and it seems to interact with the Thr92Ala polymorphism in the modulation of IR.

INTRODUCTION

Thyroid hormones are critical to the development, growth and metabolism of virtually all tissues (1-3). The iodothyronine deiodinases types 1 (D1), 2 (D2) and 3 (D3) are selenoenzymes of an oxidoreductase family that catalyze iodine removal from the outer (D1 and D2) or inner ring (D1 and D3) of thyroid hormones. Whereas D1 and D2 convert T4 to the metabolically active hormone T3, D3 inactivates both T4 and T3 (2, 3). In humans, D2 is the most important deiodinase for intracellular T3 generation in target tissues (1, 3) and, together with D1, is responsible by 80% of peripheral T3 (4).

The gene that codifies D2 (*DIO2*) is expressed in thyroid, pituitary, brain, heart, placenta, skeletal muscle and adipocytes (5-10). D2 activity is tightly regulated at transcriptional (11, 12), post-transcriptional (12, 13) and post-translational levels (14, 15), thereby supporting the hypothesis that this enzyme plays an important homeostatic role in metabolism.

Type 2 diabetes mellitus (T2DM) is a heterogeneous group of disorders usually characterized by varying degrees of insulin insufficiency and insulin resistance (IR), which result in increased blood glucose concentrations (16). Ultimately, IR results either from inappropriately increased hepatic gluconeogenesis or decreased glucose disposal rate in tissues such as skeletal muscle, adipose tissue and liver (17).

It is well known that glucose homeostasis can be affected by thyroid status (18, 19). Thyroid hormone influences insulin action in skeletal muscle and adipose tissue in part by upregulating the expression of the glucose transporter 4 (GLUT4), and therefore increasing glucose uptake (20-22). An increase in both hepatic endogenous glucose disposal and insulin mediated-glucose uptake is observed in patients with hyperthyroidism as compared with euthyroid subjects (23-25). In contrast, studies in

hypothyroidism conditions show a decrease in both glucose disposal (24) and insulin mediated-glucose uptake in muscle, and also an impaired ability of insulin to stimulate glucose disposal related to insulinaemia (24, 26). In animal models, experimental induction or spontaneous forms of thyroid dysfunction are also associated with impaired glucose tolerance (26, 27). Mice with targeted disruption of *dio2* gene have higher insulin levels during glucose tolerance tests and reduced glucose uptake during insulin tolerance tests, consistent with the occurrence of IR (28). Taking these observations into account, it is plausible that a lower intracellular D2-generated T3 in skeletal muscle could create a state of relative intracellular hypothyroidism, decreasing the expression of genes involved in energy use, such as GLUT4, and thus resulting in increased IR (29, 30).

Previous studies have demonstrated that polymorphisms in the *DIO2* gene might interfere in the phenotypic expression of D2 (8, 31). Interestingly, a study described that a *DIO2* single-nucleotide polymorphism (SNP), in which a threonine (Thr) changes to alanine (Ala) at codon 92 (Thr92Ala, rs225014, A/G), was associated with IR in obese Caucasian women and with a 20% lower glucose disposal rate in nondiabetic Caucasian subjects (32). The Ala92Ala genotype was also associated with a more pronounced IR in T2DM patients, and with a decreased enzyme activity in tissue biopsy samples (8). Even though a number of other studies failed to demonstrate an association between the Thr92Ala polymorphism and glycemic traits or T2DM in some populations (33-35), the association between this polymorphism and risk for T2DM was reinforced by data from a recent systematic review and meta-analysis (36). However, the mechanism of reduced D2 activity in Ala92Ala subjects is still not clear since two studies failed to identify any changes in the *ex vivo* biochemical properties of the mutant enzyme (8, 37), suggesting that this variant could be only a marker for abnormal *DIO2* expression. Thus, it seems

reasonable to speculate that the lower D2 activity that justifies the association of the Thr92Ala polymorphism with IR is the result of a linkage disequilibrium between this polymorphism and a functional polymorphism yet to be identified elsewhere in the *DIO2* gene (30).

Therefore, the aim of this study was to determine whether other SNP's in the *DIO2* gene, individually or in combination with the Thr92Ala polymorphism, could contribute to the manifestation of IR phenotype.

MATERIALS AND METHODS

Study population

In the first phase of this study, we used direct automated sequencing of the *DIO2* gene to search for SNPs in 12 T2DM patients with different degrees of IR and in 2 nondiabetic subjects. These 12 patients had extreme values of Homeostasis Model Assessment-IR (HOMA-IR) index in our T2DM population and were selected from two subgroups according to their values: 6 patients with low HOMA-IR index (≤1.3, mean 0.79 ± 0.39) and 6 patients with high HOMA-IR index (≥12.9, mean 19.04 ± 5.35). In addition, the *DIO2* gene was also sequenced in 2 nondiabetic subjects randomly selected from the nondiabetic blood donors. All T2DM patients and nondiabetic subjects were self-defined as white. The results generated by sequencing the *DIO2* gene in T2DM patients were compared with those sequences of the nondiabetic subjects and with sequences of the human *DIO2* gene available in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). The sequences obtained for nondiabetic subjects were similar to those published in GenBank.

In the second phase of the study, allele and genotype frequencies of a potentially informative *DIO2* variant (rs225017 T/A) found by sequencing were compared between 1077 T2DM patients and 516 nondiabetic subjects. The criteria used for chose this variant for further analysis were: 1) the polymorphism was a known to be neutral variation; 2) it should involved change between chemically different aminoacids or was localized at regulatory regions such as promoter or 3'- unstranlated region (3'UTR); 3) it not had a rare frequency (minor allele frequency less than 5% in the literature or GenBank); and 4) the frequency of the variant should be different between analyzed groups (in this case, low and high HOMA-IR groups) (38-40). Additionally, haplotypes constructed from the combination of the rs225017 polymorphism with the Thr92Ala polymorphism were also compared between T2DM patients and nondiabetic subjects.

The 1077 patients with T2DM were selected from a multicenter study that started recruiting patients in Southern Brazil in 2002. The latter project sought to study risk factors for T2DM and its complications. Initially, it included four centers located at teaching hospitals in the Brazilian State of Rio Grande do Sul, namely Grupo Hospitalar Conceição, Hospital São Vicente de Paula, Hospital Universitário de Rio Grande and Hospital de Clínicas de Porto Alegre. The detailed description of that study can be found elsewhere (41). The nondiabetic group was composed by 516 healthy volunteers attending the blood donation facility at Hospital de Clínicas de Porto Alegre.

The information obtained from the study did not influence the patients' diagnosis or treatment. The protocol was approved by the Hospital ethical committees, and all patients and nondiabetic subjects gave their written informed consent.

Clinical and anthropometric profiles and laboratory analyses

A standard questionnaire was used to collect information from all patients about age, age at T2DM diagnosis, and drug treatment. All patients underwent physical and laboratory evaluations. They were weighed (barefoot and wearing light outdoor clothing) and had their height measured. Body mass index (BMI) was calculated as weight (kg) / height $(m)^2$. Blood pressure (BP) was measured twice, in the sitting position, with a 5-min rest between measurements, using a mercury sphygmomanometer (Korotkoff phases I and V). The mean of the two measurements was used to calculate systolic and diastolic BP. Arterial hypertension was defined as BP ≥140/90 mmHg or use of antihypertensive drugs regardless of BP at the time of assessment.

Serum samples were collected from all T2DM patients for laboratory testing after a 12-h fast. Glucose levels were determined by a glucose oxidase method; creatinine by the Jaffé reaction; glycated hemoglobin (A1C) by an ion-exchange HPLC procedure (Merck-Hitachi L-9100 glycated hemoglobin analyzer, Merck, Darmstadt, Germany; reference range: 2.7 – 6.0%); and total plasma cholesterol, HDL-cholesterol, and triglycerides, by enzymatic methods. LDL-cholesterol was calculated using the Friedewald equation. Serum insulin was measured by radioassay (Elecsys® Systems 1010/2010/modular analytics E170, Roche Diagnostics, Indianapolis, IN). Insulin sensitivity was estimated by HOMA-IR index [fasting insulin (milliunits per milliliter) x fasting glucose (millimoles per liter)/22.5] (42). The mean HOMA-IR index values of control subjects in our laboratory were 1.84 ± 1.02 (43). A standard questionnaire was used to collect information from all nondiabetic subjects regarding age, sex, skin color, and presence of comorbidities.

All TDM2 patients and nondiabetic subjects are white. The ethnic group was defined on the basis of self-classification and subject classification (skin color, nose and lip shapes, hair texture and family history).

Molecular analysis

DNA was extracted from peripheral blood leukocytes by a standardized salting-out procedure. A search for variants of *DIO2* gene was performed in 14 subjects (12 type 2 diabetic patients and 2 nondiabetic subjects) by direct sequencing of all exons, partial intron sequences (500bp near to exon-intron junctions), and 5'UTR and 3'UTR sequences (1000bp each, including the Selenocysteine Insertion Sequence Element – ESECIS - in the 3'UTR). Screening this limited number of individuals may fail to detect some rarer polymorphisms; however, this number is adequate to identify representative variants which are sufficiently polymorphic to warrant association studies. Direct sequencing in an automated ABI 3100 *Avant Genetic Analyzer* (Life Technologies, Foster City, CA; USA) was performed using ABI Prism Big Dye Terminator Cycle Sequence Ready reaction kit (Life Technologies), following the manufacturer's protocol, and using primers depicted in **Supplementary Table 1**.

Among the SNPs identified through the sequencing of the *DIO2* gene in 12 T2DM patients with low or high HOMA-IR values and in 2 nondiabetic subjects, we selected the rs225017 (T/A) polymorphism in the 3'UTR region for subsequent genotyping in all diabetic patients and nondiabetic subjects. This SNP together with the Thr92Ala (rs225014) polymorphism were determined using primers and probes contained in Human Custom TaqMan Genotyping Assays 40x (Assays-By-Design Service, Life Technologies). Primers and probes sequences used for genotyping are

depicted in **Supplementary Table 1**. Reactions were conducted in 96-well plates, in a total reaction volume of 5 μL using 2 ng of genomic DNA, TaqMan Genotyping Master Mix 1x (Life Technologies), and Custom TaqMan Genotyping Assay 1x. Plates were then placed in a real-time PCR thermal cycler (7500 Fast Real-Time PCR System; Life Technologies) and heated for 10 minutes at 95°C, followed by 45 cycles of 95°C for 15 seconds and 62°C for 1 minute. Fluorescence data files from each plate were analyzed using automated allele-calling software (SDS 2.1; Life Technologies).

RNA secondary structure prediction

RNA MFold Software Version 3 was used for mRNA secondary structure prediction by the free energy minimization model (44). The *DIO2* mRNA sequence (*DIO2* isoform a, variant 1 – ref. NM_013989.4) was used to generate a set of possible structures at suboptimal free energy status. We compared the structures generated from mRNA sequences containing a combination of the rs205017 and the rs225014 (Thr92Ala) polymorphic alleles and wild-type alleles.

Statistical analyses

Results are expressed as mean ± SD, % or median (minimum-maximum values). Allelic frequencies were determined by gene counting, and departures from the Hardy-Weinberg equilibrium (HWE) were verified using χ^2 tests. Linkage disequilibrium (LD) between the rs205017 and Thr92Ala polymorphisms was examined using Lewontin's D' |D'| and r^2 measures (45, 46). The haplotypes constructed from the combination of these two *DIO2* polymorphisms and their frequencies were inferred using the Phase 2.1

program, which implements a Bayesian statistical method (46, 47). We also used this program to compare the distributions of *DIO2* haplotypes between T2DM patients and nondiabetic controls through permutation analyses of 1,000 random replicates (46).

Clinical and laboratory characteristics were compared using ANOVA, unpaired Student's *t* test or χ^2, as appropriate. Variables with skewed distribution were logarithmically transformed before analyses. To verify if the rs225017 polymorphism was independently associated with T2DM, a multiple logistic regression analysis was performed with T2DM as the dependent variable and age, gender, and rs225017 polymorphism as independent variables. Linear regression analysis was performed to verify the independent association between the rs225017 polymorphism and IR parameters after adjusting for covariates (age, gender, BMI and use of T2DM medication). Haplotype interaction between the rs225017 and Thr92Ala polymorphisms in modulating fasting insulin and HOMA-IR index was tested by general linear model univariate analyses (GLM), after adjusting for covariates. In these interaction analyses, each of the polymorphisms was modeled as dichotomous variables.

All analyses were performed using SPSS version 18.0 (SPSS, Chicago, IL, USA). P values less than 0.05 were considered statistically significant.

RESULTS

DIO2 screening and identification of candidates polymorphisms for association with insulin resistance

An interim study comprising samples of 12 T2DM patients and 2 nondiabetic subjects was used to search for polymorphisms in the *DIO2* gene. This first phase study

identified 448 amplicons (data not shown). Although no mutation was detected in the data set, 5 previously described SNPs were identified (**Supplementary Figure 1**). These SNPs included two polymorphisms in the 5' flanking region (rs199598135, rs12885300), one polymorphism in exon 3 (rs225014 - Thr92Ala), and two in the 3'UTR (rs225015, rs225017).

Table 1 shows the clinical characteristics of the 12 T2DM patients selected for the screening of the *DIO2* gene as well as the occurrence of the 5 identified *DIO2* polymorphisms in these patients. **Supplementary Table 2** depicts the frequencies of these polymorphisms in the 12 T2DM patients. The presence of the genotype TT in the rs225017 (A/T) polymorphism was more frequent in patients with high HOMA-IR index (6 in 6) as compared to patients with low HOMA-IR index (2 in 6). In addition, this polymorphism was much more frequent in our sample than that described in GenBank (0.66 *vs*. 0.36; **Supplementary Table 2**). For these reasons, it was selected for further analysis in an association study.

Variants in the DIO2 gene are associated with increased risk for T2DM in a case-control study

The baseline characteristics of the 1077 T2DM patients and 516 nondiabetic subjects included in the study were as follows: mean age was 59.3 ± 10.3 years *vs*. 46.2 ± 10.3 years ($P < 0.001$), and males comprised 47% and 63% of the sample ($P < 0.001$), respectively.

Genotyping and allelic frequencies of the rs225017 polymorphism in 1077 T2DM patients and 516 nondiabetic individuals are described in **Table 2**. The rs225017 T allele frequency of this *DIO2* polymorphism did not differ significantly between T2DM patients and nondiabetic subjects (0.51 vs. 0.48, P=0.196). In addition, the genotype frequency T/T was 26.9% in patients with T2DM and 24.6% in nondiabetic subjects (P=0.452), and this result did not change after logistic regression analysis adjusting for age and gender (OR = 1.15, 95% CI=0.86 - 1.55; P=0.354).

Table 2 describes the frequencies of the Thr92Ala polymorphism in this sample population. Although the frequency of Ala92 allele was higher in T2DM patients (0.41) than in nondiabetic subjects (0.37), the difference did not achieve formal statistical significance (P=0.065). Nevertheless, the genotype frequency was significant (16.7% vs. 12%, P=0.05), and after logistic regression analysis adjusting for age and gender, the presence of the genotype Ala/Ala was significantly associated with risk for T2DM (OR = 1.59, 95% CI=1.10 - 2.31; P=0.010). Because the data regarding the Thr92Ala polymorphism has been already published (29), we will only explore the data about this variant in the context of its interaction with the rs205017 polymorphism. Allelic and genotype frequencies of both analyzed polymorphisms were in HWE in both T2DM patients and nondiabetic subjects (P > 0.05).

We used a Bayesian statistical method to estimate the frequencies of different haplotypes produced by the combination of the Thr92Ala and rs225017 polymorphisms in patients with T2DM and nondiabetic subjects **(Supplementary Table 3)**. The first letter of the haplotypes refers to the Thr92Ala polymorphism and the second to the rs225017 polymorphism. Permutation analysis showed that the distributions of the four inferred haplotypes were statistically different between diabetic patients and nondiabetic subjects (P=0.001) (**Supplementary Table 3**). Interestingly, after adjusting for age and

gender, the homozygous genotype frequency of the Ala/T haplotype was significantly higher in T2DM patients as compared with nondiabetic subjects (14.0% vs. 9.0%; OR = 1.70, 95% CI = 1.11 - 2.61; P=0.015) (**Table 3**).

To determine whether the rs225017 (A/T) and rs225014 (Thr92Ala) polymorphisms were in linkaged disequilibrium (LD) in nondiabetics and T2DM patients we used Bayesian method. The analysis showed that the rs225017 polymorphism is in partial LD with the rs225014 polymorphism (|D'| = 0.811; r^2 = 0.365). These results were confirmed in HAPMAP Project, (http://hapmap.ncbi.nlm.nih.gov/).

DIO2 polymorphisms are associated with insulin resistance in patients with T2DM

Table 4 summarizes the clinical and laboratory characteristics of T2DM patients grouped according to the different genotypes of the rs225017 polymorphism. Assuming a recessive model of inheritance, patients with A/A or A/T genotypes were grouped and compared with patients carrying the T/T genotype. T2DM duration (P = 0.009), gender proportion (P = 0.038) and use of metformin alone (P = 0.027) or metformin + sulfonylureas (P = 0.015) for treatment of T2DM were differentially distributed between patients with the T/T genotype and A allele carriers. It is worth mentioning that none of the variables showed in **Table 4** attained statistical significance when assuming dominant (A/A *vs*. A/T-T/T) or additive (A/A *vs*. T/T) models of inheritance (data not shown).

A subgroup of 227 T2DM patients who were not receiving insulin therapy and had serum creatinine levels <114.4 μmol/l was further analyzed for IR measurements. This subgroup was representative of the whole sample: the mean age was 59.3 ± 9.3

years (P = 0.803 for the comparison with the whole sample), the mean T2DM duration was 11.2 ± 8.2 years (P = 0.580), the mean A1C was 7.3 ± 1.7% (P = 0.12), and the mean BMI was 28.9 ± 4.7 kg/m^2 (P = 0.89). Males comprised 45.3% (n = 103) of the sample (P = 0.45).

In this subgroup of patients, the median fasting plasma insulin level in patients with the T/T genotype was higher than in patients carrying the A allele (15.71 vs. 10.57, P = 0.005), whereas fasting plasma glucose levels did not differ significantly among these genotypes (P = 0.144). Moreover, T/T patients showed higher HOMA-IR index than those patients carrying the A allele (5.20 vs. 3.50, P = 0.005). Because insulin sensitivity is known to be influenced by multiple independent factors, multiple linear regression analyses were performed with HOMA-IR index ($\log_{10}$ HOMA-IR) or insulin ($\log_{10}$ fasting insulin) as dependent variables. The T/T genotype of the rs225017 polymorphism remained significantly associated with HOMA-IR [standardized coefficient β for T/T genotype = -0.366, 95% CI (-0.654 – -0.078); P = 0.013] and insulin levels [standardized coefficient β for T/T genotype = -0.268, 95% CI (-0.441– -0.096); P = 0.002], after adjusting for age, gender, BMI and use of medication for T2DM (metformin or sulfonylureas).

Taking these results into account, we aimed to test whether any haplotype constituted by the rs225017 and Thr92Ala polymorphisms could influence differently fasting plasma insulin levels or HOMA-IR index as compared with the effect of each polymorphism analyzed separately. To test this hypothesis, we performed GLM analyses using fasting insulin levels or HOMA-IR index as the dependent variables, and age, gender, BMI, use of medication for T2DM and the polymorphic combinations of the two *DIO2* variants as the independent variables. These interaction analyses are depicted in **Table 5**. Patients carrying the Ala/Ala-T/T genotype showed higher fasting

insulin values as compared to patients with other genotype combinations, adjusting for covariables (F=11.072, P=0.001). Likewise, patients carrying the Ala/Ala-T/T genotype showed higher HOMA-IR index than patients with other genotype combinations (F=4.740; P=0.010).

DIO2 mRNA secondary structure prediction

We used the MFold software to obtain initial insight into whether the combination of the two analyzed *DIO2* polymorphisms causes a significant change in the secondary structure of the *DIO2* mRNA (**Figure 1**). Although the wild-type (Thr/A) and the mutated (Ala/T) haplotypes showed small differences in their free energies (Dg), their structure are similar.

DISCUSSION

Insulin resistance is a disturbance that results from increased hepatic gluconeogenesis and decreased glucose disposal rate in metabolic active tissues, and it plays a major role in the pathogenesis of T2DM (48). Environmental and genetic factors are involved in its pathogenesis; nonetheless, major genetic factors remain yet to be identified (49-51). Taking into account that thyroid hormones seem to have an important role in IR development, and that D2 is the most important deiodinase for intracellular T3 generation in target tissues, the *DIO2* gene is candidate gene for IR pathogenesis. Here, through sequencing of the *DIO2* gene, we found 5 polymorphisms that could be associated with IR. Among them, homozygosis for the major allele of the rs225017 (A/T) polymorphism was associated with IR in T2DM patients. Moreover, patients

carrying a combination constituted by the T/T genotype of the rs225017 polymorphism and the Ala/Ala genotype of the Thr92Ala polymorphism showed increased HOMA-IR index as compared with patients with other genotypes combinations, suggesting that they interact in the modulation of IR.

D2 is tightly regulated at transcriptional, post-transcriptional, translational and post-translational levels (2, 52). Theoretically, *DIO2* polymorphisms could affect different points of regulation, generating changes in mRNA stability or expression, alterations in catalytic sites and activity or even in the mechanism of selenocysteine insertion. Previous studies have demonstrated that the Ala/Ala genotype of the *DIO2* Thr92Ala (rs225014) polymorphism was associated with IR in nondiabetic and T2DM patients (8, 30, 32, 53), and with increased risk to T2DM in a meta-analysis study (OR = 1.4, 95% CI=1.03-1.94, P=0.03) (29). The Ala/Ala genotype was also associated with lower D2 activity in thyroid biopsy samples, but *in vitro* studies did not detect any significant change in the biochemical properties of the polymorphic enzyme, suggesting that this variant could be only a marker of abnormal *DIO2* expression (8).

Given that the Thr92Ala polymorphism probably is not the functional polymorphism that explaining alone the described association between the *DIO2* gene and IR, we searched for other informative polymorphisms in the *DIO2* gene that could be associated with IR or T2DM. No mutations were found in *DIO2* gene, but a polymorphism in 3'UTR (rs225017) was individually associated with both fasting plasma insulin levels and HOMA-IR index in T2DM patients. In the presence of the Ala92Ala genotype, the rs225017 variant was also associated with risk to T2DM and even higher IR. Polymorphisms located at the 3'UTR are usually associated with mRNA stability and, in selenoenzymes, the 3'UTR contains the selenocysteine insertion sequence (SECIS), which is very important for looping formation in the mechanism of

cis-acting mRNA structure of selenocysteine insertion (54, 55). Thus, the rs205017 polymorphism in 3'UTR could be destabilizing the *DIO2* mRNA or obstructing the mechanism of selenocysteine insertion and, thereby, reducing the amount of D2 available for the activation of T3. In this context, polymorphic D2, generated from the Ala/T haplotype sequence, might generate less T3 in skeletal muscle and adipocytes, which, consequently, could create a local state of intracellular hypothyroidism, decreasing the expression of genes involved in metabolism, such as GLUT4, and leading to IR (30).

Recently, the *DIO2* rs6574549 other polymorphism in 3'UTR was associated with elevated fasting insulin levels and higher insulin action but not with T2DM in Pima Indians (56), also which could be seen as a candidate for the functional variant associated with IR. This variant is in LD with the Thr92Ala (56) and rs205017 (57) (HAPMAP Project, http://hapmap.ncbi.nlm.nih.gov/) polymorphisms. Therefore, further studies are necessary to determine: 1) which polymorphism is the functional variant interacting with the Thr92Ala polymorphism in the modulation of IR; 2) if there is another functional polymorphism in the *DIO2* gene yet to be identified; and 3) if these polymorphisms have combined effects on D2 function. The results presented here add to the understanding of the molecular interactions controlling IR and might partially explain the association between the Thr92Ala polymorphism and this pathology.

Some factors unrelated to the rs205017 and Thr92Ala polymorphisms could have interfered with the findings of the present study. First, medications for T2DM treatment could have played a role because some are known to affect insulin sensitivity. However, we minimized such a possibility by excluding insulin-treated patients from the group of 227 patients analyzed for IR. Furthermore, the rs205017 variant and the Ala/Ala-T/T haplotype remained independently associated with HOMA-IR index and

plasma insulin levels after adjusting for medications for T2DM. In addition, none of these patients were using thiazolidinediones drugs, agents that change insulin sensitivity. Second, IR was assessed by calculation of HOMA-IR index rather than the reference method, the euglycemic-hyperinsulinemic clamp (42, 58, 59). Although HOMA-IR index is only an estimate of IR, it is simple to perform and shows a good correlation with the reference method (42, 58), therefore, is considered a good approach for cohort and epidemiological studies (59). Third, we cannot rule out the possibility of stratification bias in our sample, even though we analyzed only self-defined white subjects, thus reducing the risk of false positive/negative associations due to this bias. In addition, the 227 T2DM patients analyzed for IR were representative of the whole sample (n = 1077) with respect to age, T2DM duration, A1c, gender, BMI and presence of arterial hypertension. The frequencies of the rs205017 and Thr92Ala polymorphisms were also similar between these 227 patients and the whole T2DM sample (data not shown). Fourth, screening only 12 T2DM patients and 2 nondiabetic subjects for *DIO2* polymorphism might have failed to detect some rarer polymorphisms; however, this number was enough to identify most representative variants which are sufficiently polymorphic to warrant association studies. Finally, our results could represent a type 1 error. However, the scientific plausibility of the reported association provides evidence against type 1 error.

In summary, the *DIO2* rs225017 (A/T) polymorphism is a new polymorphism associated with IR in white T2DM patients, and seems to interact with the Thr92Ala polymorphism in the modulation of this characteristic. Further studies are needed to evaluate the functional and epidemiological importance of the rs205017 polymorphism in IR and T2DM.

DECLARATION OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Table 1. Characteristics of patients with T2DM included in the *DIO2* polymorphisms screening study

Patients	Gender	Age	BMI	Duration of T2DM (years)	Candidate Polymorphisms					HOMA-IR index	Fasting insulin
					rs199598135 (-/T)	rs12885300 (Gly/Asp)*	rs225014 (Thr/Ala)	rs225015 (A/G)	rs225017 (A/T)		
High HOMA-IR index											
#1	M	55	24	13	T	Gly	Ala	A	T	23.40	29.08
#2	M	78	31.3	25	-	Gly	Ala	A	T	20.09	39.53
#3	M	45	26.6	4	-	Gly	Ala	G	T	19.07	89.88
#4	F	52	43.2	12	-	Asp	Ala	A	T	7.78	17.62
#5	M	38	34.1	3	-	Gly	Thr	A	T	7.54	19.35
#6	F	66	29.9	15	-	Gly	Ala	G	T	6.20	18.34
Low HOMA-IR index											
#7	F	48	26.3	2	-	Gly	Ala	A	T	1.33	3.80

#8	M	64	23.5	11	-	Asp	Thr	A	A	1.22	4.77
#9	M	64	28	25	-	Gly	Ala	G	T	0.88	4.63
#10	M	59	26.3	7	-	Asp	Thr	G	A	0.48	1.69
#11	F	67	27	2	-	Gly	Thr	A	A	0.46	1
#12	M	49	28	4	-	Asp	Thr	A	A	0.32	1

* This polymorphism is also called Orf-a Gly3Asp and changes aminoacids only in the D2 protein codified by the isoform a - variant 2 of the *DIO2* mRNA. In the other DIO2 variants, it occurs in a regulatory region. BMI, body mass index; HOMA-IR, homeostasis model assessment – insulin resistance; T2DM, type 2 diabetes mellitus.

Table 2. Genotype and allele frequencies of the *DIO2* rs225017 (T/A) and rs225014 (Thr92Ala) polymorphisms in T2DM patients and nondiabetic individuals

	TDM2 patients (n=1077)	Nondiabetic subjects (n=516)	P
rs225017			
Genotype frequency			
A/A	279 (25.9)	147 (28.5)	0.452
A/T	508 (47.2)	242 (46.9)	
T/T	290 (26.9)	127 (24.6)	
Allele frequency			
A	0.495	0.519	0.196
T	0.505	0.481	
Thr92Ala			
Genotype frequency			
Thr/Thr	384 (35.7)	195 (37.8)	0.050
Thr/Ala	513 (47.6)	259 (50.2)	
Ala/Ala	180 (16.7)	62 (12)	
Allele frequency			
Thr	0.595	0.629	0.065
Ala	0.405	0.371	

Data are n (%) or proportion. P values were computed by χ^2 tests.

Table 3. Frequencies of *DIO2* combined genotypes in T2DM patients and nondiabetic subjects

Haplotypes	Nondiabetic subjects	TD2M patients	Unadjusted OR (95% CI); P	Adjusted OR (95% CI); P
Thr/Thr-A/A (wild type), Thr/Thr-A/T, Thr/Ala-A/T, Thr/Ala-A/A or Thr/Ala-A/T	373 (0.72)	765 (0.71)	1	1
Thr/Thr-T/T or Thr/Ala-T/T	81 (0.16)	132 (0.12)	0.796 (0.588-1.077); 0.796	0.863 (0.595-1.252); 0.430
Ala/Ala-A/A or Ala/Ala-A/T	16 (0.03)	33 (0.03)	1.007 (0.547-1.853); 0.982	1.185 (0.582-2.409); 0.640
Ala/Ala-T/T	46 (0.09)	147 (0.14)	1.560 (1.096-2.222); 0.014	1.698 (1.107-2.605); 0.015
Total	516	1077	-	-

Data are shown as n (%). Multiple logistic regression analyses with age, sex and genotypes as independent variable and T2DM as the dependent variable.

Table 4. Clinical and laboratory characteristics of T2DM patients broken down by the *DIO2* rs225017 (T/A) polymorphism

	Genotypes		
	A/A or A/T (n = 798)	T/T (n = 279)	P
Age (Years)	59.6 ± 9.8	58.2 ± 10.3	0.051
Duration of diabetes (Years)	12.1 ± 9.0	10.5 ± 7.9	0.009
Gender (% males)	49.0	42.0	0.038
BMI (kg/m^2)	28.8 ± 4.8	29.3 ± 5.3	0.117
Total cholesterol (mmol/l)	5.4 ± 1.2	5.5 ± 1.2	0.419
Systolic BP (mm Hg)	141.9 ± 23.7	141.8 ± 21.8	0.953
Diastolic BP (mm Hg)	84.8 ± 12.7	85.6 ± 13.1	0.823
HDL cholesterol (mmol/l)	1.17 ± 0.3	1.16 ± 0,3	0.753
LDL cholesterol (mmol/l)	1.29 ± 1.1	1.31 ± 1.1	0.533
Serum creatinine (µmol/l)[a]	81.4 (26.5-937)	79.6 (35.4-540)	0.127
Triglycerides (mmol/l)[a]	2.00 (0.3-13.2)	1.99 (0.5–8.7)	0.821
Fasting plasma glucose (mmol/l)	9.3 (2.9-21.9)	9.8 (3.3-20.5)	0.144

A1C (%)	7.1 ± 2.0	7.3 ± 1.8	0.343
Fasting insulin (UI/ml)[a,b]	10.6 ± 1.0	15.7 ± 1.0	0.005
HOMA-IR index[a,b]	3.5 (0.3-19.5)	5.2 (0.6-20)	0.005
SU / Met / SU + Met (%)	31.1 / 36.0 / 14.3	34.8 / 45.9 / 22.9	0.249 / 0.027 / 0.015

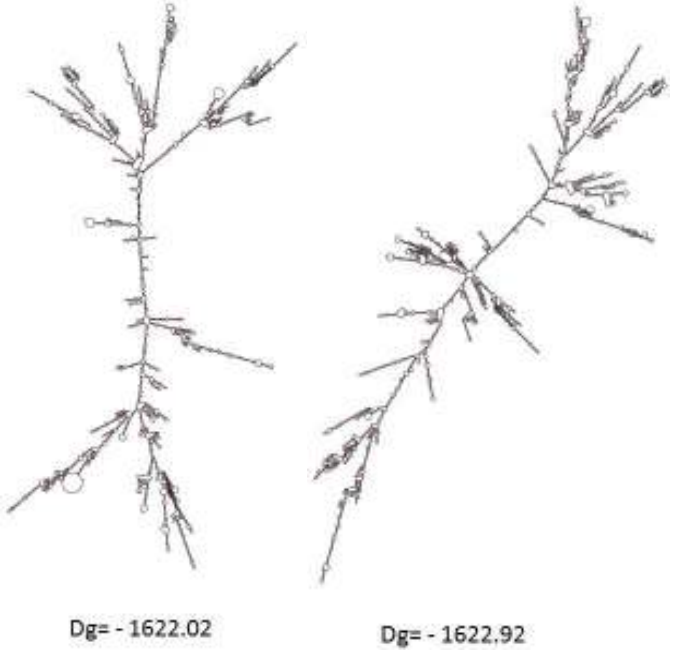
Data are mean ± SD, median (minimum – maximum values) or %. A1C, glycated hemoglobin; BMI, body mass index; HOMA-IR, homeostasis model assessment – insulin resistance; Met, metformin; SU, sulfonylureas; WHR, waist-to-hip ratio; P values were computed by χ^2 or ANOVA tests, as appropriate. [a] Variables which were logarithmically transformed before analyses. [b] For comparisons of fasting insulin levels and HOMA-IR index among rs225017 genotypes, we analyzed only 227 individuals (162 individuals harboring the A/A or A/T genotypes and 65 individuals harboring the T/T genotype).

Table 5. Interaction analyses between the *DIO2* rs225017 (T/A) and rs225014 (Thr92Ala) polymorphisms on fasting plasma insulin and HOMA-IR index

	rs225017						
	A/X			T/T			
Thr92Ala	Thr/X (n=118)	Ala/Ala (n=44)	P[a]	Thr/X (n=35)	Ala/Ala (n=30)	P[a]	F; P[b]
Age (years)	59.7 ± 9.9	57.2 ± 10.5	0.177	58.2 ± 10.6	58.1 ± 10.0	0.941	-
Gender (% males)	49.0	42.0	0.439	39.0	49.0	0.417	-
BMI (kg/m^2)	28.8 ± 4.9	28.6 ± 4.5	0.865	29.4 ± 5.5	29.3 ± 5.1	0.959	-
SU/Met/SU + Met (%)	31.3/35.6/14.0	28.6/50.0/14.0	0.830/0.270/0.997	35.3/51.5/22.0	34.3/40.3/24.0	0.907/0.196/0.803	-
Fasting insulin (UI/ml)[c,d]	13.1 ± 0.9	16.1 ± 1.5	0.794	13.9 ± 0.8	17.8 ± 1.1	0.011	11.072; 0.001
HOMA-IR index[c,e]	3.5 (0.3-19.5)	2.8 (1.6-13.6)	0.916	3.4 (0.6-13.5)	6.1 (0.9-20.1)	0.034	4.740; 0.010

Data are mean ± SD, median (minimum – maximum values) or %. BMI, body mass index; HOMA-IR, homeostasis model assessment – insulin resistance; Met, metformin; SU, sulfonylureas. [a] Data were analyzed using Student's t-test. [b]F and P values obtained from the general linear model - interaction analyses, after adjusting for age, gender, BMI and use of medication for T2DM. [c] Variables which were logarithmically transformed before analyses. [d] Adjusted R squared for fasting plasma insulin = 0.135. [e] Adjusted R squared for HOMA-IR = 0.111.

D2 92Thr 3UTRA (Wild-type) D2 92Thr 3UTRT



D2 92Ala 3UTRA D2 92Ala 3UTRT (Mutated)

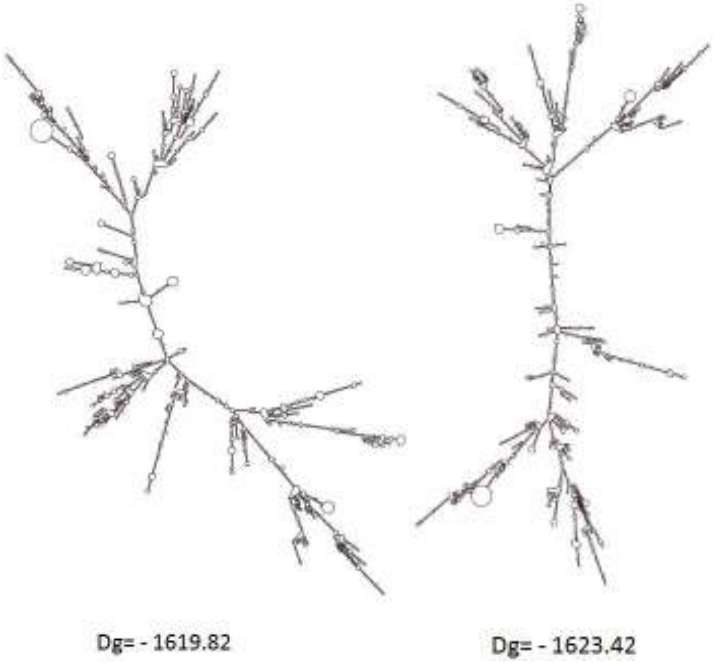


Figure 1. *DIO2* mRNA structure prediction.

DIO2 mRNA secondary structures predicted from different haplotypes constituted by the rs225014 (Thr92Ala) and rs225017 (T/A) polymorphisms. Dg = free energy minimization model.

SUPPLEMENTARY TABLES AND FIGURES

Supplementary Table 1. Primer and probe sequences used for sequencing analyses of the *DIO2* gene or genotyping of *DIO2* polymorphisms

	Sequences	Site	Size
Sequencing analyses [a]			
#1	F 5'- TAGGTCACAGATCTTACAAAG - 3' / R 5'- CAGTGAGTCTCCCTGACGCCT – 3'	-1000 to -500	500 pb
#2	F 5'- GGCTGCAGAGAGAGGCACTT - 3' / R 5'- TGGAGTGTGCCCATCAATTC - 3'	-500 to +1	500 pb
#3	F 5'- CCCCACCCCCTTTATCACCA - 3' / R 5'- GAGGTCAAGTGGCTGAGCCA - 3'	+1 to 491	491 pb
#4	F 5'- GCTTGGAAATGAAAGTAGAA - 3' / R 5'- GAATGACCGAGTCATAGAGA - 3'	301 to 807	506 pb
#5	F 5'- CACAAGGGAACTGACTCAGG - 3' / R 5'- ACTCCCAAATCACAGCAAGA - 3'	680 to 1197	517 pb
#6	F 5'- TTACGGGGTAGCCTTTGAAC - 3' / R 5'- TTTTCTTCTGGTCTCAAAGC - 3'	800 to 1289	489 pb
#7	F 5'- TCCAAGTCCACTCGCGGAGA - 3' / R 5'- CCTCAGCCTCCCATCAAGCA - 3'	840 to 1325	485 pb
#8	F 5'- TCGTGGGGAGAGCAAAGAAT - 3' / R 5'- ATTTTGTTGAGGAGCCAGGG - 3'	5600 to 6097	497 pb
#9	F 5'- TCGTGGGGAGAGCAAAGAAT - 3' / R 5'- ATTTTGTTGAGGAGCCAGGG - 3'	6000 to 6500	500 pb
#10	F 5'- TCTTCAGTGACTATACAGAATG - 3' / R 5'- AATGTAGACCAGCAGGAAGT - 3'	8658 to 9155	497 pb
#11	F 5'- CAAACTGGTGGAAGAGTTCT - 3' / R 5'- CCTGTCTTTCAGTAAGCCAA - 3'	9104 to 9603	499 pb
#12	F 5'- CCTATTGGCTTACTGAAAGA - 3' / R 5'- AATTTCTGGGGTATGAAGAC - 3'	9580 to 10069	489 pb

#13	F 5'- ATATTTGTAATTGTGAGGGG - 3' / R 5'- GTCAATGGAAATTCCATGAT - 3'	10426 to 10921	495 pb
#14	F 5'- CACATTCACACTGTTGTCCTT - 3' / R 5'- CCTCTACCTCAAAATAATGAGT - 3'	11675 to 12170	495 pb
#15	F 5'- CCTACTTTGTATAGCTAAGTGAC - 3' / R 5'- GAACAAATGTCCAGATTCAT - 3'	12705 to 13184	479 pb
#16	F 5'- TTGAAAACAAACTTCTCGCA - 3' / R 5'- CACATCCCCCAATCCTAATA - 3'	13681 to 14179	498 pb
Genotyping [b]			
rs225014 (Thr92Ala)	F 5'-GGTACCATTGCCACTGTTGTCA-3' / R 5'- GTCAGGTGAAATTGGGTGAGGAT-3'	984	100 pb
	FAM-5'- ATGTCTCCAGTGCAGAA-3' / VIC-5'-TTTGGTTCTTGCACACCTAGTTCT-3'		
rs225017 (A/T)	F 5'- TTTGGTTCTTGCACACCTAGTTCT -3' /	3338	100 pb
	R 5'- AAAATGGATAGAAAAAAACTAAAGTTGAAA ATACA 3'		
	FAM-5'- CACTCTTTTCTCATTTCAGA -3' / VIC-5'- CACTCTTTTCTCAATTCAGA -3'		

F = forward primers and R = reverse primers. [a] For sequencing analysis, primers were designed using human *DIO2* gene (NM_000793.5) and Vector NTI® Software (Invitrogen). [b] For genotyping of polymorphisms, primers and probes were designed by Life Technologies (Custom TaqMan Genotyping Assays).

Supplementary Table 2. Frequencies of polymorphisms identified through sequencing of the *DIO2* gene

Identification	Region	Absolute frequency[a]	Relative frequency	Described frequency[b]
rs199598135	5'-flanking	1/12	0.08	ND
rs12885300	5'-flanking	8/12	0.66	0.43
rs225014	Exon 3	7/12	0.58	0.35
rs225015	3'-UTR	4/12	0.33	0.21
rs225017	3'-UTR	8/12	0.66	0.36

[a] Absolute frequencies observed on the 12 T2DM patients screened for *DIO2* variants. [b] Frequencies described on GenBank from white population. ND = non-described in GenBank.

Supplementary Table 3. Haplotypes of *DIO2* gene according to the presence of T2DM

Haplotypes	Nondiabetic subjects (n = 1032)	T2DM patients (N = 2154)
Thr / A (wild-type)	0.49 (509)	0.49 (1062)
Thr / T	0.15 (159)	0.18 (388)
Ala / A	0.07 (69)	0.04 (78)
Ala / T (mutated)	0.28 (295)	0.29 (626)

The first letter of the haplotype refers to the Thr92Ala polymorphism and the second letter to the rs225017 polymorphism. P value obtained from permutation analysis comparing the haplotype distributions between diabetic patients and nondiabetic subjects = 0.001. n = number of chromosomes.

Supplementary Figure 1. **Candidate polymorphisms identified by sequencing of the *DIO2* gene.** The vertical arrows show the five *DIO2* polymorphisms identified through sequencing. ESECIS, Selenocysteine Insertion Sequence Element; Ex1, Ex2 and Ex3 represents exons in *DIO2* gene. 3'UTR = 3'-untranslated region.