Association of two synonymous splicing-associated CpG single nucleotide polymorphisms in calpain 10 and solute carrier family 2 member 2 with type 2 diabetes

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Abstract. Coding synonymous single nucleotide polymorphisms (SNPs) have attracted little attention until recently. However, such SNPs located in epigenetic, CpG sites modifying exonic splicing enhancers (ESEs) can be informative with regards to the recently verified association of intragenic methylation and splicing. The present study describes the association of type 2 diabetes (T2D) with the exonic, synonymous, epigenetic SNPs, rs3749166 in calpain 10 (CAPN10) glucose transporter (GLUT4) translocator and rs5404 in solute carrier family 2, member 2 (SLC2A2), also termed GLUT2, which, according to prior bioinformatic analysis, strongly modify the splicing potential of glucose transport-associated genes. Previous association studies reveal that only rs5404 exhibits a strong negative T2D association, while data on the CAPN10 polymorphism are contradictory. In the present study DNA from blood samples of 99 Greek non-diabetic control subjects and 71 T2D patients was analyzed. In addition, relevant publicly available cases (40) resulting from examination of 110 Personal Genome Project data files were analyzed. The frequency of the rs3749166 A allele, was similar in the patients and non-diabetic control subjects. However, AG heterozygotes were more frequent among patients (73.24% for Greek patients and 54.55% for corresponding non-diabetic control subjects; P=0.0262; total cases, 52.99 and 75.00%, respectively; P=0.0039). The rs5404 T allele was only observed in CT heterozygotes (Greek non-diabetic control subjects, 39.39% and Greek patients, 22.54%; P=0.0205; total cases, 34.69 and 21.28%, respectively; P=0.0258). Notably, only one genotype, heterozygous AG/CC, was T2D-associated (Greek non-diabetic control subjects, 29.29% and Greek patients, 56.33%; P=0.004; total cases, 32.84 and 56.58%, respectively; P=0.0008). Furthermore, AG/CC was strongly associated with very high (≥8.5%) glycosylated plasma hemoglobin levels among patients (P=0.0002 for all cases). These results reveal the complex heterozygotic SNP association with T2D, and indicate possible synergies of these epigenetic, splicing-regulatory, synonymous SNPs, which modify the splicing potential of two alternative glucose transport-associated genes.

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Introduction

RNA splicing is a fundamental process, which contributes to the structural and functional complexity of proteins and influences their regulatory role and tissue specificity (1,2). Splicing enhancers in exons are considered to be responsible for the inclusion of exonic sequences in the gene transcript. There is growing evidence that polymorphisms in high impact exonic splicing enhancers (ESEs) strongly influence the activity of disease-associated genes and modify their association with different pathological conditions (3). Bioinformatic resources are available for evaluating the efficiency of ESEs (4).

It has been previously demonstrated that a major role of intragenic DNA methylation is associated with the regulation of alternative splicing (5,6). It should therefore be expected that polymorphisms, which modify a G or a C in a CpG dinucleotide, affect the epigenetic profile in exonic sequences that are most frequently found to be methylated (7). In sites of tentative DNA methylation, particularly when located in ESEs, this would lead to allele-specific methylation differences (8). In view of the recently demonstrated impact of DNA methylation on the splicing process (6), it is also expected that exonic CpG polymorphisms may further affect splicing. The presence of ESEs and their relative potential are predictable by bioinformatic analysis. Recent experimental evidence verified the consistency of the computational results with experimentally observed exon inclusion using a minigene (9). It is also evident that CpG-single nucleotide polymorphisms (SNPs) in

prominent ESEs of disease-associated genes are of particular importance (10,11). Based on this evidence, various studies have focused on genetic variations (SNPs) at CpGs, which may be responsible for predisposition to various pathological conditions, including type 2 diabetes (T2D) (11,12).

T2D is a metabolic disorder characterized by high glucose blood levels associated with insulin resistance and relatively low levels of insulin. Together with obesity, blood hypertension and hyperlipidemia, T2D is one of the most frequent conditions associated with metabolic syndrome, which is currently considered a major cause for cardiovascular disease. Genetic association studies for the identification of SNPs associated with these diseases are performed by genome-wide association study (13). However, the distinct epigenetic/splicing-associated role of these SNPs has not, to the best of our knowledge, been addressed, despite previous evidence that the expression of different splicing isoforms is a major factor for disease association even in the heterozygous state (14,15).

In view of the above, a bioinformatic analysis of synonymous SNPs in all T2D-associated genes (11) was performed in the present study to identify prominent CpG-SNPs, which introduce major modifications in the splicing potential of exonic sequences, which may be responsible for T2D. This analysis identified two principle CpG-SNPs, rs5404 in solute carrier family 2, member 2 (SLC2A2), and rs3749166 in calpain (CAPN10), a membrane protease, which is involved in glucose transporter (GLUT)4 translocation (16). rs3749166 (A>G) is located in exon 11 of the *CAPN10* gene and rs5404 (C>T) in exon 5 of the SLC2A2 gene. The two CpG-SNPs introduce pronounced changes in the ESE score (splicing potential) of the corresponding exonic sequences in these genes. The association of CAPN10 SNP with T2D in particular, has been addressed in previous studies (17-22).

In the present study, the association of these two epigenetic CpG-SNPs were analyzed, which introduced the greatest changes of the splicing potential in the corresponding genes, with T2D and other metabolic syndrome-associated pathological conditions (arterial hypertension and obesity). In addition, the possibility that this association might be observed only in the heterozygotic state of these SNPs was investigated.

Materials and methods

Study population. The investigated population included 99 non-diabetic control participants (Table IA) and 71 T2D patients (Table IB). Participants were classified as having T2D based on the American Diabetic Association criteria (23) as follows: i) ≥126 mg/dl fasting plasma glucose concentration; ii) glycosylated plasma hemoglobin (HbA1c) ≥6.5%; iii) insulin use; iv) use of other diabetes medication. All participants provided their medical family history, smoking habits and dietary information, followed by written informed consent. Their names were anonymized prior to study completion. The methods followed in the present study were performed according to the Declaration of Helsinki.

The present study was approved by the Bioethics Committee of Aristotle University Medical School (Thessaloniki, Greece; protocol no. 2629; 19 April 2011), the Scientific Council of Thessaloniki Panagia General Hospital (Thessaloniki, Greece; protocol no. A9825; 9 June 2011) and the Research Committee of Aristotle University, Operational Program 'Education and Lifelong Learning' of the National Strategic Reference Framework (NSRF) - Research Funding Program: Heracleitus II (project no. 87113).

Anthropometric and biochemical analysis. Anthropometric measurements, including weight and height were obtained according to standardized protocols. The epidemiological profile consisted of age, gender, metabolic family history, smoking status, dietary conditions, and accompanying diseases (arterial hypertension and hyperlipidemia). Participants were classified as having an accompanying disease (arterial hypertension and hyperlipidemia) when the use of antihypertensive or antihyperlipidemic medication was reported respectively, independently of their biochemical lipid profile determination. Information regarding the type of medication (tablets and insulin) and potential diabetic complications were recorded for the diabetic patients.

The biochemical analysis included determination of fasting plasma glucose, HbA1c, total serum cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol and serum triglycerides. Peripheral blood samples (2 ml) from all 170 participants for molecular genetic analysis were collected in tubes containing EDTA and centrifuged at 4,500 x g for 20 min at room temperature. Buffy coat leukocytes were then isolated and stored at -20˚C.

DNA extraction and genotype analysis. Genomic DNA was extracted from the buffy coat fraction prepared as described above using PureLink Genomic DNA kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's instructions. DNA integrity was verified by gel electrophoresis (70 V/cm for 20 min) using 0.8% agarose gel and ethidium bromide staining. DNA purity was determined by the optical density $(OD)_{260}/OD_{280}$ nm absorption ratio using an Eppendorf Biophotometer. Genomic sequences containing SNPs (rs3749166 and rs5404) were amplified by DNA polymerase chain reaction (PCR) using Platinum Taq DNA polymerase (Invitrogen; Thermo Fisher Scientific, Inc.). The PCR conditions for rs3749166 amplification were as follows: 94˚C for 2 min, 35 cycles of 94˚C for 45 sec, 60˚C for 45 sec and 72˚C for 1.5 min followed by 72˚C for 10 min. A forward primer (5'-CAGGTCCCAGAGGGTGGAA-3') and a reverse primer (5'-CAGGTAGGTGGAGGGCACAA-3') were used for amplifying a 153-bp fragment containing SNP rs3749166. A 344-bp fragment containing SNP rs5404, was amplified by PCR using a forward primer (5'-TCAGGGAGGGGCTTTCATTC-3') and a reverse primer (5'-CAGTCAGGGAGGGACGAGA-3') under the following conditions: 94˚C for 2 min, 35 cycles of 94˚C for 45 sec, 58˚C for 45 sec and 72˚C for 1.5 min followed by 72˚C for 10 min. Primer design was facilitated by Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), an online primer designing tool (24). Twelve microliters of each PCR product were separated (70 V/cm for 20 min) on a 2% agarose gel and visualized using ethidium bromide staining. In addition, the PCR products were purified using a PureLink PCR Purification kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The sequence of the purified PCR products was verified by commercial sequence analysis (VBC-Biotech Service GmbH, Vienna,

Table I. Genotypes and epidemiological parameters (age, gender, BMI, metabolic, family history, smoking status, dietary conditions and accompanying diseases) of non-diabetic control subjects (Table A) and T2D patients (Table B).

A, Non-diabetic control subjects

S/No.	n	Age (years)	Gender	BMI	rs3749166	rs5404	FG (mg/dl)	HbA1c (%)	Chol (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	TGL (mg/dl)	FH	Smoking status	No diet	Diseases
1	1	65	M	23	G/G	C/C	110	5.9	106	80	36	95		+	+	
2	3	47	F	25	A/A	C/C	85	5.3	178	139	46	150		+		HL
3	4	58	F	21	A/G	C/C	84	5.4	223	182	59	146				
4	6	71	F	22	A/G	C/T	110	6.4	220	182	63	131				HL
5	7	80	F	28	A/A	C/C	106	6.0	163	141	42	69				HT
6	9	77	F	26	A/A	C/C	96	5.6	353	305	51	190				HL
7	10	60	F	29	A/G	C/T	118	6.2	153	111	40	174	+			HT/HL
8	12	58	M	34	A/G	C/C	94	5.6	177	143	33	139		+		HT
9	13	54	M	25	A/G	C/T	104	5.7	267	219	67	177				
10	14	50	F	35	A/A	C/C	98	5.6	298	256	53	160				HL
11	16	66	F	24	G/G	C/C	110	5.9	185	153	60	104				HT/HL
12	19	50	F	35	G/G	C/T	82	5.6	225	194	31	126		+	+	
13	20	68	F	32	A/G	C/C	88	5.5	215	184	53	103				HT
14	21	75	M	28	A/A	C/C	87	5.6	112	92	28	76		+		HT
15	23	50	M	22	A/G	C/T	87	5.2	239	206	61	106	+			
16	25	76	F	29	A/G	C/T	98	5.6	236	186	49	204				HT/HL
17	26	65	F	26	A/G	C/T	107	5.8	187	152	57	118	+			HT/HL
18	27	54	F	29	G/G	C/C	107	5.9	250	218	56	106		+		HL
19	28	57	F	46	A/G	C/T	115	6.0	239	200	51	143			+	HT/HL
20	29	80	F	24	A/G	C/C	83	5.3	188	161	63	73				HT/HL
21	30	80	F	27	A/A	C/C	85	5.4	212	180	58	100				HT/HL
22	31	77	M	31	A/G	C/C	89	5.6	178	154	38	82				HT
23	32	66	M	26	A/G	C/T	92	5.6	229	188	50	159				
24	37	54	F	28	A/A	C/C	110	5.8	230	198	40	124	+			HT/HL
25	38	69	M	29	A/A	C/T	101	5.9	202	153	40	209		+		HT/HL
26	39	52	F	35	A/A	C/T	95	5.6	171	119	40	220				HL
27	40	74	F	23	G/G	C/C	100	5.7	172	143	69	79				HT/HL
28	43	45	F	38	A/G	C/T	100	5.7	216	190	38	96				
29	44	56	M	38	A/A	C/C	78	5.6	179	144	50	128				
30	48	71	F	27	A/G	C/T	101	5.8	171	122	33	215				HT/HL
31	50	79	M	23	A/G	C/C	91	5.6	167	134	46	123				HT/HL
32	51	45	F	34	A/G	C/T	88	5.6	242	199	49	167	+			HL
33	52	52	F	31	A/A	C/C	94	5.6	211	162	41	208				HL
34	54	58	M	36	A/G	C/T	111	5.8	154	113	40	167				HT
35	56	61	F	31	A/G	C/C	101	6.2	257	216	52	155				

Table I. Continued.

S/No.	n	Age (years)	Gender	BMI	rs3749166	rs5404	FG (mg/dl)	HbA1c (%)	Chol (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	TGL (mg/dl)	FH	Smoking status	No diet	Diseases
36	58	76	F	28	A/G	C/C	107	5.8	259	228	73	83				HT
37	59	69	F	31.5	A/G	C/C	103	5.7	202	172	48	103				HT
38	60	73	F	25	A/G	C/C	90	5.4	222	190	53	109				HT
39	61	45	F	34	G/G	C/C	89	5.2	173	144	51	97				
40	63	66	M	30	A/A	C/T	117	6.4	170	138	41	121		+		HT/HL
41	64	60	F	30	A/G	C/C	93	5.6	170	131	40	155				HT/HL
42	66	65	F	28	A/G	C/C	98	5.6	193	150	41	171				
43	67	45	F	17	G/G	C/T	98	5.0	176	147	53	93				
44	68	63	F	28	A/G	C/C	90	5.1	214	185	57	92				HL
45	70	50	F	33	A/G	C/T	105	6.4	217	180	48	137			+	HL
46	71	62	F	28	A/G	C/C	109	6.0	263	220	62	152				HL
47	75	60	F	29	G/G	C/C	88	5.6	225	192	48	114				HT
48	76	52	M	25	G/G	C/C	91	5.5	197	160	54	134				
49	77	50	F	25	A/A	C/T	99	5.6	210	168	42	168				HL
50	78	58	F	25	A/G	C/C	88	5.3	249	214	81	93				HL
51	81	70	F	29	G/G	C/C	117	5.8	212	172	49	150	+			HL
52	84	55	F	32	G/G	C/C	95	5.4	218	187	49	103				HT/HL
53	85	53	F	25	A/A	C/C	91	5.5	223	183	67	132				HT
54	87	65	F	34	G/G	C/C	106	5.8	240	202	51	139				HT/HL
55	88	49	F	33	A/G	C/T	98	5.6	213	170	33	185		+	+	HT/HL
56	89	73	F	28	A/A	C/C	108	6.3	216	188	52	89				
57	90	57	M	32	A/A	C/C	91	5.5	205	157	30	208		+		HT/HL
58	92	73	F	21	A/G	C/T	109	5.9	261	215	55	175				HT/HL
59	94	66	F	30	A/G	C/C	115	6.3	163	132	52	105				HT/HL
60	99	68	M	30	A/A	C/C	85	5.5	213	163	45	202				HL
61	100	58	F	30	A/A	C/C	95	5.6	232	203	44	100				
62	101	53	F	27	A/G	C/T	106	5.7	218	177	64	140				
63	102	59	F	23	A/G	C/C	95	5.3	169	142	45	89				HT
64	104	78	M	24	A/A	C/C	100	5.7	211	186	55	70				HT
65	106	64	F	33	A/G	C/T	108	6.4	208	164	43	177				HT
66	107	47	F	27	A/A	C/T	105	5.7	239	196	57	155				HL
67	108	43	F	28	A/A	C/T	89	5.4	159	130	44	103		+		
68	109	71	M	27	A/G	C/C	101	5.7	171	122	33	215				HT
69	110	57	F	32	A/G	C/T	104	5.8	220	155	40	286				HL
70	111	60	F	26	G/G	C/C	99	5.3	167	144	49	65				
71	112	65	F	26	A/G	C/C	93	5.4	201	165	76	104				HT/HL
72	113	47	F	29	G/G	C/C	102	5.7	216	180	61	122				
73	114	73	F	24	A/G	C/C	120	6.2	216	188	52	89				

Table I. Continued.

S/No.	n	Age (years)	Gender	BMI	rs3749166	rs5404	FG (mg/dl)	HbA1c (%)	Chol (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	TGL (mg/dl)	FH	Smoking status	No diet	Diseases
74	118	46	F	29	A/A	C/T	88	5.5	148	88	30	267				HT/HL
75	120	58	F	26	A/G	C/C	104	5.7	263	138	61	61				HL
76	121	56	F	32	G/G	C/C	87	5.6	187	149	70	120				
77	153	60	F	33	G/G	C/C	87	5.3	235	200	64	112		+	+	HT/HL
78	155	66	M	26	A/G	C/C	124	5.7	256	200	45	232		+	+	HT/HL
79	156	50	F	26	A/G	C/C	93	5.2	256	176	60	337		+		HL
80	158	54	F	24	A/A	C/T	104	5.7	257	218	62	131				HT
81	159	63	F	25	A/A	C/C	115	5.8	181	148	64	99				HL
82	161	49	F	22	A/G	C/T	93	5.3	213	178	100	75		+	+	
83	165	50	F	29	A/G	C/T	85	5.1	158	84	31	339				HL
84	166	50	M	29	A/G	C/T	107	5.7	258	215	32	174		+		HL
85	171	52	M	28	A/A	C/T	101	5.7	213	175	36	151		+		
86	173	51	M	32	A/G	C/T	78	5.3	191	153	38	151		+	+	
87	174	50	M	28	A/A	C/T	96	5.0	171	139	38	120				
88	176	80	F	32	A/G	C/C	121	5.9	158	112	43	183				HT/HL
89	177	71	M	28	A/G	C/C	105	5.8	220	175	77	144				HT/HL
90	178	56	F	30	A/G	C/T	108	5.8	182	152	53	93				HT
91	179	67	M	29	A/G	C/C	105	5.7	179	149	34	112				
92	180	65	F	26	A/G	C/C	91	5.7	198	168	73	73				HT/HL
93	189	50	F	31	A/G	C/T	82	5.2	197	168	58	87			+	
94	190	50	M	31	A/A	C/T	95	5.4	286	228	51	187				HL
95	192	58	F	58	A/A	C/T	93	5.6	244	210	68	100			+	HT/HL
96	194	52	F	25	A/G	C/T	85	5.2	219	194	44	81			+	HL
97	195	61	F	27	A/G	C/C	82	5.6	242	202	69	93				HT/HL
98	196	77	F	39	A/A	C/C	108	6.2	218	185	71	90			+	HT/HL
99	197	61	F	35	A/G	C/C	94	5.6	182	49	145	122			+	HT/HL

B, T2D patients

S/No.	n	Age (years)	Gender	BMI	Age of diagnosis	rs3749166	rs5404	FG (mg/dl)	HbA1c	Chol (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	TGL (mg/dl)	FH	Smoking status	Medication	No diet	DC	Diseases
1	2	62	M	30	50	A/G	C/C	128	9.4	154	125	39	109					DNA	HT/HL
2	5	60	F	41	35	A/G	C/C	183	8.9	156	128	39	102				+	DNA	HT/HL
3	8	80	F	32	80	A/G	C/C	143	11	177	143	39	134			T	+		HT/HL
4	11	54	F	27	40	A/G	C/C	142	9.5	190	159	55	104			T/IN			
5	15	80	F	23	75	A/A	C/C	128	9.8	135	108	40	98	+		IN		DNA	HT

Table I. Continued.

S/No.	n	Age (years)	Gender	BMI	Age of diagnosis	rs3749166	rs5404	FG (mg/dl)	HbA1c	Chol (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	TGL (mg/dl)	FH	Smoking status	Medication	No diet	DC	Diseases
6	17	71	F	44		A/A	C/C	144	7.4	150	117	37	129	+			+	DNA	HT/HL
7	18	65	F	24		A/G	C/C	206	9.0	204	168	47	136			T			HL
8	22	60	M	28		A/G	C/C	93	6.1	281	188	39	429			T			HL
9	24	66	F	30		A/G	C/T	110	7.1	191	164	50	86			T		DNA	HT
10	33	57	F	24		A/A	C/C	177	7.8	219	189	55	97			T/IN			HT/HL
11	34	78	F	41	65	G/G	C/T	155	7.5	157	124	40	126	+	+	T			HT
12	35	63	M	36	50	A/A	C/C	254	9.9	252	198	32	1077	+		T	+		HT/HL
13	36	71	F	33	54	A/G	C/C	139	7.8	216	175	54	153			T/IN	+	DNA	HL
14	41	64	M	30	50	A/G	C/T	286	8.8	219	173	31	202	+		T/IN	+	DNA	HT/HL
15	42	80	M	18	60	A/G	C/T	346	10.5	192	162	51	100	+		T		DNA	
16	45	72	F	30	65	A/G	C/C	83	6.4	126	98	29	115			T			
17	46	62	F	31	62	A/A	C/C	168	7.4	223	184	52	148	+		Diet			HT/HL
18	47	73	F	33	60	A/A	C/T	185	8.0	189	156	48	117			T		DNA	HT
19	49	71	M	28	65	A/A	C/C	111	7.6	219	180	45	153			T			HT/HL
20	53	58	F	30	57	A/G	C/C	295	8.7	201	154	43	195			Diet			HL
21	55	67	M	25	55	A/A	C/C	295	10.6	205	162	36	182		+			DNA	HT/HL
22	57	66	F	29		A/G	C/C	109	7.4	200	153	36	200			T			HL
23	62	79	F	30		A/G	C/T	151	7.8	169	141	45	99			T/IN		DNA	
24	65	59	M	36		A/A	C/C	130	6.8	125	84	29	178			T			
25	69	63	F	29	40	A/G	C/C	89	7.2	178	133	37	190			T			
26	72	62	F	26		A/G	C/C	228	8.6	178	133	50	175			IN			HL
27	73	69		30		A/A	C/C	171	7.5	166	132	57	114			T			HT/HL
28	74	54	F	24		A/G	C/C	210	8.6	290	216	32	339	+		T			
29	79	51	M	28		A/G	C/C	127	7.6	181	127	26	246			T/IN			HT/HL
30	80	70	M	39		A/A	C/T	134	7.7	152	106	33	200			IN	+	DNA	HT/HL
31	82	68	F	34	40	A/G	C/T	155	8.2	243	186	61	224			IN			HL
32	83	45	F	60	45	A/G	C/C	111	7.8	240	180	27	275			Diet	+		HL
33	86	81	F	28	68	A/G	C/C	132	7.4	171	132	41	156			IN		DNA	HT
34	91	62	F	34	62	A/G	C/T	98	7.2	312	241	41	313	+		Diet	+		HT
35	93	59	F	36	58	A/G	C/C	91	7.2	193	153	40	161		+	Diet			HT
36	95	52	F	40	50	A/G	C/T	119	7.0	212	172	53	148			T			HT
37	96	79	F	30	60	G/G	C/C	145	7.6	169	141	49	94	+		T			HT
38	97	45	F	30	37	A/G	C/C	149	7.2	144	102	28	182	+	+	T			HT
39	98	45	F	22	37	A/G	C/C	269	8.8	188	162	60	73	+	+	IN	+		
40	103	62	F	38	52	A/G	C/T	152	7.8	145	80	45	284			T	+	DNA	HT
41	105	67	M	32	57	A/G	C/C	233	8.8	235	182	37	232		+	T/IN			
42	115	65	M	19		A/G	C/C	422	8.9	182	149	41	125	+		T		DNA	
43	116	79	F	32	75	A/G	C/T	151	7.8	151	120	36	124			T		DNA	HT

Table I. Continued.

S/No.	n	Age (years)	Gender	BMI	Age of diagnosis	rs3749166	rs5404	FG (mg/dl)	HbA1c	Chol (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	TGL (mg/dl)	FH	Smoking status	Medication	No diet	DC	Diseases
44	117	74	F	38	67	A/A	C/T	164	7.8	130	93	35	152			T/IN			HT/HL
45	119	45	F	56		A/G	C/C	167	7.6	191	134	36	250	+	+	Diet			HL
46	151	74	F	31		A/G	C/C	102	7.2	239	206	62	106	+		Diet	+		HT/HL
47	152	66	F	30	62	A/G	C/C	126	6.8	152	120	34	127	+	+	T	+		HT
48	154	78	M	32	58	A/G	C/C	179	9.1	123	80	33	183	+		T/IN	+	DNA	HT/HL
49	157	73	M	33	58	A/G	C/C	138	7.7	108	85	38	77			T	+	DNA	HT
50	160	59	F	48	59	A/G	C/C	148	7.5	190	156	43	130	+		T	+		HT/HL
51	162	68	F	33	45	A/G	C/C	165	8.7	168	122	37	197	+		IN		DNA	HT
52	163	63	M	28	58	A/G	C/C	257	10.3	164	130	38	134			T		DNA	HT/HL
53	164	74	F	30	64	G/G	C/C	124	5.8	181	130	62	93			Diet			HT/HL
54	167	72	F	26	46	A/G	C/C	134	7.0	162	137	49	77			T			HT/HL
55	169	67	F	28	49	A/G	C/C	125	7.4	167	130	47	141			T		DNA	HT/HL
56	170	73	F	34	48	G/G	C/C	235	10.1	177	134	31	187			T/IN		DNA	
57	172	73	F	35	60	A/G	C/C	192	12.5	153	98	28	248	+		T/IN	+	DNA	HT/HL
58	175	79	F	33	69	A/G	C/C	217	8.8	179	95	30	391			T	+		HT/HL
59	181	78	F	33	62	A/G	C/C	341	8.5	212	160	55	207			IN			
60	182	80	M	29	60	A/G	C/T	130	8.5	171	126	37	192			T/IN		DNA	HT/HL
61	183	80	F	29	75	A/G	C/C	171	7.5	152	117	35	143			T		DNA	HT/HL
62	184	66	F	62	51	A/G	C/T	68	7.8	190	160	47	107			IN			HL
63	185	72	M	47	55	A/G	C/C	269	8.8	243	150	37	429			T/IN		DNA	HT/HL
64	186	78	M	30	75	A/A	C/C	127	7.4	170	145	43	82			T			HT
65	187	57	F	26	53	A/G	C/C	240	9.5	256	196	33	271	+	+	T	+		HL
66	188	77	M	30	60	A/A	C/C	154	8.3	229	193	40	141	+		T		DNA	HT
67	191	53	F	32	51	A/G	C/C	114	6.9	216	191	54	74	+		T			HT
68	193	67	M	26		A/A	C/C	155	7.2	211	181	58	95		+		+		HT/HL
69	198	67	F	30	32	A/G	C/T	167	6.8	228	193	57	122	+		T	+	DNA	HT/HL
70	199	64	F	31	55	A/G	C/C	174	8.2	219	180	47	148	+		IN		DNA	HL
71	200	80	M	29	68	A/G	C/C	163	7.5	155	125	41	113			T		DNA	HT

The + symbol refers to smokers, individuals with a positive family history and those individuals not following a specific diet in the smoking status, FH and no diet columns, respectively. S/No., serial number; n, sample number; BMI, body mass index; F, female; M, male, FG, fasting glucose; HbA1c, glycosylated plasma hemoglobin; Chol, total cholesterol; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; TGL, triglycerides; FH, family history; T, tables; IN, insulin; T/IN, tables and insulin; No diet, no dietary compliance; DC, diabetic complications; DNA, diabetic neuropathy and angiopathy; HT, arterial hypertension; HL, hyperlipidemia; HT/HL, arterial hypertension and hyperlipidemia.

Table II. Statistical analysis of epidemiological parameters of individuals included in Table I.

Parameter	Non-diabetic controls[a] (n=99)	T2D patients[b] (n=71)	χ^2	P-value
Male	24	21	0.605	0.4368
Female	75	50		
Age (years)	60.62±10.11	66.94±9.65		<0.0001
Body mass index (kg/m^2)	28.96±5.34	32.30±7.98		0.0013
Age of T2D diagnosis		56.37±10.95		
T2D duration (years)		11.65±8.33		
Fasting glucose (mg/dl)	98.31±10.45	170.32±6.63		<0.0001
Glycosylated hemoglobin (%)	5.65±0.31	8.13±1.21		<0.0001
Total cholesterol (mg/dl)	208.57±38.52	188.96±40.46		0.0016
Low-density lipoprotein cholesterol (mg/dl)	147.14±35.00	169.78±36.81		<0.0001
High-density lipoprotein cholesterol (mg/dl)	50.93±13.06	42.14±9.46		<0.0001
Trigycerides (mg/dl)	137.18±54.49	179.45±134.09		0.0052
Family history (positive)	6	25	23.565	<0.0001
Smoking status	20	10	1.065	0.3021
Not following dietary instructions	13	20	5.977	0.0014
Diet		8		
Medication (tablets)		35		
Medication (insulin)		10		
Medication (tablets and insulin)		13		
No medication or dietary intervention		5		
Diabetic complications (neuropathy, angiopathy)		30		
Diabetic complications and disease duration (>4 years)		24		0.0014
Diabetic complications and disease duration (≤4 years)		1		
Accompanying diseases	72	59	2.516	0.1127
Accompanying diseases (arterial hypertension)	17	19	2.278	0.1313
Accompanying diseases (hyperlipidemia)	24	12	1.335	0.2479
Accompanying diseases (arterial hypertension and hyperlipidemia)	31	28	1.204	0.2725

[a]Of the 48 PGP non-diabetic controls, 27 are male and 21 are female. The mean age of the 48 PGP non-diabetic controls with one or the two polymorphisms is 59.4 years (SD=10.5). [b]Of the 23 PGP T2D patients, 21 are male and 2 are female. The mean age of the 23 patients with one or the two polymorphisms is 56.7 years (SD=11.2). T2D, type 2 diabetes; PGP, Personal Genome Project; SD, standard deviation.

Austria) using the forward primer for rs3749166 and the reverse primer for rs5404). Nucleotide sequence analysis was performed using the Chromas software (version 2.6.2).

Personal Genome Project (PGP) data. To validate the results of the analysis, the allele frequencies of the rs5404 and rs3749166 polymorphisms were evaluated using public genome and exome data, available through the PGP repository (25). Cases matching the patient and non-diabetic control profiles of the present study were selected and 40 additional cases (35 non-diabetic controls and 5 T2D patients) were included, containing allele information of the rs3749166 polymorphism. In addition, 71 cases (48 non-diabetic controls and 23 T2D patients) with allele information of the rs5404 polymorphism were evaluated. Among these, 35 non-diabetic controls and 5 T2D patients contained genetic data for the two polymorphisms. Of the 71 PGP individuals, 32.4% were female and the mean age was 58 years (standard deviation, 10.85).

Statistical analysis. Graphpad online tool (https://www.graphpad.com) was used to perform statistical analyses. Student's t-test was used to compare groups of continuous variables, and the χ^2 and Fisher's test were used to compare the proportions of genotypes or alleles. A two-tailed P<0.05 was considered to indicate a statistically significant difference. The difference of the ESE scores between the major S_A and minor S_a alleles was calculated as the $\Delta Score = |S_A - S_a|$.

Results

Clinical data and statistical analysis of epidemiological parameters. Statistical analysis of the data from T2D patients and non-diabetic control subjects (Table II) demonstrated that among the T2D patients, fasting glucose levels and HbA1c were significantly higher (P<0.0001); however, there was no correlation with accompanying diseases, such as arterial hypertension or hyperlipidemia. By contrast, LDL and triglyceride

Table III. Statistical evaluation of (A) rs3749166 and (B) rs5404 SNP frequencies and genotypes among total and Greek T2D patients and non-diabetic controls. (C) Association of observed rs3749166 and rs5404 genotype combinations with disease among total, and Greek T2D patients and non-diabetic control subjects.

A, rs3749166 genotypes

Subject	AA, n (%)	AG, n (%)	GG, n (%)	χ^2	P-value
Non-diabetic control (total)	40 (29.85)	71 (52.99)	23 (17.16)	11.09	0.0039
T2D patients (total)	15 (19.74)	57 (75.00)	4 (5.26)		
Non-diabetic control (Greek)	29 (29.29)	54 (54.55)	16 (16.16)	7.28	0.0262
T2D patients (Greek)	15 (21.13)	52 (73.24)	4 (5.63)		

B, rs5404 genotypes

Subject	TT, n (%)	CT, n (%)	CC, n (%)	χ^2	P-value
Non-diabetic control (total)	0 (0)	51 (34.69)	96 (65.31)	4.967	0.0258
T2D patients (total)	0 (0)	20 (21.28)	74 (78.72)		
Non-diabetic control (Greek)	0 (0)	39 (39.39)	60 (60.61)	5.369	0.0205
T2D patients (Greek)	0 (0)	16 (22.54)	55 (77.46)		

C, rs3749166 and rs5404 combined genotypes

Subject	GG/CC, n (%)	GG/CT, n (%)	AG/CC, n (%)	AG/CT, n (%)	AA/CC, n (%)	AA/CT, n (%)
Non-diabetic control (total)	18 (13.43)	5 (3.73)	44 (32.84)	27 (20.15)	23 (17.16)	17 (12.69)
T2D patients (total)	3 (3.95)	1 (1.32)	43 (56.58)	14 (18.42)	12 (15.79)	3 (3.95)
P-value	0.031	0.4211	0.0008[a]	0.7614	0.7973	0.0491
Non-diabetic control (Greek)	14 (14.14)	2 (2.02)	29 (29.29)	25 (25.25)	17 (17.17)	12 (12.12)
T2D patients (Greek)	3 (4.22)	1 (1.40)	40 (56.33)	12 (16.90)	12 (16.90)	3 (4.22)
P-value	0.039	1.000	0.004[b]	0.169	0.963	0.100

P-values were evaluated with respect to the disease association of each genotype combination relative to the remaining genotype combinations. [a]OR=2.67; [b]OR=3.11. T2D, type 2 diabetes; OR, odds ratio.

levels were significantly higher among T2D patients (P<0.0001; P=0.0052) and HDL levels were significantly lower (P<0.0001). The observed age difference among T2D patients and non-diabetic control subjects was significant (P<0.0001), potentially because the majority of individuals with T2D are diagnosed at an older age (data not shown). All other parameters, such as smoking status, did not differ among T2D patients and non-diabetic control subjects in the present study.

Genotype frequencies for rs3749166 and rs5404 SNPs in T2D patients and non-diabetic control subjects. The rs3749166 polymorphism was detected by PCR amplification (data not shown) and sequencing of a 153-bp PCR fragment, which included the SNP (Fig. 1A). Similarly, a 344-bp fragment, including the rs5404 SNP was amplified by PCR (data not shown) and analyzed by sequencing (Fig. 1B).

The rs3749166 and rs5404 frequencies for the Greek T2D patients and non-diabetic controls are summarized in Table III. Statistical analysis of these data revealed that only the heterozygous rs3749166 genotype (AG, partially epigenetic) was associated with T2D, while the epigenetic genotype (GG) appeared to be protective for the disease (P=0.0262; Table IIIA). A more significant positive correlation was obtained when the PGP data were incorporated into the study (P=0.0039; Table IIIA).

Analysis of the rs5404 polymorphism from the two sets of data revealed that the homozygous TT genotype was not observed, although the CT frequency was significant (21.28% in T2D patients and 34.69% in non-diabetic control subjects) and that the T genotype may be protective for the disease (Table IIIB; P=0.0205 and P=0.0258).

Finally, the association of these splicing-affecting genotype combinations with TD2 was analyzed. The results are presented in Table IIIC and reveal that only the AG/CC genotype is strongly associated with T2D in all cases examined [Greek: P=0.004 and odds ratio (OR), 3.11; PGP: P=0.0008 and OR, 2.67]. Furthermore, the GG/CC and AG/CT genotypes may be protective for the disease. In addition, the T allele was infrequent among individuals who were homozygous for rs3749166 (GG/CT epigenetic genotype).

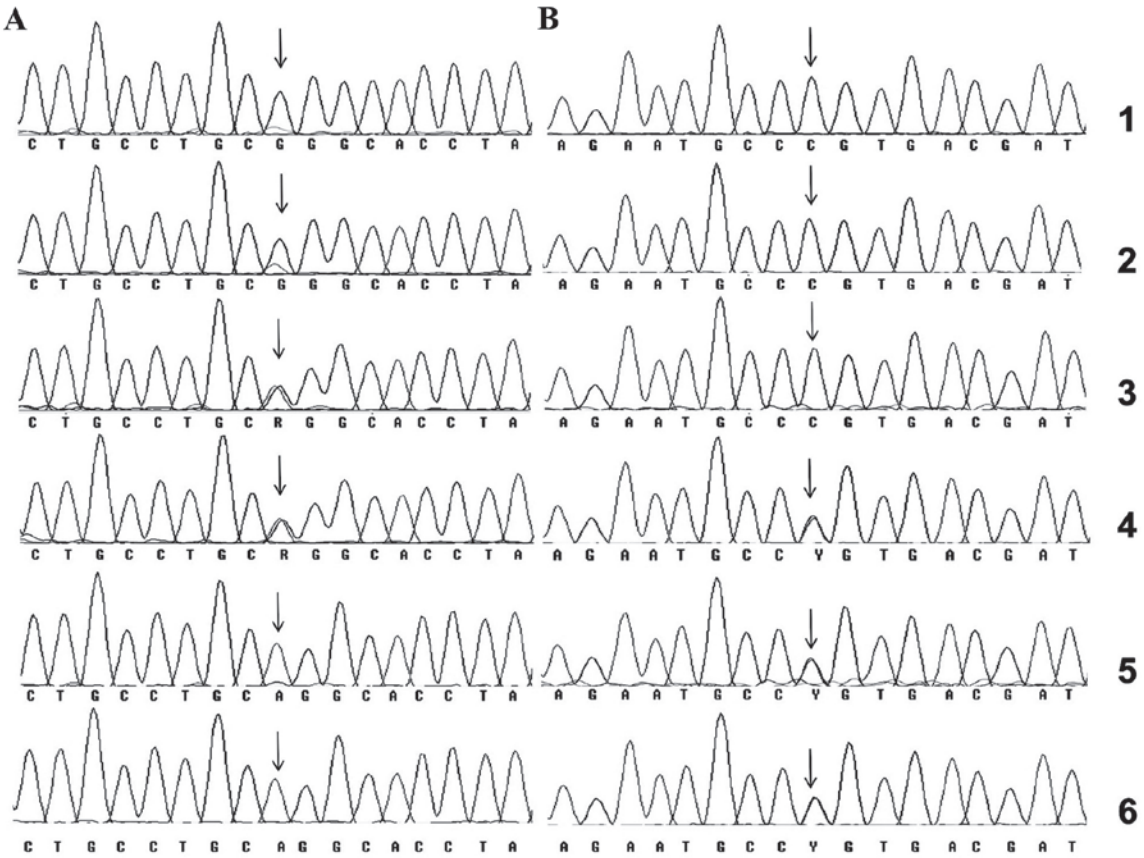

Figure 1. (A) Nucleotide sequence of the amplified fragments containing the rs3749166 (A>G) calpain 10 polymorphism for samples 34, 40, 187, 200, 46 and 117 (rows 1-6, respectively). (B) Nucleotide sequences of the amplified fragments of rs5404 (C>T) solute carrier family 2 member 2 for the samples 3, 68, 74, 19, 47 and 77 (rows 1-6, respectively). rs3749166 and rs5404 polymorphic sites are indicated using arrows.

Table IV. AG/CC genotype combination among all individuals and T2D patients, relative to the HbA1c levels (≥8.5%). T2D patients are shown in parenthesis.

	AG/CC	AA/CC	GG/CC	AG/CT	AA/CT	GG/CT
HbA1c ≥8.5%	19 (19)	3 (3)	1 (1)	3 (3)	0	0
HbA1c <8.5%	50 (21)	26 (9)	16 (2)	34 (9)	15 (3)	3 (1)
Total	69 (40)[a]	29 (12)	17 (3)	37 (12)	15 (3)	3 (1)

[a]P=0.0002, OR=5.10 (P=0.0306, OR=1.30). T2D, type 2 diabetes; HbA1c, glycosylated plasma hemoglobin; OR, odds ratio.

Association of the rs3749166/rs5404 genotype combinations with glucose metabolism. Another common characteristic among carriers of the AG/CC genotype (disease-associated) is the presence of high HbA1c levels (≥8.5%) (Table IV; P=0.0002, OR, 5.10) although neither of the polymorphisms was found to be independently associated with the T2D criteria (elevated fasting glucose levels and HbA1c).

Discussion

Elucidating the impact of epigenetic synonymous SNPs, particularly those involved in the regulation of alternatively spliced exons, is critical for understanding the pathogenesis of complex diseases. The polymorphisms included in the present study were selected on the basis of their epigenetic character and because they are the only synonymous SNPs strongly modifying the splicing-associated exonic enhancers associated with glucose transport (11). CAPN10 and GLUT2 participate in complementary transporter systems, which might be expected to act in a concerted manner. CAPN10 is a T2D-associated protease, which facilitates insulin-stimulated GLUT4 translocation via its activity on the distal secretory pathway (16). Although the association of rs3749166 with T2D has been the subject of various reviews and meta-analytic studies, it is still questioned if it may influence the development of T2D independently or in combination with other CAPN10 gene polymorphisms (17,18). Furthermore, the second SNP investigated in the present study, rs5404 in SLC2A2, has been evaluated in association with T2D (19-22); however, the obtained results were contradictory. In certain studies (20,21) a

Table V. Evaluation of the ESE modifications introduced by the rs3749166 (A>G) and rs5404 (C>T) SNPs using ESE finder [Cartegni *et al* (4)].

Gene	SNP	Exon type	Splice site/SR protein binding	Major allele ESE finder score	Minor allele ESE finder score	ΔScore[a]
CAPN10	rs3749166	Alternative	Exon 11 splice site (3SS_U2_human)	10.350	-3.220	13.570
SLC2A2	rs5404	Constitutive	SRp40	3.793	6.324	2.531
			SF2/ASF (IgM-BRCA1)	2.337	3.769	1.402
			SF2/ASF	3.162	3.778	0.616

[a]ΔScore: Difference of the ESE scores between the major and minor allele. Lower splicing score limit according to ESE finder: 3SS_U2_human=6.632; SRp40=2.670; SF2/ASF(IgM-BRCA1)=1.867; SF2/ASF=1.956. ESE, exonic splicing enhancers; SNP, single nucleotide polymorphism; CAPN10, calpain 10; SLC2A2, solute carrier family 2, member 2.

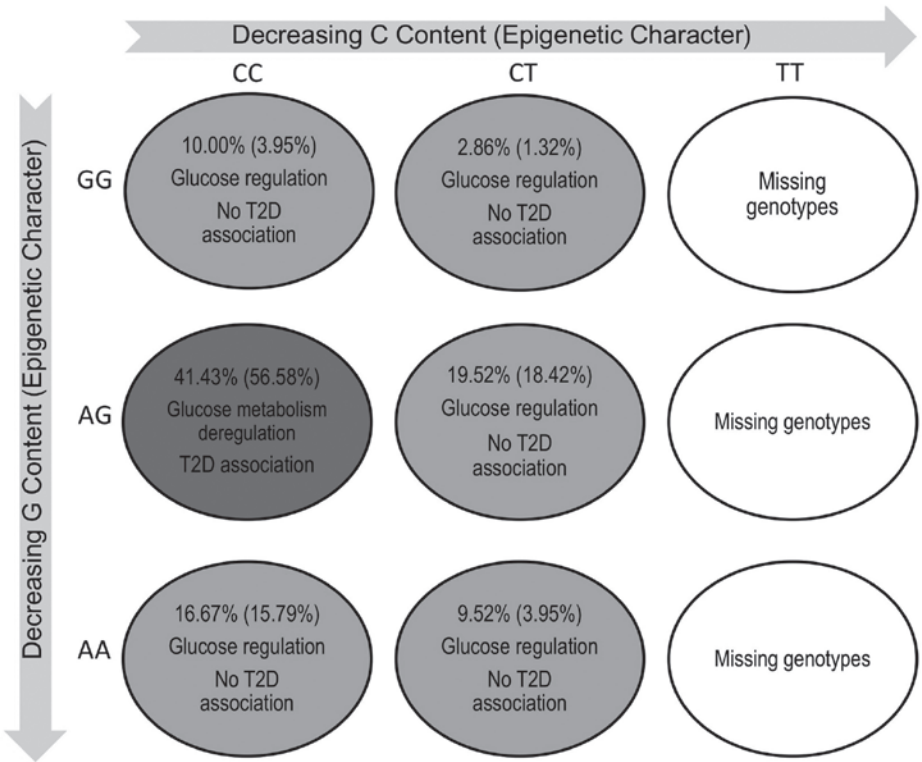

Figure 2. Frequencies of combined observed and expected genotypes resulting from rs3749166 and rs5404 single nucleotide polymorphism in all analyzed cases. The T2D patient frequencies are presented in parenthesis. The genotypes are presented in order of decreasing epigenetic character. Missing combined genotypes are also included. T2D, type 2 diabetes.

significant risk was observed among homozygotes, which was similar to the present results, while in another study (19) the minor allele was found to be associated with increased disease risk and with reduced postprandial glucose levels. To the best of our knowledge, the present study is the first to provide a comprehensive concurrent analysis of two SNPs (rs3749166 and rs5404) under investigation, which appear to be critical for the splicing of genes involved in complementary glucose transport systems.

Computational analysis has shown that the two polymorphisms interfere with splicing regulation (11). However, according to the data reported by Karambataki *et al* (11) and summarized in Table V (4), these SNPs modify the binding potential of splicing factors in different ways. rs3749166 (A allele) in CAPN10 strongly modifies the binding site of 3SS_U2 splicing enhancer of alternatively expressed exon 11 and, thus, may lead to the production of more than one splicing product in AG heterozygotes. By contrast, in its heterozygotic state, rs5404 (T allele) in SLC2A2 modifies the response of the ESE elements in this sequence to serine/arginine-rich (SR) proteins, particularly SRp40 and SF2/ASF (IgM-BRCA1) (26). As the two SNPs modify CpG sequences, they also perturb epigenetic regulation for the homozygotic genotypes AA and TT (but not GG and CC, or AG and CT genotypes); however, they do not introduce functionally significant single amino acid modifications (coding synonymous).

The present results, summarized in Fig. 2, indicate that the most protective genotype for T2D is the fully epigenetic genotype. In accordance with the above-mentioned analysis, the heterozygous rs3749166 and rs5404 genotypes are also differently associated with T2D. Carriers of the AG/CC genotype (heterozygous for rs3749166) are significantly more frequent among the T2D patients and exhibit particularly high levels of HbA1c, probably indicating resistance to pharmaceutical intervention for T2D. Similar findings regarding the negative effect associated with the synthesis of two different isoforms have been recently reported in association with heterozygous SNPs causing alternative splicing. For example, Tian *et al* (27) reported a number of disorders associated with alternative exon expression and splicing. In addition, Kurzawski *et al* (14) reported on the effect of epigenetic SNP rs5030952 in CAPN10, which exhibits a heterozygotic association with post-transplant diabetes mellitus.

By contrast, the presence of the apparently protective rs5404 SNP (CT genotype in SLC2A2) is potentially associated with the modified response of CT heterozygotes to different stimuli (based on the data from ESE score analysis at least one novel ESE is formed, which significantly responds to SRp40 proteins). This appears to be particularly significant for carriers of the rs3749166 CAPN10 AG genotype (the combined AG/CT genotype is not T2D-associated, and its carriers do not exhibit particularly high levels of HbA1c. The advantage of epigenetic regulation provided by the C allele and, thus, the ESE response may be lost among TT homozygotes. This could be a possible explanation for the absence of TT homozygotes regardless of the relatively high total frequency of the T allele (21.28%, non-Mendelian genetics).

These findings provide the hypothesis that mutations modifying the response to splicing regulatory mechanisms (epigenetic and ESE) may be associated with strong negative functional changes, and exhibit complex, nonlinear disease associations (28). Provided that functionally significant epigenetic SNPs are frequent (11), this type of genetic variation is expected to have a strong impact on disease and evolution.

A major obstacle in investigating complex pathological conditions, such as metabolic syndrome, is the limited understanding of the regulatory factors involved in the expression of interacting components. Recent evidence indicates the key role of alternative RNA expression in developmental changes (29), and the production of coding and non-coding RNA sequences. Another factor is the complex epigenetic modifications, which may also lead to the expression of different RNA isoforms. The current results indicate likely synergies between synonymous splicing-regulatory epigenetic SNPs, which modify the splicing potential of two different glucose transport-associated genes, and reveal that bioinformatic analysis and careful investigation of the SNPs under investigation may become a powerful tool for identifying potentially significant genetic modifications with respect to splicing.

In conclusion, the results presented above indicate for the first time, to the best of our knowledge, the correlation and disease association of two synonymous epigenetic SNPs, which participate in the regulation of the glucose transport system and introduce exclusively splicing-associated modifications. Taken together, these results reveal that T2D is subject to deregulation by complex splicing mechanisms, which may exhibit heterozygous disease association or protection, depending on the splicing-affecting genetic variation. A detailed bioinformatic analysis of the changes introduced by SNPs would facilitate the understanding of the impact of functional changes introduced by genetic variation.

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