Brief Genetics Report

Systematic Search for Single Nucleotide Polymorphisms in the FOXC2 Gene

The Absence of Evidence for the Association of Three Frequent Single Nucleotide Polymorphisms and Four Common Haplotypes With Japanese Type 2 Diabetes

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FOXC2, a forkhead/winged helix transcription factor, represents a promising candidate gene for type 2 diabetes since transgenic mice that specifically overexpress this gene in adipocytes are lean and insulin sensitive. To determine whether there are single nucleotide polymorphisms (SNPs) in this gene that are associated with type 2 diabetes, sequences of the coding and ~1 kb of 5′ flanking regions in 24 Japanese type 2 diabetic subjects were initially analyzed using PCR direct sequencing, and the regions containing the identified polymorphisms were then examined. In 200 control subjects, three frequent SNPs were found (g. −512C>T [32.3%] and −350G>T [13.0%] in the 5′ flanking region and +1548C>T [10.0%] in the 3′ flanking region). Linkage disequilibria were found between all three pairs of these SNPs. Of the eight possible haplotypes defined by these SNPs, only four were found. When the frequencies of these SNPs and the four common haplotypes between 195 type 2 diabetic and 200 control subjects were compared, no association was evident. The +898C>T (Pro300Ser), +907C>A (Leu303Met), 1167_1169delCCA (389delHis), and +1251C>A (Ala417Ala) identified in the coding region were rare, although +907C>A could be higher in type 2 diabetic subjects (1.5%) than in control subjects (0.3%). Thus, the SNPs identified in the FOXC2 gene are unlikely to have major effects on susceptibility to Japanese type 2 diabetes. *Diabetes* 52: 562–567, 2003

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BAT, brown adipose tissue; IRS-1, insulin receptor substrate-1; PPARγ, peroxisome proliferator–activated receptor γ; SNP, single nucleotide polymorphism; Taq, thermus aquaticus; UCP-1, uncoupling protein-1; WAT, white adipose tissue.

Type 2 diabetes is characterized by insulin resistance in insulin target tissues and an impaired insulin secretion from pancreatic β-cells (1). The gene mutations identified thus far account for specific types of diabetes as single genetic factors and constitute only a small proportion of all type 2 diabetic cases (2). Common type 2 diabetes is thought to be a polygenic disease, and its major genetic factors remain to be elucidated (3). It has recently been reported that single nucleotide polymorphisms (SNPs) in calpain-10, peroxisome proliferator–activated receptor γ (PPARγ), and adiponectin are associated with type 2 diabetes (4–6).

FOXC2, a forkhead/winged helix transcription factor, has been identified as a key regulator of adipocyte metabolism (7). Transgenic mice that specifically overexpress the FOXC2 gene in white and brown adipocytes show a lean and insulin-sensitive phenotype. In these mice, intra-abdominal white adipose tissue (WAT) deposition is reduced with a change to brown fat–like histology, whereas interscapular brown adipose tissue (BAT) is hypertrophic. Serum triglycerides, free fatty acids, glucose, and insulin levels are all lowered. The enhanced expression of BAT-specific genes such as uncoupling protein-1 (UCP-1) and PPARγ coactivator-1 (PGC-1), and genes involved in the insulin signaling pathway in WAT, such as the insulin receptor and insulin receptor substrate-1 (IRS-1) could account for this improvement. Since an enhanced FOXC2 gene expression appears to counteract insulin resistance and obesity, this represents a promising candidate for a type 2 diabetes susceptibility gene.

In view of this, we initiated a systematic search for SNPs in the FOXC2 gene of Japanese subjects. The FOXC2 gene has only one exon based on the human draft sequence for the FOXC2 gene on chromosome 16 (GenBank accession no. NT_024788) and the human FOXC2 cDNA sequence (no. NM_005251). We initially examined this exon 1 that encodes the entire molecule and the ~1 kb of 5′ flanking region in 24 type 2 diabetic patients using PCR direct

TABLE 1
Clinical features of the control and type 2 diabetic subjects

	Control subjects	Type 2 diabetic subjects
n (M/F)	200 (108/92)	195 (95/100)
Age (years)	54.3 ± 8.1	60.0 ± 11.6
Age of onset (years)		48.4 ± 12.3
Duration of diabetes (years)		11.7 ± 9.1
Height (cm)	161.5 ± 8.1	157.9 ± 8.9
BW (kg)	60.3 ± 10.0	58.4 ± 12.0
Max BW (kg)		68.4 ± 14.7
δBW (kg)		9.9 ± 6.8
Age of max BW (years)		44.3 ± 14.5
BMI (kg/m^2)	23.0 ± 2.8	23.4 ± 3.7
Max BMI (kg/m^2)		27.3 ± 4.7
δBMI (kg/m^2)		4.0 ± 2.7
HbA_{1c} (%)	4.8 ± 0.3	7.7 ± 1.7
Treatment (diet/OHA/insulin)		46/104/45

Data are means ± SD. BW, body weight; Max BW, maximum body weight; δBW, Max BW − BW; δBMI, Max BMI − BMI; OHA, oral hypoglycemic agents.

sequencing. This screening of 24 subjects permits the detection of an allele whose frequency in patients is 3.3% with a power of 80%, and 4.7% with a power 90%. Since we found that the human draft sequence of FOXC2 contained one extra C at 1061 (NT_024788), resulting in a frameshift mutation, we used the one base shorter number beyond +1060 as the correct reference numbers of this gene. This sequence matched with the human FOXC2 cDNA sequence (no. NM_005251). The sequencing of both strands revealed the presence of two SNPs, −512C>T and −350G>T, in the 5′ flanking sequence; one synonymous SNP, +1251C>A (Ala417Ala) and one missense SNP, +907C>A (Leu303Met) in the coding region; and +1548C>T in the 3′ untranslated region. When the regions that included these identified SNPs were sequenced in a total of 195 type 2 diabetic subjects and 200 control subjects, the other two rare mutations, namely +898C>T (Pro300Ser) and a deletion of CCA between 1167 and 1169 (1167_1169delCCA, 389delHis), were also found as described below.

We determined the sequences of the regions containing these six SNPs and 1167_1169delCCA in 200 control subjects (See Table 1 for clinical features). The frequencies of these polymorphisms were determined to be −512C>T (32.3%), −350G>T (13.0%), +898C>T (0%), +907C>A (0.3%), 1167_1169delCCA (0.5%), +1251C>A (0%), and +1548C>T (10.0%) (Table 2). The frequencies of the genotypes in each of the SNPs with >5% frequencies, namely −512C>T, −350G>T, and +1548C>T, are also shown (Table 3). We then examined the linkage disequilibrium between any two of these three frequent SNPs. Linkage disequilibrium was found between all of the three pairs (Table 4). The estimated frequencies of haplotypes defined by −512C>T and +1548C>T were f(C-C) = 0.577, f(C-T) = 0.100, f(T-C) = 0.323, and f(T-T) = 0.000. The estimated frequencies of haplotypes defined by −512C>T and −350G>T were f(C-G) = 0.547, f(C-T) = 0.130, f(T-G) = 0.323, and f(T-T) = 0.000. The estimated frequencies of haplotypes defined by −350G>T and +1548C>T were f(G-C) = 0.771, f(G-T) = 0.099, f(T-C) = 0.129, and f(T-T) = 0.001. We then determined the estimated haplotype frequencies defined by these three SNPs, and only four of the eight possible haplotypes were detected (Table 5). The estimated haplotype frequencies defined by −512C>T, −350G>T, and +1548C>T were f(C-G-C) = 0.447, f(T-G-C) = 0.323, f(C-T-C) = 0.130, and f(C-G-T) = 0.100.

Next we sequenced the regions containing the identified polymorphisms in 171 additional type 2 diabetic subjects, and compared the allele frequencies of these polymorphisms between a total of 195 type 2 diabetic patients and 200 nondiabetic control subjects (see Table 1 for clinical features). No significant differences in the allele frequencies of −512C>T, −350G>T, +898C>T, +907C>A, 1167_1169delCCA, +1251C>A, and +1548C>T were detected between type 2 diabetic and control subjects (Table 2). The +907C>A (Leu303Met) was found in 6 of 390 alleles in type 2 diabetic subjects, but in only 1 of 400 alleles in the control subjects, suggesting that this missense mutation could be higher in type 2 diabetes, but it should be noted that the allele frequency was quite low. Genotype frequencies of the SNPs with >5% frequencies, namely −512C>T, −350G>T, and +1548C>T, are not significantly different between type 2 diabetic and control subjects (Table 3).

The estimated haplotype frequencies were then determined for type 2 diabetes. In the 195 type 2 diabetic subjects, significant linkage disequilibria were found to be the same as for the control subjects, and the estimated haplotype frequencies were also similar to those in control subjects. The estimated frequencies of haplotypes defined by −512C>T and +1548C>T were f(C-C) = 0.592, f(C-T) = 0.105, f(T-C) = 0.303, and f(T-T) = 0.000. The

TABLE 2
Allele frequency of SNPs in the FOXC2 gene for control and type 2 diabetic subjects

SNP	Control subjects	Type 2 diabetic subjects	*P*
−512C>T	129/400 (32.3%)	118/390 (30.3%)	0.591
−350G>T	52/400 (13.0%)	53/390 (13.6%)	0.834
+898C>T (Pro300Ser)	0/400 (0%)	1/390 (0.3%)	0.494
+907C>A (Leu303Met)	1/400 (0.3%)	6/390 (1.5%)	0.066
1167_1169delCCA(389delHis)	2/400 (0.5%)	0/390 (0%)	0.499
+1251C>A (Ala417Ala)	0/400 (0%)	1/390 (0.3%)	0.494
+1548C>T	40/400 (10.0%)	41/390 (10.5%)	0.816

Fisher's exact probability test was used for the statistical analysis. Allele frequencies represent minor alleles different from the reference sequence (GenBank accession no. NT_024788). The nucleotide number of each SNP is counted from A of the start codon as 1, with the caution that the one base shorter numbers than those from the human draft sequence for the FOXC2 gene are used beyond +1060 as correct reference numbers.

TABLE 3
Genotype frequencies of each SNP with a >5% frequency in the FOXC2 gene for control and type 2 diabetic subjects

	Control subjects			Type 2 diabetic subjects				
SNPs	AA	Aa	aa	AA	Aa	aa	χ^2	P
−512C>T	94	83	23	93	86	16	1.25	0.535
−350G>T	151	46	3	146	45	4	0.175	0.916
+1548C>T	161	38	1	156	37	2	0.362	0.834

χ^2 Test was used for the statistical analysis. The nucleotide number of each SNP is counted from A of the start codon as I, with the caution that the one base shorter numbers than those from the human draft sequence for the FOXC2 gene are used beyond +1060 as correct reference numbers. A, major allele; a, minor allele different from the reference sequence gene (GenBank accession no. NT_024788).

estimated frequencies of haplotypes defined by −512C>T and −350G>T were f(C-G) = 0.561, f(C-T) = 0.136, f(T-G) = 0.303, and f(T-T) = 0.000. The estimated frequencies of haplotypes defined by −350G>T and +1548C>T were f(G-C) = 0.759, f(G-T) = 0.105, f(T-C) = 0.136, and f(T-T) = 0.000. The estimated haplotype frequencies defined by the three SNPs were f(C-G-C) = 0.456, f(T-G-C) = 0.303, f(C-T-C) = 0.136, and f(C-G-T) = 0.105 (Table 5).

We detected three frequent SNPs with >5% allele frequencies, namely g −512C>T, −350G>T, and +1548C>T. Linkage disequilibria were found between all three pairs of these SNPs. None of these three SNPs or the four common haplotypes defined by them were associated with type 2 diabetes, suggesting that it is unlikely that these SNPs of the FOXC2 gene have major effects on susceptibility to Japanese type 2 diabetes. It should be noted that a small effect of these polymorphisms on susceptibility cannot be excluded, since the sample size was limited in the present study.

Our findings show that Japanese subjects had four major haplotypes determined by the three SNPs located in the region in linkage disequilibrium, namely, g. −512C>T, −350G>T, and +1548C>T. These three SNPs would be useful as haplotype tag SNPs in searching for an association with specific phenotypes in type 2 diabetes, especially in cases where a large number of samples are involved. Most recently, Gabriel et al. (8) reported that only three to five common haplotypes in each haplotype block, in which historical recombination is rare, generally capture 90% of the chromosomes. These common haplotypes represent potentially attractive markers for association studies.

We identified six novel SNPs and a deletion, namely −512C>T, −350G>T, +898C>T, +907C>A, 1167_1169delCCA, +1251C>A, and +1548C>T in the FOXC2 gene. These have not been found in either the J-SNP (http://snp.ims.u-tokyo.ac.jp) or the NCBI databases (http://www.ncbi.nlm.nih.gov/). Of these, two SNPs, −512C>T and −350G>T, were found in the 5′ flanking region. When the sequences around these two SNPs were assessed using the TFSEARCH computer program (http//www.cbrc.jp/research/db/TFSEARCH.html), the results indicated that −350G>T might affect a putative binding site for MyoD, whereas no significant DNA elements were found around −512.

Two missense mutations, +898C>T (Pro300Ser) and +907C>A (Leu303Met), and a deletion, 1167_1169delCCA (389delHis), in the coding region of the FOXC2 gene were found in the present study. Since these mutations were rare, a structural defect in this gene is not a major determinant of susceptibility to type 2 diabetes. Whether these mutations may affect type 2 diabetes susceptibility in a small number of subjects merits further investigation, especially including an analysis of their family members.

TABLE 4
Linkage disequilibrium between two of the three SNPs in the FOXC2 gene and haplotype frequencies in control subjects

Haplotype	Estimated HF	Expected HF	D	D′	χ^2	P
−512 +1548					21.2	$<10^{-5}$
C - C	0.577	0.610	−0.033	−1		
C - T	0.100	0.067	+0.033	+1		
T - C	0.323	0.290	+0.033	+1		
T - T	0.000	0.033	−0.033	−1		
−512 −350					28.5	$<10^{-7}$
C - G	0.547	0.589	−0.042	−1		
C - T	0.130	0.088	+0.042	+1		
T - G	0.323	0.281	+0.042	+1		
T - T	0.000	0.042	−0.042	−1		
−350 +1548					5.50	0.019
G - C	0.771	0.783	−0.012	−0.917		
G - T	0.099	0.087	+0.012	+0.917		
T - C	0.129	0.117	+0.012	+0.917		
T - T	0.001	0.013	−0.012	−0.917		

To examine a deviation from linkage equilibrium between 2 SNPs, a χ^2 test was performed as described by Imanishi et al. (18). The nucleotide number of each SNP is counted from A of the start codon as 1, with the caution that the one base shorter numbers than those from the human draft sequence for the FOXC2 gene are used beyond +1060 as correct reference numbers. HF, haplotype frequency; D, linkage disequilibrium parameter; D′, relative linkage disequilibrium value.

TABLE 5
Estimated haplotype frequencies defined by the three frequent SNPs in the FOXC2 gene

SNPs −512 −350 +1548	Control subjects (n = 200)	Type 2 diabetic subjects (n = 195)
C G - C	0.447	0.456
T G - C	0.323	0.303
C T - C	0.130	0.136
C G - T	0.100	0.105
Total frequency	1.000	1.000

Haplotype frequencies between three SNPs were estimated based on the Arlequin program (16). The frequencies of four major haplotypes are shown. The frequencies of the other four minor haplotypes were $<10^{-5}$. When the linkage disequilibrium was analyzed using the likelihood ratio test in EH program (15), linkage disequilibrium was apparent in each group (χ^2 = 46.0, $P = 2.43 \times 10^{-9}$ in control subjects and χ^2 = 45.6, $P = 2.94 \times 10^{-9}$ in type 2 diabetic subjects). The nucleotide number of each SNP is counted from A of the start codon as 1, with the caution that the one base shorter numbers than those from the human draft sequence for the FOXC2 gene are used beyond +1060 as correct reference numbers.

TABLE 6
Primers used for PCR and sequencing of 5′ flanking region of the FOXC2 gene promoter

Primers (5′–3′)	5′ position of each primer	Buffer and DNA polymerase for PCR
PCR		
pr1F CCAAACCCCACAAAACTGCTCGCAGCGACG	−1248	Advantage GC genomic polymerase mix
pr1R GTAATTCTGCTCGCTCAGGTAGGGCACCAC	+72	
Sequencing		
pr1F CCAAACCCCACAAAACTGCTCGCAGCGACG	−1248	
1RN1 TCAGGTAGGGCACCACTCC	+58	
pr2F GGCCCCCATAATTAGGAAA	−938	
pr2R ATCTCTCCCAAAGACCTTG	−841	
pr3F TCTTAGAGCCGACGGATTC	−645	
pr3R CCCGGAACTTTGAGCCAAT	−560	
pr4F GTCCTGGAGCCAGCGAGGA	−283	
pr4R TTTCAGCGGACCGGGCGGA	−198	

The 5′ positions are shown by defining the translation start site as +1. The 5′ sequences were determined based on the comparison between the human draft sequence for the FOXC2 gene on chromosome 16 (GenBank accession no. NT_024788) and the human FOXC2 cDNA sequence (no. NM_005251). One large fragment was amplified by PCR with primers indicated as PCR and each region was sequenced with ones indicated as sequencing as described in RESEARCH DESIGN AND METHODS. F, forward; R, reverse; N, nested.

In these mutations, the +907C>A (Leu303Met) is most promising since this missense mutation was found in 6 of 390 alleles in type 2 diabetic subjects, but in only 1 of 400 alleles in control subjects. Interestingly, mutations in the coding region of the FOXC2 gene are known to cause lymphoedema-distichiasis, an autosomal dominant form of primary lymphoedema with onset of lower-limb swelling at puberty or later. Small insertions or deletions and nonsense mutations in the coding region of this gene have been occasionally reported (9–11).

In summary, we report here a systematic search for SNPs in the FOXC2 gene and the identification of three frequent SNPs in linkage disequilibrium, in addition to four major haplotypes defined by these SNPs. No association of these SNPs or haplotypes with type 2 diabetes was evident. The issue of how these SNPs affect FOXC2 gene expression in adipocytes and whether SNPs in this gene are associated with type 2 diabetes in other ethnic groups remains unclear. Further study will be required to clarify these points.

RESEARCH DESIGN AND METHODS

This study involved an initial screening of 24 unrelated type 2 diabetic outpatients from the Ehime University Hospital and the Ehime Prefectural Hospital. The subjects were selected based on the fact that they showed the typical characteristics of type 2 diabetes, such as age of onset between 40 and 60 years, treatment with diet alone or oral hypoglycemic agents, and the presence of first-degree relatives with type 2 diabetes. Diabetes was diagnosed based on the American Diabetes Association criteria as reported in 1998 (12). The entire FOXC2 gene was initially sequenced in these 24 diabetic patients. For the association study, the regions containing the SNPs identified were sequenced in 171 additional unrelated type 2 diabetic subjects and 200 nondiabetic subjects. The 200 nondiabetic control subjects did not have a history of diabetes or first-degree relatives with diabetes, and they had a normal glucose tolerance as evidenced by a 75-g oral glucose tolerance test. All patients and control subjects were informed of the purpose of the study and their consent was obtained. The study was approved by the ethics committee of the Ehime University Hospital and Ehime Prefectural Hospital. The clinical characteristics of the 195 type 2 diabetic subjects and the 200 control subjects are summarized in Table 1. The age of the control subjects was comparable to the age of onset of the type 2 diabetic subjects.

PCR direct sequencing was performed as described previously with the following modifications (13,14). Genomic DNA was extracted from leukocytes using a DNA Isolation Kit for Mammalian Blood (Boehringer Mannheim, Indianapolis, IN). The 5′ flanking region and exon 1 of the FOXC2 gene were individually amplified using primers, as described in Tables 6 and 7. The 5′ flanking region was amplified using the Advantage GC genomic polymerase mix by following the manufacturer's protocol (Clontech Laboratories, Palo Alto, CA). Genomic DNA (100 ng) was amplified in a 50-μl reaction mixture, which included 1 μl of Advantage GC polymerase mix, 10 pmol of each primer, and 10 nmol of each dNTP. After the first denaturing for 1 min at 95°C, PCR was carried out for 35 cycles with denaturing at 94°C for 30 s, annealing and extension at 68°C for 3 min, with a final extension period of 3 min. The coding region (exon 1) was amplified by touchdown PCR. Genomic DNA (50 ng) was amplified in a 25-μl reaction mixture, which included 0.625 units of thermus aquaticus (Taq) or EXTaq DNA polymerase (Takara Shuzo Biomedical Group, Shiga, Japan), 5 pmol of each primer, 5 nmol of each dNTP, and 5% DMSO. When this condition was not applicable, a PCR Optimizer kit (Invitrogen) was used. The PCR mixture was the same as above except for the buffers indicated in Table 2. After the first denaturing for 3 min at 94°C, the touchdown PCR was carried out for 15 cycles with denaturing at 94°C for 1 min, annealing at 65–51°C for 2 min by reducing 1°C each cycle, and extension at 72°C for 3 min. An additional 25 cycles were done with denaturing at 94°C for 1 min, annealing at 50°C for 2 min, and extension at 72°C for 3 min, with a final extension period of 7 min. The amplified PCR products were electrophoresed on a 1.5–2% agarose gel and then stained with Cyber Green I (BioWhittaker Molecular Application, Rockland, ME) to confirm the size of each molecule.

These PCR products were sequenced using forward and/or reverse primers, after purification using a Multiscreen PCR filter (Millipore, Bedford, MA). The sequencing reaction was carried out using Taq Dye Deoxy and ABI Prism terminator cycle sequencing kits (PE Applied Biosystems, Foster City, CA). The products were then purified using an Autoseq G-50 column (Amersham Pharmacia Biotech, Piscataway, NJ). These products were then electrophoresed on an ABI Gene analyzer 3100 system (PE Applied Biosystems). Both strands of the entire FOXC2 gene were sequenced for the initial screening of the 24 type 2 diabetic subjects to detect any unknown SNPs. Sequences of plus strands where the identified SNPs were located were then checked for the association study, since these strands permit a more precise identification of these SNPs. The other strand was also sequenced, when required.

The χ^2 test was used for statistical analysis unless otherwise indicated. Haplotype frequencies between two and three SNPs were estimated based on the EH program (15), and the Arlequin program (16), respectively. The relative linkage disequilibrium value (D') for two polymorphisms was defined as the ratio of the linkage disequilibrium parameter (D) to the possible maximum linkage disequilibrium parameter (17). To examine deviations from linkage equilibrium between the two polymorphisms, a χ^2 test was performed as described by Imanishi et al. (18). The linkage disequilibrium among three SNPs was analyzed using the likelihood ratio test in EH program (15).

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TABLE 7
Primers used for PCR and sequencing of the coding region of the FOX 2 gene

Amplified region	Primers (5′–3′)	5′ position of each primer	Buffer and DNA polymerase for PCR
1			
PCR			
1F	CTCTCTCGCTCTCAGGGC	−81	I
7R	ATCCCCGCGCTGTACTGCT	+155	
Sequencing			
1FN2	TCTCAGGGCCCCCCTCGCT	−72	
7RN6	GCGCTGTACTGCTCCGGGT	+149	
2			
PCR and sequencing			
7F	GCAGAATTACTACCGGGCT	+63	I*
1R	TGCTTGTTCTCCCGGTAGA	+341	
3			
PCR and sequencing			
2F	CCTACAGCTACATCGCGCT	+221	ET*
2R	CTCCTTGGACACGTCCTTC	+528	
4			
PCR			
3F	CTACAACATGTTCGAGAACG	+456	A*
3R	TGATGTTCTCCACGCTGAA	+826	
Sequencing			
3FN	TGTTCGAGAACGGCAGCTT	+464	
3RN	TTCTCCACGCTGAAGCCAG	+821	
5			
PCR			
4F	CAAGGTGGAGACGCTGAG	+678	I*
9R	TCCAGGCCCTGAGCGCAC	+965	
Sequencing			
4FN2	AGACGCTGAGCCCCGAGAG	+686	
9R	TCCAGGCCCTGAGCGCAC	+965	
6			
PCR			
9FN	GCCGCTCCCCCTGCCCTA	+897	I
4RN2	CGCTCGGGTGGTCCGAGAG	+1090	
Sequencing			
9FN	GCCGCTGGCGCTGCCTA	+897	
4RN3	GGTGGTCCGAGAGGGCCT	+1081	
7			
PCR			
5F	GATGAGCCTGTACACCGG	+1004	
5R	GAACATCTCCCGCACGTT	+1362	
Sequencing			
5F	GATGAGCCTGTACACCGG	+1004	I*
5RN	CACGTTGGGGAAAGTTTGC	+1350	
8			
PCR			
6F	GTATCTCAACCACAGCGG	+1272	ET*
8R2	GGGTCTGAGAAAAGGTTGG	+1636	
Sequencing			
6FN	CAACCACAGCGGGGACCT	+1278	
8RN	GAAAAGGTTGGTGGACATG	+1628	

The 5′ positions are shown by defining the translation start site as +1 with the caution that the one base shorter numbers than those from the human draft sequence for the FOXC2 gene on chromosome 16 (GenBank accession no. NT_024788) are used beyond +1060 as correct reference numbers of this gene. The coding region was determined based on the comparison between the human draft sequence and the human FOXC2 cDNA sequence (no. NM_005251). Each of the eight fragments was amplified by touchdown PCR with primers indicated as PCR and sequenced with ones indicated as sequencing as described in RESEARCH DESIGN AND METHODS. The nested primers required for sequencing are indicated as N. *ExTaq DNA polymerase was used instead of Taq. To denote the buffer used, A and I are based on the name of the Invitrogen Optimized TM buffer. ET, ExTaq buffer (Takara). F, forward; R, reverse.

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