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Rare variants identified in the *HNF-4*α β-cell-specific promoter and alternative exon 1 lack biological significance in maturity onset diabetes of the young and young onset Type II diabetes

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Abstract

Aims/hypothesis. The recently identified alternative promoter (P2) of *HNF-4*α is the major *HNF-4*α transcription start site in pancreatic beta cells. The significance of the P2 promoter was shown by the identification of a mutation in the IPF-1 binding site of the alternative promoter which cosegregated with diabetes in a large MODY family. The role of the P2 promoter and the associated alternative exon 1 in both MODY and polygenic Type II (non-insulin-dependent) diabetes mellitus is not known. Linkage to this region in studies of Type II diabetes makes the P2 region a strong candidate for a role in Type II diabetes susceptibility.
Methods. To assess the role of the P2 region we screened MODY, young-onset Type II diabetic subjects, and probands from Type II diabetes families linked to chromosome 20 for variants of the P2 promoter and associated exon of *HNF-4*α.

Results. Two variants were found that were not present in the control subjects. The –79C/T substitution was present in a MODY family but did not perfectly cosegregate with diabetes. A –276G/T substitution was identified in two UK young-onset diabetes probands but did not co-segregate with diabetes. Reporter gene studies did not indicate changes in transcriptional activity caused by either the –79C/T or –276G/T single nucleotide substitutions.
Conclusion/interpretation. We found no evidence to suggest that variation in the P2 proximal promoter region and associated alternative exon 1 of *HNF-4*α contribute to young onset Type II diabetes susceptibility in Northern Europeans. [Diabetologia (2002) 45:1344–1348]

Keywords: *HNF4-*α, *HNF-1*α, *HNF-1*β, IPF-1, maturity onset diabetes of the young, Type II diabetes mellitus, P2 promoter.

Mutations in the transcription factor genes hepatic nuclear factor *(HNF) -1*α, *HNF-1*β and *HNF-4*α and insulin promoter factor 1(*IPF-1*) cause the majority of cases of MODY [1]. The mechanism by which *HNF-1*α, *HNF-1*β, *HNF-4*α, *IPF-1* and other genes interact to result in normal beta-cell function is uncertain.

Recently it has been recognised that in the beta cell the predominant isomer of *HNF-4*α is *HNF-4*α7 which utilises a far upstream promoter of *HNF-4*α, P2, with an alternative exon 1 that in humans is 45 kb 5′ to the previously identified P1 promoter [2, 3, 4]. The P2 promoter contains functional binding sites for

HNF-1α, *HNF-1β* and *IPF-1* [3, 4]. This suggests that *HNF-1α*, *HNF-1β* and *IPF-1* could regulate *HNF-4α* through the P2 promoter in the pancreas, in contrast to the situation in the liver where *HNF-4α* regulates *HNF-1α*. Consistent with this, *HNF-1α* was recently found to be essential for P2 promoter activity and nucleosomal histone hyperacetylation in mice [4]. Furthermore, we identified a large MODY family with a *–146T/C* nucleotide substitution in the *IPF-1* binding site of the P2 promoter, which cosegregated with diabetes (LOD3.25) [3]. This mutation was shown to reduce transcriptional activity in a beta-cell line by reducing the binding of *IPF-1* [3].

HNF-4α is located on chromosome 20q12-q13.1. Several groups have reported linkage of Type II (non-insulin-dependent) diabetes mellitus to this region. These include studies of US Caucasians, Finnish and French Type II diabetes families [5, 6, 7, 8, 9, 10, 11]. As a strong candidate gene, *HNF-4α* exons and promoter P1 have been screened in a number of these studies but not the P2 promoter. Common variants in the P1 promoter and the downstream exons did not explain the observed linkages to this region [8, 11].

The role of genetic variation in the P2 promoter and the associated exon in non-MODY Type II diabetes has not been studied. Given the evidence for linkage to the genomic region of *HNF-4α* and its crucial regulatory role in the pancreas it represents a strong candidate for a role in Type II diabetes susceptibility. Although we identified one co-segregating mutation in one of seven MODY families [3], the role of the P2 promoter and associated exon has not been evaluated in further MODY families in whom a mutation has not been found. We therefore screened Northern European subjects with Type II diabetes or MODY for variation in the P2 promoter and associated exon of *HNF-4α*.

Subjects and methods

Subjects. The first group we studied comprised: 25 MODY probands that were previously found to have no mutations in the five known MODY genes [12, 13, 14]: one South African of Indian origin, one Spaniard, two Italians, two Danes, two Swedes, four Finns and 13 French probands meeting MODY criteria (autosomal dominant inheritance of Type II diabetes with at least one family member diagnosed before 25 years of age) (Table 1). Of these 25 families, seven had LOD scores at the *HNF4α* locus of less than –2.0. All other families were either too small for linkage analyses (*n*=8) or could not be excluded at LOD less than –2.0 (LODs ranged from –1.92 to +1.01). In one family we identified a mutation in the *HNF4α* P1 promoter but given this promoter does not act in the beta cell the proband from this family was included.

The second group included: Young onset (non-MODY) Type II diabetic subjects: 48 white diabetic subjects from the United Kingdom of British or Irish origin, who were GAD negative with at least one Type II diabetic first degree relative, and 38 French young onset Type II diabetic subjects, in whom linkage to chromosomal region 20q13 had been found [7] (Table 1).

The third group included: 48 white non-diabetic subjects from the United Kingdom (HbA$_{1C}$<6.0%) (Table 1). All subjects gave informed consent and the investigations were carried out in accordance with the Declaration of Helsinki.

Sequencing. We PCR-amplified and sequenced the P2 promoter, encompassing the HNF-1 and IPF-1 binding sites, and the associated alternative exon 1 using genomic DNA [3]. The following overlapping primer pairs were designed and used separately by the collaborating laboratories:

Forward 5′-CCAGGTTGGACTCTCACCTCTC-3′ and Reverse 5′-GTGTCCCATGGCCTCCCAAAG-3′ amplifying bases –383 to 116 of the P2 promoter and exon (42786617 to 42787115 from the p-terminal of chromosome 20); or Forward 5′-TGACACACCCATAGTTTGGAG-3′ and Reverse 5′-ATGGCCTCCCAAAGCTGAC-3′ amplifying bases –418 to 109 of the P2 promoter and exon (42786582 to 42787108 (UCSC Human Genome Project Working Draft, 22 December 2001 assembly (hg10) http://genome.cse.ucsc.edu/)).

Cell lines, transfections and reporter gene assays. Luciferase reporter gene constructs were generated by introducing PCR fragments containing the appropriate *HNF-4α* P2 promoter sequence into vectors pGL2-Basic (–79C/T variant) or pGL3-BasicII (Promega) (–276G/T variant). For the –79C/T constructs, bases –54 to –371 of the P2 promoter were PCR amplified with forward primer 5′-TTTGAGCTCACCTCTCCAGCCC-CTTCT-3′ and two different reverse primers: 5′-GGACTCG-AGCACAGCAGCAGCACAGCCACCAGGAACCCG-3′ for the T variant and 5′-GGACTCGAGCACAGCAGCAG-3′ for

Table 1. Clinical characteristics of the subjects examined according to diabetes status

Clinical characteristic	MODY X	UK young onset diabetic subjects (non-MODY)	French young onset diabetic subjects (non-MODY)	UK non-diabetic subjects
N	25	48	38	48
Male/female	11/14	30/18	21/17	26/22
Age[a] (years)	16 (12–27)	36.0 (32.0–39.5)	36.0 (28.5–39.8)	70 (62–74)
BMI	24.7 (21.8–27.7)	29.6 (28.0–33.5)	26.1 (23.4–29.3)	28.0 (24.4–31.6)
Treatment with diet (%)	9.5	35.4	63.2	–
Treatment with OHA (%)	14.3	47.9	26.3	–
Treatment with insulin (%)	76.2	16.7	10.5	–

[a] Age at diagnosis for Diabetic subjects, age at study for non-diabetic subjects

Data are *n*, % or medians (interquartile range)

Table 2. Frequency of the *HNF4α* P2 variants identified in the four study groups

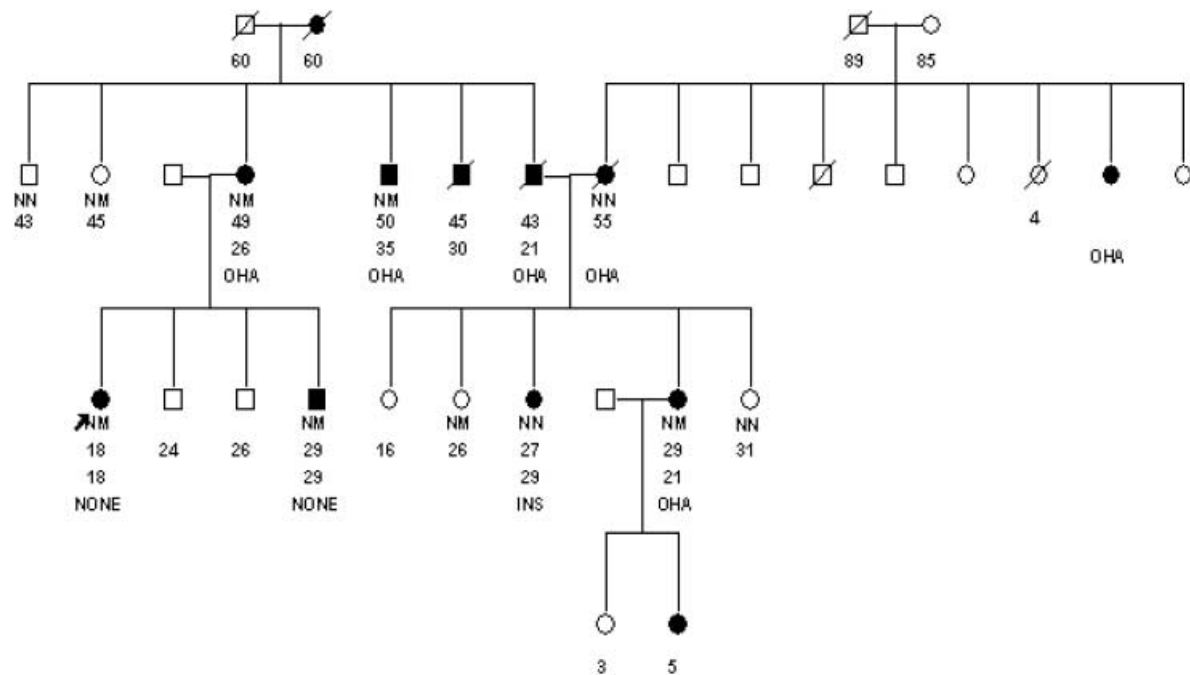| Mutation	Allele frequency			
	MODY X	UK young onset diabetic subjects (non-MODY)	French young onset diabetic subjects (non-MODY)	UK non-diabetic subjects
–79C/T	C: 0.98 T: 0.02	C: 1.00 T: 0.00	C: 1.00 T: 0.00	C: 1.00 T: 0.00
–276G/T	G: 1.00 T: 0.00	G: 0.98 T: 0.02	G: 1.00 T: 0.00	G: 1.00 T: 0.00

Fig. 1. Pedigree of MODYX family showing pattern of inheritance of the C/T nucleotide substitution at –79. *Solid symbols* represent affected and *open symbols* unaffected individuals. Individual mutation status is given: N = normal, M = C/T mutation. For affected subjects: age at testing followed by age at diagnosis followed by treatment regime (OHA oral hypoglycaemic agents, INS = insulin) are given. For unaffected subjects age at testing is given. The proband is indicated by an *arrow*

the C variant. For the –276G/T variant, constructs were made containing nucleotides –1 to –346 (P2/-346) or nucleotides –1 to –2200 (P2/-2200) of the P2 promoter to exclude the possibility that the mutation influences more distal unidentified regulatory elements. PCR was carried out with forward primers 5′-CCGCTCGAGCGGCCCTGTCCTCAAATTG-3′ for P2/-346 and 5′-CCGCTCGAGGGAAGGCAATGTGAGA-3′ for P2/-2200 and the reverse primer 5′-CCCAAGCTTGGGCCAAGCCCACCCAGC-3′.

To increase the possibility of testing the mutation in a representative cellular context, two cell lines were used to test the functional effect of each mutation. INS-1 and FT0-2B cells [15] were cultured and transfected [3]. Min6 and CaCo2 cells were cultured in DMEM with 50 mmol/l β-mercaptoethanol and 15% foetal calf serum. Transfections of MIN6 and CaCo2 cells were done using 0.4 µg of *HNF-4α*-P2 luciferase reporter gene constructs using Effectene (Qiagen) at a 1:10 ratio to DNA.

Results

We identified one variant in the MODYX subjects: a –79 C/T nucleotide substitution (Table 2). The variant did not co-segregate completely with diabetes but was present in five of the seven diabetic family members screened and was also present in two of the four non-diabetic family members screened (Fig. 1). The –79 C/T variant was not seen in any of the non-diabetic subjects from the UK.

The –79C/T variant is contained within the 5′ untranslated region of *HNF-4α* transcripts originating from the P2 promoter, and is conserved in mice and humans. To analyse its effect on transcriptional activity, we mutated this site in a luciferase reporter gene construct containing the P2 promoter sequence. Transient transfections in mouse Min6 beta-cells and human colon-derived CaCo2 cells failed to show differences in transcriptional activity between the constructs containing C or T at position –79 (Fig. 2).

In 2 of 48 UK young onset (non-MODY) Type II diabetic subjects we identified a –276 G/T nucleotide substitution (Table 2). However, in each case it was inherited from an unaffected parent and was not present in an affected first degree relative. The –276 G/T variant was not present in either the 38 French young
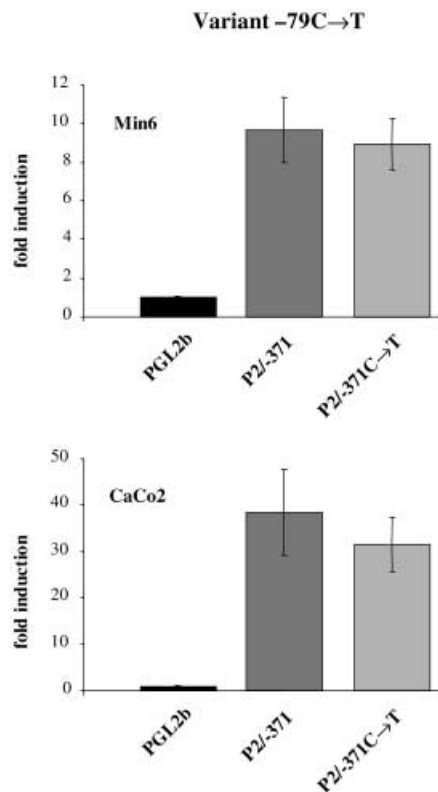
Variant −79C→T

Fig. 2. Luciferase reporter gene assays of transiently transfected Min6 and CaCo2 cells using P2 promoter constructs containing either the C(P2/-371) or the T(P2/-371C→T) variants at position −79 where −371 indicates the 5′ extent of the promoter used in the construct relative to the translation start site. Fold induction refers to the activity of the pGL2-Basic vector. Standard error bars were calculated from three different experiments, each representing duplicate calculations of two independent clones for each construct
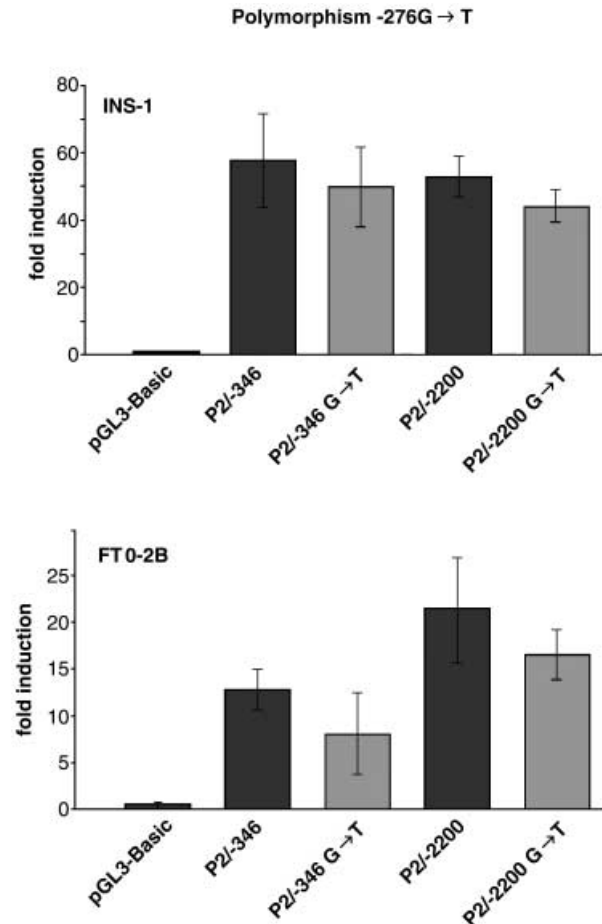
Polymorphism −276G → T

Fig. 3. Luciferase reporter gene assays of transiently transfected INS-1 and FT0–2B cells using the following reporter gene constructs of the P2 promoter sequence of *HNF-4α* in front of the luciferase gene: P2/-346, P2/-2200 as well as P2/-346G→T and P2/-2200G→T containing the polymorphism −276 G→T (where −346 or −2200 indicates the 5′ extent of the promoter used in the construct relative to the translation start site). Fold induction refers to the activity of the pGL3-Basic vector. The error bars indicate standard deviations of six calculations

onset (non-MODY) Type II diabetic subjects, or in the 48 UK nondiabetic subjects. The previously reported −146T/C substitution was not present in any of the subjects studied.

The −276 T variant occurs in a potential metal responsive element (TGCRCNC) in the opposite strand [16]. To assess whether variation at this site affects transcriptional activity, we undertook luciferase reporter gene assays of transiently transfected insulinoma (INS-1) and hepatoma (FT0-2B) cell lines. We used constructs containing the −276 G or T variant in fragments of the P2 promoter containing bases −1 to −346 or −1 to −2200 in front of the luciferase gene. No difference in fold activity was seen between the constructs containing the G variant and those containing the T variant in either of the cell lines (Fig. 3).

Discussion

Our results do not support the hypothesis that variation in the far upstream P2 promoter is an important

cause of MODY or young onset Type II diabetes even in families showing linkage to this region. It remains possible that variation in the P2 promoter and associated exon is a minor contributor to Type II diabetes susceptibility, although given their low frequency, variants will only contribute to a small proportion of the linkage results. However, the role of the P2 promoter in the aetiology of Type II diabetes should be investigated in cohorts of late-onset Type II diabetes subjects. Although we have excluded up to 418 bp of the P2 promoter region and the associated exon from a role in our cohorts it remains possible that variation in distant unidentified transcriptional control elements of this promoter could be important in Type II diabetes susceptibility.

In conclusion, variation in the *HNF-4α* P2 promoter and the corresponding exon are not a common cause of MODY or early-onset Type II diabetes in-

cluding those from families linked to chromosome 20. It is not likely that variation in the P2 region of *HNF-4α* accounts for the previously identified linkage between this region and Type II diabetes although it cannot be excluded from a role in late onset Type II diabetes.

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