Research Paper

Comparative understanding of *UTS2* and *UTS2R* genes for their involvement in type 2 diabetes mellitus

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Several reports have shown that urotensin 2 (*UTS2*) and its receptor (*UTS2R*) are involved in glucose metabolism and insulin resistance, which lead to development of type 2 diabetes mellitus (T2DM) in humans. In the present study, we annotated both bovine *UTS2* and *UTS2R* genes and identified 5 single nucleotide polymorphisms (SNPs) for the former gene and 14 mutations for the latter gene. Four mutations were genotyped on a Wagyu x Limousin reference population, including 6 F_1 bulls, 113 F_1 dams and ~250 F_2 progeny. Among 12 phenotypes related to fat deposition and fatty acid composition, we observed that the *UTS2* gene was significantly associated with the amount of skeletal saturated fatty acids, while its receptor (*UTS2R*) gene had significant effects on amounts of saturated and monounsaturated fatty acids, Δ^9 desaturase activity for converting 16:0 into 16:1, muscle fat (marbling) score and *Longissimus Dorsi* muscle area. However, in this population, these markers were not associated with subcutaneous fat depth or percent kidney, pelvic and heart fat. We also found that mutations in the promoter regions altered the promoter activities in both genes and coding SNPs might affect the mRNA stability in the *UTS2R* gene. Overall, our present study provides the first evidence that both *UTS2* and *UTS2R* genes regulate skeletal muscle fat accumulation and fatty acid metabolism, thus indicating their potential pathological functions related to obesity and T2DM in humans.

Key words: Type 2 diabetes mellitus, *UTS2*, *UTS2R*, skeletal fat accumulation, fatty acid composition.

1. Introduction

Three members of the urotensin II family of peptides have been discovered in mammals, including urotensin 2 (UTS2), urotensin 2 receptor (UTS2R) and urotensin 2 domain containing (UTS2D). UTS2 is cyclic peptide with an 11 amino acid mature peptide in human, but it has been recognized as a hormone in the neurosecretory system of teleost fish for over 30 years [1]. In addition, UTS2 is a potent vasoconstrictive peptide that regulates both endothelium-dependent and independent vasodilation [2]. The human orphan G protein-coupled receptor 14 (GPR14) was cloned in 1995 [3] and then confirmed to function as the UTS2 receptor (UTS2R) [4]. Interestingly, the UTS2R shares highest identity with the somatostatin receptor SSTR4 [3]. A novel urotensin II-related peptide, now named the urotensin 2 domain containing (*UTS2D*) was also isolated in human, mouse and rat [5]. UTS2D binds and activates the urotensin 2 receptor, suggesting that it is the endogenous and functional ligand for UTS2R.

Among these three genes, both *UTS2* and *UTS2R* have been reported to affect glucose metabolism and insulin resistance, a core pathological characteristic of patients with type 2 diabetes mellitus (T2DM). In Hong Kong Chinese, the GGT haplotype (-605G, 143G and 3836T) in the *UTS2* gene is associated with higher plasma level of urotensin 2 and insulin, and higher homeostasis model assessment of insulin resistance index and beta-cell function, while the AC haplotype (-11640A and -8515C) in the *UTS2R* gene has a higher amount of plasma glucose 2 h after a 75 g oral glucose load [6]. In human diabetic tissue, Langham and colleagues [7] found that expression of both *UTS2* and *UTS2R* are increased 45- and almost 2,000-fold in comparison to control nephrectomy tissue, respectively (P<0.0001) using quantitative real-time polymerase chain reaction. In the healthy rat, infusion of synthetic rat urotensin 2 inhibits both insulin release induced by glucose and insulin responses induced by carbachol, glucagon-like peptide-1, and a calcium channel agonist [8]. However, in streptozotocin-induced diabetic rats, long-term

treatment with palosuran, a UTS2R antagonist, improved survival, increased insulin, and slowed the increase in glycemia, glycosylated hemoglobin, and serum lipids [9]. Therefore, the urotensin 2 system plays a unique role both in insulin secretion and in the renal complications of diabetes.

Studies have shown that the fat droplets accumulated in human skeletal muscle are a major contributor to insulin resistance [10]. For example, in male Pima Indians, negative relationships were found between amounts of triglyceride in skeletal muscle and physiological and supraphysiological insulin levels, and nonoxidative glucose disposal (r = -0.44 – -0.53, P < 0.01) [11]. In a European population, muscle lipid was correlated with percent body fat (r = 0.50, p = 0.028), waist:hip ratio (r = 0.74, p < 0.001), visceral fat (r = 0.62, p = 0.004) and insulin sensitivity (r = -0.53, p = 0.016) [12]. Just recently, Suzuki and colleagues [13] reported that enhanced muscle mass might have positive effects on fat utilization, thus reducing the metabolic problems and insulin resistance associated with obesity and T2DM. In addition, both *UTS2* and *UTS2R* mRNA are expressed in skeletal muscle as well as in lung, pancreas, kidney and liver [14]. Therefore, the objective of the present study was to determine whether both *UTS2* and *UTS2R* genes contribute to muscle lipid metabolism using cattle as a model organism.

2. Materials and Methods

Animals, body fat deposition and fatty acid composition.

A Wagyu–Limousin F_2 reference population was used in the present study, including 6 F_1 bulls, 113 F_1 dams and ~250 F_2 progeny. The entire core of the *longissimus dorsi* was sampled from these F_2 progeny and relative amounts of saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA), three indexes of Δ^9 desaturase activity as $R_1 = (14:1/14:0) \times 100\%$; $R_2 = (16:1/16:0) \times 100\%$ and $R_3 = (18:1/18:0) \times 100\%$, conjugated linoleic acid mg/100 g dry meat (CLA), cholesterol mg/100 g dry meat (CHOL), *Longissimus Dorsi* area (LDA in in^2) and muscle fat (marbling) score (MFMS) were measured. In addition, subcutaneous fat depth (SFD) and percent kidney-pelvic-heart fat (KPH) were also recorded in the population. Muscle fat (marbling) score was determined at the interface of the 12th and 13th ribs and was evaluated by subjective comparison of the amount of fat within the longissimus muscle with photographic standards (National Livestock and Meat Board 1981). Subcutaneous fat depth was recorded at the 12th rib at a point three-fourths the width of the longissimus muscle from its chine bone end. The amount of KPH was estimated and recorded as a percentage of carcass weight. Development and management of the Wagyu–Limousin reference population and measurement and definition of these phenotypes were described previously [15,16].

Gene, mutation, genotyping and association.

Both cDNA and genomic DNA sequences of the bovine *UTS2* and *UTS2R* were retrieved from the GenBank databases using a comparative approach, as previously described [17]. Alignment between cDNA and genomic DNA sequences was used to determine genomic organization for each of these two genes. A total of 11 pairs of primers (Table 1) were designed based on the genomic DNA sequences and used to screen genetic polymorphisms in the promoter, coding and untranslated regions of both bovine *UTS2* and *UTS2R* genes. Approximately 50 ng of genomic DNA each from six Wagyu x Limousin F_1 bulls was amplified in a final volume of 10 µl that contained 12.5 ng of each primer, 150 µM dNTPs, 1.5 mM MgCl$_2$, 50 mM KCl, 20 mM Tris-HCl and 0.25U of Platinum Taq polymerase (Invitrogen, Carlsbad, CA). The following PCR conditions were used: 94°C for 2 min, 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, followed by a final 5 min extension at 72°C. PCR products were then sequenced on ABI 3730 sequencer in the Laboratory for Biotechnology and Bioanalysis (Washington State University) using a standard protocol and polymorphisms were detected.

A total of 5 mutations were identified in *UTS2* and 14 in *UTS2R* gene, respectively. Two SNPs (single nucleotide polymorphisms), one in the promoter and one in intron 2 of *UTS2*, and two mutations, one INDEL (insertion/deletion) in the promoter and one coding SNP of *UTS2R*, were selected for genotyping on a Sequenom iPLEX assay using services provided by the Children's Hospital Oakland Research Institute, Oakland, California. The phenotypic data for all measurements described above were analyzed in a fixed effects model that included the effects of year, gender, age at harvest (linear) and the genotype for each marker using the GLM (general linear model) procedure of SAS v9.1 (SAS Institute Inc., Gary, NC). Pair-wise comparisons of least squares means were performed using a protected t-test.

Promoter activity assay.

Among the genetic polymorphisms discovered above, three SNPs were located in the promoter region of *UTS2* and one insertion/deletion in the promoter of *UTS2R* gene, respectively. The effects of these mutations on promoter activities were then examined using a Dual-Luciferase Report Assay System (Promega, Madison, WI). The forward and reverse

gene-specific primers that were used to amplify the promoter regions (Table 1) were then engineered with a 5′ *Bgl*II and 3′ *Hind*III site plus a 5′ tail of CTTC, respectively, for directional cloning into the *Bgl*II/*Hind*III site of pGL3-basic (Premega, Madison, WI). Two types of haplotypes at three polymorphic sites in the *UTS2* promoter: A-C-A and C-A-G; and two types of INDELs in the *UTS2R* promoter: TA insertion and TA deletion were prepared for the promoter constructs. The human lung carcinoma H1299 cells were transfected with each of the recombinant pGL3 plasmids containing the constructs described above. pRL-CMV plasmid was also co-transfected into H1299 cells as a transfection control. The human cells were collected 28 hours post-transfection and firefly luciferase and *Renilla* luciferase activities were measured with the Dual Luciferase Reporter Assay system according to the manufacturer's protocol. Light emission was quantified with a Multilabel Counter (Wallace 1420 Victor 2, Turku, Finland). Triplicate data were collected from three independent experiments. The ratios of firefly luciferase activity to *Renilla* luciferase activity were calculated and used to compare the differences in activity between two constructs for each gene.

Table 1. Primers designed for mutation detection in the bovine *UTS2* and *UTS2R* genes.

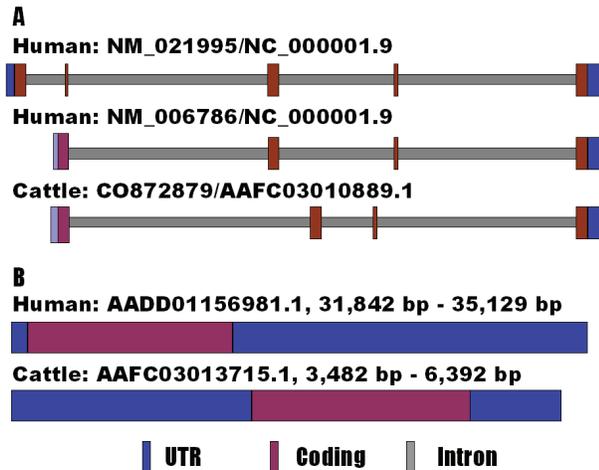| Target region	Primer sequences (5'-3')	Size in bp	Annealing Tm
UTS2			
Promoter	Forward: GCCTTGAGATTGAATTTTTGCTGTG Reverse: AAATTTACTGTCTTGTTGCCTAGTG	676	61°C
Exon 1 (relic)	Forward: TTTTGTACACTAGGCAACAAGACAG Reverse: TGAGACATGCCTTAAGAATCCTCAGA	540	61°C
Exon 1	Forward: GGGATGATATGAGGTCATAGGATAAT Reverse: TCTGAGACATGCCTTAAGAATCC	409	57°C
Exon 2	Forward: CTCCTCCAGGGGATCTTCTCAAC Reverse: TAATGCTCTTCTTCCCTCCCCTTG	506	61°C
Exon 3	Forward: TTTGTGAACTCTCGGCGGCTAGAAA Reverse: GGTCCTTGGACCCAGTGAAGATAA	557	61°C
Exon 4	Forward: ATCCCATGAAACAGCAAGAAAACC Reverse: CAACCACTCATAGTATCTGCAAAACA	404	61°C
UTS2R			
Promoter	Forward: AGTCACCATCACCAAAATCATCCA Reverse: CGGACTCGGATTCAGATTGTCAGT	676	61°C
Exon 1A	Forward: GAGAGGCCTTTGAACTTGCACTGT Reverse: TAGACGTACATGGAGGCAGAGGTG	631	61°C
Exon 1B	Forward: GGCATGGCAGGCAATGTGTA Reverse: ATGCCCAGGATGAGGTAGAGCAC	599	61°C
Exon 1C	Forward: GTGGTCATCGGGCTGCTCTAC Reverse: CCACTCCCCACAGCCTACCC	531	61°C
Exon 1D	Forward: CAGCCAGCAAGCCACTGAGAC Reverse: GGGTCCTGCCTCCTTTGACAC	601	61°C

3. Results and Discussion

Comparative annotation of *UTS2* and *UTS2R* genes.

The cDNA sequences of human *UTS2* (NM_021995.1, transcript variant 1) and *UTS2R* (NM_018949.1) genes were used as references for BLAST searches against GenBank "nr" database, thus retrieving orthologous cDNA sequences for both bovine genes (CO872879 for *UTS2* and NM_001040484.1 for *UTS2R*). BLAST searches using the bovine cDNA sequences as queries then obtained their genomic DNA sequences (AAFC03010889 for *UTS2* and AAFC03013715 for *UTS2R*) from the bovine genome sequencing database (Build 3.1).

For the bovine *UTS2* gene, alignment between the cDNA and the genomic DNA sequences revealed that it consists of four exons only, which correspond to the transcript variant 2 of the human ortholog (NM_006786) (Figure 1A). However, alignment

between the human transcript variant 1 (NM_021995) and the same bovine genomic DNA contig detected a relic for exon 1 of the variant, but it seems that its expression in cattle is totally destroyed by an unusual intron splicing site of CT instead of GT (see AAFC03010889.1:*g.9800C*). In fact, GenBank databases show that the *UTS2* variant 1 does not exist in either mouse (NM_011910) or rat (NM_019160).

A
Human: NM_021995/NC_000001.9

Human: NM_006786/NC_000001.9

Cattle: CO872879/AAFC03010889.1

B
Human: AADD01156981.1, 31,842 bp - 35,129 bp

Cattle: AAFC03013715.1, 3,482 bp - 6,392 bp

UTR   Coding   Intron

Figure 1. Genomic organizations of *UTS2* (A) and *UTS2R* (B) between human and cattle.

In mammals, the *UTS2R* is an intronless gene. However, the current GenBank information shows that the bovine *UTS2R* gene contains a 5′UTR of 89 bp and a 3′UTR of 384 bp (NM_001040484.1), while the human ortholog has none of them (NM_018949.1). Therefore, we decided to re-annotate the gene in both human and cow using an electronic rapid amplification of cDNA ends (e-RACE) approach. Basically, we used the genomic DNA sequences flanking the coding sequences as queries for BLAST searches to determine both 5′UTR and 3′UTR ends of the genes against the EST (expressed sequence tag) databases. The e-RACE approach retrieved an EST BF513269 for the 5′UTR end and AA621666 for the 3′UTR end of the human *UTS2R* gene. The same approach also obtained an EST EE388038 for the 5′UTR end and CK775836 for the 3′UTR end of the bovine ortholog. As a result, we complied a tentative full-length cDNA sequence of 3,288 bp for the human gene (see AADD01156981.1 from position 31,842 bp to 35,129 bp) and 2,911 bp for the bovine gene (see AAFC02086367.1 from position 3,482 bp to 6,392 bp). The former cDNA sequence has a 5′UTR of 95 bp, a coding sequence of 1170 bp and a 3′UTR of 2,023 bp, while the latter cDNA sequence contains a 5′UTR of 1,270 bp, a coding sequence of 1,155 bp and a 3′UTR of 486 bp (Figure 1B). In addition, a polyadenylation site

(AAUAAA) was also identified in the newly annotated cDNA sequences of both human and bovine *UTS2R* genes.

Genetic polymorphisms.

Direct sequencing of PCR products on 6 F_1 bulls identified three SNPs in the promoter and two SNPs in intron 2 of the *UTS2* gene: AAFC03010889.1:*g.9408A>C*, *g.9552C>A*, *g.9628G>A*, *g.13294G>A* and *g.13900A>C*, respectively. Interestingly, we observed in these 6 F_1 bulls that three promoter SNPs and two intronic SNPs have no historical recombination by forming two haplotypes (ACA and CAG) in the former region and two haplotypes (GA and AC) in the latter region. Therefore, only one SNP from the promoter and one SNP from the intron 2 were chosen for genotyping.

For the bovine *UTS2R*, one insertion/deletion (INDEL) with two nucleotides of *TA* (AAFC03013715.1:*g.2935-36TA>--*) was detected in the promoter region, while 13 SNPs were identified in the coding and 3′UTR regions of the gene, including AAFC03013715.1:*c.6446T>C*, *c.6506C>T*, *c.6593T>C*, *c.6749G>A*, *c.6830T>C*, *c.6842A>G*, *c.7232G>A*, *c.7359C>T*, *g.7466G>C*, *g.7632A>G*, *g.7692C>T*, *g.7714G>A* and *g.7720G>A*, respectively. Although the first eight SNPs in the bovine *UTS2R* gene are coding SNPs, none are missense mutations. However, the Mfold web server [18] predicted that these 13 SNPs do cause mRNA secondary structure changes in the bovine gene (Figure 2). In addition to the promoter INDEL, only one of these 13 SNPs was chosen for genotyping, because they form just two haplotypes as described in Figure 2.

Associations of *UTS2* and *UTS2R* with body fat deposition.

The association analysis involved four mutations, i.e., AAFC03010889.1: *g.9628G>A* and *g.13900A>C* in the bovine *UTS2* gene and AAFC03013715.1:*g.2935-36TA>--* and *c.6506C>T* in the bovine *UTS2R* gene, which were genotyped on a Sequenom iPLEX assay. As two SNPs in the former genes had no combination in these F_2 progeny by forming two haplotypes AA and CG, only three markers were used in the data analysis. The phenotypes that had at least one suggestive (P=0.05 – 0.06) or significant (P<0.05) association are listed in Table 2. The *UTS2* gene had a significant effect on one trait only, i.e., the relative amount of saturated fatty acids. In particular, animals with *GG* genotypes had 1.39 units (P=0.0365) less SFA than animals with *AA* genotypes. For the bovine *UTS2R* gene, the promoter INDEL polymorphism was significantly associated with MUFA (P=0.0452), R_2 (P=0.0177) and LDA

(P=0.0488) (Table 2). The homozygous animals with the insertion allele (*TA/TA*) had 1.65 units higher MUFA, but 1.70% lower R_2 activity and 0.56 in² smaller LD muscle area than the homozygous animals with TA deleted (Table 2). The coding SNP in the bovine *UTS2R* gene had significant impacts on both SFA and MUFA, plus a suggestive effect on MFMS (Table 2). The *TT* animals had an additional 0.64 units of muscle fat deposition and 1.82 units of MUFA, but 1.75 less units of SFA than the *CC* animals.
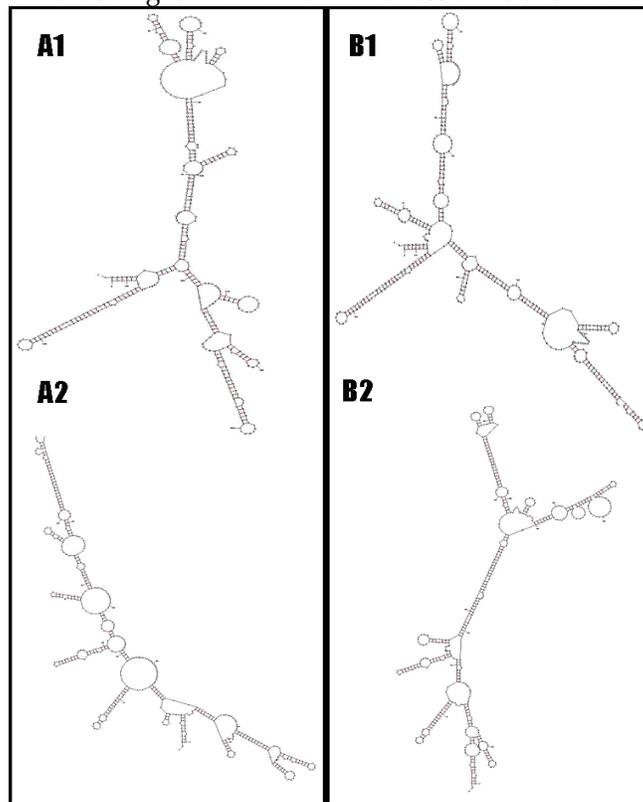
Figure 2. The *UTS2R* mRNA local secondary structures between haplotype TCTGTAGCGACGG and haplotype CTCACGATCGTAA based on two-segment analysis (A1 vs. B1 and A2 vs. B2).

Table 2. Associations of *UTS2* and *UTS2R* genes with muscle fat phenotypes*

| Phenotype	UST2 *g.9628G>A*		UST2R *g.2935-36TA>--*		UST2R *c.6506C>T*	
SFA	A/A	43.55±0.181[a]	-- / --	44.40±0.618[a]	CC	44.39±0.311[a]
	A/G	43.56±0.224[a]	TA/ --	43.73±0.238[a]	CT	43.44±0.168[b]
	G/G	42.16±0.518[b]	TA/TA	43.24±0.175[a]	TT	42.64±0.305[c]
	P	0.0365	P	0.0823	P	0.0004
MUFA	A/A	50.28±0.193[a]	-- / --	48.95±0.651[a]	CC	49.49±0.330[a]
	A/G	50.44±0.239[a]	TA/ --	50.28±0.250[ab]	CT	50.40±0.178[b]
	G/G	51.31±0.552[a]	TA/TA	50.60±0.185[b]	TT	51.31±0.323[c]
	P	0.2102	P	0.0452	P	0.0006
R_2	A/A	14.90±0.174[a]	-- / --	16.16±0.585[a]	CC	15.21±0.306[a]
	A/G	14.28±0.215[a]	TA/ --	14.81±0.225[b]	CT	14.58±0.165[a]
	G/G	14.85±0.496[a]	TA/TA	14.46±0.166[b]	TT	14.45±0.299[a]
	P	0.0762	P	0.0177	P	0.1394
LDA	A/A	13.18±0.136[a]	-- / --	13.55±0.450[ab]	CC	13.40±0.234[a]
	A/G	13.21±0.168[a]	TA/ --	13.51±0.176[a]	CT	13.25±0.128[a]
	G/G	13.14±0.385[a]	TA/TA	12.99±0.128[b]	TT	12.78±0.229[a]
	P	0.9847	P	0.0488	P	0.1187
MFMS	A/A	6.10±0.115[a]	-- / --	5.83±0.382[a]	CC	5.86±0.197[a]
	A/G	6.14±0.142[a]	TA/ --	5.89±0.150[a]	CT	6.07±0.108[ab]
	G/G	6.16±0.326[a]	TA/TA	6.26±0.109[a]	TT	6.50±0.193[b]
	P	0.9669	P	0.1075	P	0.0558

*Each genotype was presented with LSM±SE (least square means ± standard error) and

means within a column without common superscripts are significantly different (P<0.05)

among three genotypes.

Mutations and promoter activities.

As indicated above, two types of haplotypes at three polymorphic sites in the *UTS2* promoter: A-C-A and C-A-G; and two types of INDELs in the *UTS2R* promoter: TA insertion and TA deletion, were prepared for the promoter constructs to investigate how these mutations affect promoter activities of these two genes. In the *UTS2* promoter, the C-A-G construct produced less promoter activities than the A-C-A construct by 27% in the H1299 cells (P=0.0234) (Figure 3). In the *UTS2R* promoter, the deletion allele yielded lower promoter activities than the insertion constructs by 16% in the same cell lines (P=0.0436) (Figure 3). A search for transcription binding sites using MatInspector [19] indicated AAFC03010889.1:*g.9408A* in the *UTS2* promoter gained a site for FAST-1 SMAD interacting proteins, while AAFC03010889.1:*g.9408C* gained a site for neuron-specific-olfactory factor. At the *g.9552C>A* polymorphic site, the former allele had a transcription binding site for AP4 and related proteins only, while the latter allele possessed one site for MAF/AP1 related factors and one site for octamer binding protein. There were a total of four binding sites for MAF and AP1 related factors, TCF11 transcription factor, TALE homeodomain class recognizing TG motifs and MYT1 C2HC zinc finger protein when an A allele is located at position AAFC03010889.1:*g.9628*. When the *g.9628A* is substituted by *g.9628G*, only one site was created for activator/repressor binding to transcription initiation site. Overall, the A-C-A haplotype in the *UTS2* promoter had two additional binding sites than the C-A-G haplotype. However, the MatInspector program failed to detect any gain/loss of transcription binding sites for the insertion or deletion allele in the promoter of *UTS2R* gene although they showed a difference in promoter activities as described above.
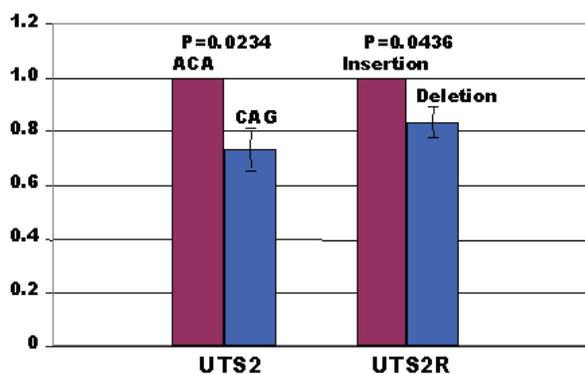
Figure 3. Affects of mutations on promoter activity in the H1299 cells.

In summary, our present study provided evidence that both *UTS2* and *UTS2R* genes are involved in skeletal muscle fat deposition and fatty acid metabolism. Such evidence is supported by the fact that none of the mutations genotyped in the population were associated with either subcutaneous fat depth (P=0.2074 – 0.9847) or the fat percentage surrounding the kidney, pelvic and heart fat (P=0.3278 – 0.7059) (data not shown). Instead, the *UTS2* gene was significantly associated with relative amount of saturated fatty acids, while its receptor had significant effects on relative amounts of saturated and monounsaturated fatty acids, Δ^9 desaturase activity for converting 16:0 into 16:1, overall fat deposition in muscle and LD muscle area (Table 2). Although mutations in the promoter regions altered the promoter activities in both genes (Figure 3) and coding SNPs might affect the mRNA stability in UTS2R gene (Figure 2), how these genes regulate fat deposition and fatty acid composition in skeletal muscle remains unknown.

Acknowledgement

Conflict of interests

The authors have declared that no conflict of interest exists.

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