

Mutations in the VNTR of the carboxyl-ester lipase gene (*CEL*) are a rare cause of monogenic diabetes

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Abstract We have previously shown that heterozygous single-base deletions in the *carboxyl-ester lipase (CEL)* gene cause exocrine and endocrine pancreatic dysfunction in two multigenerational families. These deletions were found in the first and fourth repeats of a variable number of tandem repeats (VNTR), which has proven challenging to sequence due to high GC-content and considerable length variation. We have therefore developed a screening method consisting of a multiplex PCR followed by fragment analysis. The method detected putative disease-causing insertions and deletions in the proximal repeats of the VNTR, and determined the VNTR-length of each allele.

When blindly testing 56 members of the two families with known single-base deletions in the *CEL* VNTR, the method correctly assessed the mutation carriers. Screening of 241 probands from suspected maturity-onset diabetes of the young (MODY) families negative for mutations in known MODY genes (95 individuals from Denmark and 146 individuals from UK) revealed no deletions in the proximal repeats of the *CEL* VNTR. However, we found one Danish patient with a short, novel *CEL* allele containing only three VNTR repeats (normal range 7–23 in healthy controls). This allele co-segregated with diabetes or impaired glucose tolerance in the patient's family as six of seven mutation

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carriers were affected. We also identified individuals who had three copies of a complete *CEL* VNTR. In conclusion, the *CEL* gene is highly polymorphic, but mutations in *CEL* are likely to be a rare cause of monogenic diabetes.

Introduction

The *carboxyl-ester lipase* gene (*CEL*) is expressed mainly in the acinar tissue of the pancreas (Lombardo 2001; Roudani et al. 1995) and in lactating mammary glands (Blackberg et al. 1987). The pancreatic *CEL* enzyme (E.C.3.1.1.13), also known as bile salt-stimulated or bile salt-dependent lipase, is secreted into the digestive tract where it is activated by the presence of bile salts, playing a role in cholesterol and lipid-soluble vitamin hydrolysis and absorption (Lombardo and Guy 1980). A fraction of the enzyme is also present in plasma and an interaction with plasma cholesterol and oxidized lipoproteins has been suggested (Bengtsson-Ellmark et al. 2004; Caillol et al. 1997). It is debated whether pancreatic *CEL* is transported from the duodenum to the blood (Bruneau et al. 2003a, b), or if the plasma fraction is synthesized and secreted from macrophages and endothelial cells of the blood vessels, which have been shown to express low levels of *CEL* mRNA (Li and Hui 1998). Studies in mice have suggested that macrophages expressing human *CEL* have an increased cholesteryl ester accumulation which promotes foam cell formation, leading to an increased amount of atherosclerosis lesions in the arterial vessel walls (Kodvawala et al. 2005).

Human *CEL* is nearly 10 kb of size, containing 11 exons. There is a variable number of tandem repeats (VNTR) in the coding region of exon 11. In this VNTR, 33-base pair (bp) nearly identical segments are repeated between 7 and 21 times. Sixteen repeats are present on the most common allele of examined populations (Higuchi et al. 2002; Lindquist et al. 2002; Raeder et al. 2006). The VNTR is heavily O-glycosylated in the mature protein (Wang et al. 1995). Previous studies have shown that the VNTR is not required for functional properties of the enzyme such as catalytic activity and activation by bile salts (Downs et al. 1994; Hansson et al. 1993). However, the VNTR may be necessary for proper folding, secretion and stability (Bruneau et al. 1997). An association between the total number of repeats and serum cholesterol profile has been reported (Bengtsson-Ellmark et al. 2004). In addition, preliminary results indicate that exocrine dysfunction, often seen in diabetic patients, may be linked to common single-base insertions in the *CEL* VNTR (Raeder et al. 2006).

Raeder et al. have earlier described a novel syndrome of exocrine and endocrine pancreatic dysfunction caused by mutations in *CEL* (OMIM #609812) (Raeder et al. 2006;

Vesterhus et al. 2008). Two different single-base deletions, located in repeat 1 and repeat 4 of the VNTR, were detected in two families with dominantly inherited diabetes and exocrine dysfunction. Both deletions lead to a frame shift and a premature stop codon, creating a new C-terminal end of the translated *CEL* protein. Although pancreatic lipomatosis is associated with the disease (Raeder et al. 2007), the exact pathogenic effect of the mutated *CEL* gene at the cellular level is unknown.

Sequencing the *CEL* VNTR has proven difficult by standard methods. Most of the repeated 33-bp segments harbour four Cs followed by eight Gs. This is a challenge for the DNA-replicating polymerase during PCR, and stuttering in the 3'-end is commonly observed after sequencing. Within a given allele, the repeated segments vary slightly in sequence, and there is considerable variation in the number of repeats within a population. Thus, a person usually does not exhibit identical VNTRs on the two genomic *CEL* copies that he/she carries. As a consequence, *CEL* VNTR sequences have to be read manually and extensive experience is needed to interpret the data correctly. We therefore aimed to develop an easier and more robust method for analysing the *CEL* VNTR for variants likely to have functional significance. Here, we describe a method based on multiplex PCR and fragment analysis, which detects single-base insertions/deletions in the proximal repeats of the *CEL* VNTR and simultaneously determines the total number of repeated segments in each allele. Moreover, we employed this method in the screening of MODY (OMIM# 606391) probands for *CEL* exon 11 variants.

Materials and methods

Multiplex PCR combined with fragment analysis

We designed a multiplex PCR using one unlabelled forward primer and two fluorescently labelled reverse primers (Table 1). The PCR reaction volume was 10.0 µl, containing 1× GC buffer I, 0.34 mM dNTPs, 0.2 U LaTaq Polymerase (all from TaKaRa, Otsu, Japan), 1.0 M betaine solution (Sigma-Aldrich, St. Louis, Missouri, USA), 0.5 µM *CEL*_VNTR_F primer, 0.5 µM *CEL*-VNTR_R1 primer, 0.1 µM *CEL*_VNTR_R2 primer and 10.0 ng template DNA. The PCR cycling conditions were as follows: 94°C for 1 min; then 94°C for 30 s, 61°C for 30 s and 72°C for 1 min for 38 cycles; followed by 72°C for 5 min, and cooling to 4°C. The primers were designed based on sequence information from the Ensembl database (accession number OTTHUMG00000020855). The forward primer hybridized upstream of and partly into the first repeat of the *CEL* VNTR located in exon 11 (Fig. 1a, b). The

Table 1 Primers used for multiplex PCR, standard PCR and sequencing

Name	Sequence (5'–3')	Labelling	Used for
CEL_VNTR_F	ACCGACCAGGAGGCCACCC	–	Multiplex PCR
CEL_VNTR_R1	tactcgagGGTGGCCCCGGAGTCAC	FAM	Multiplex PCR
CEL_VNTR_R2	CCTGGGGTCCCACTCTTGT	NED	Multiplex PCR
CEL-intron7F	GTCCTCACTCATTCTTCTATGGCAAC	–	PCR
CEL-exon11R	TCCTGCAGCTTAGCCTTGGG	–	PCR and sequencing
CEL-exon11F	CACACACTGGGAACCT	–	Sequencing

Capital letters represent CEL-specific nucleotides, lower case letters are mismatching nucleotides

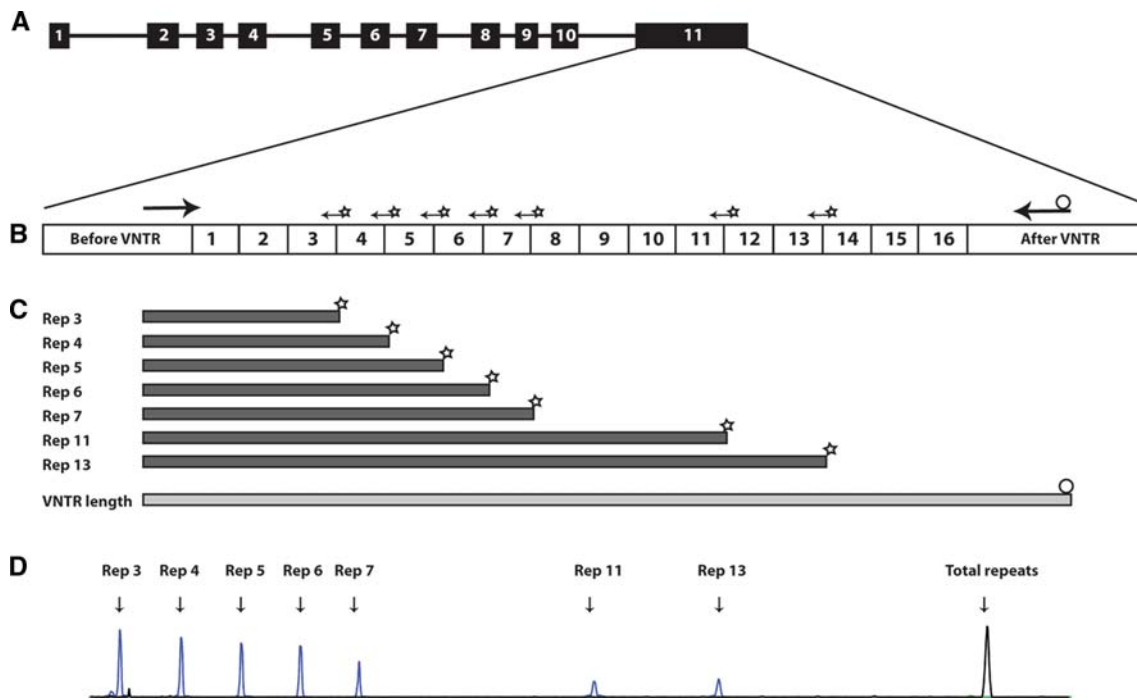


Fig. 1 An overview of the method for analyzing the *CEL* VNTR. **a** Organization of the *CEL* gene. The gene consists of 11 exons, each represented by a numbered box. Exon 11 contains the VNTR. **b** Organization of exon 11 and the amplification principle. Three different primers are used in the multiplex PCR. The unlabelled forward primer partly overlaps with the first repeat of the VNTR. A FAM-labelled (star) reverse primer binds to every repeat harbouring

the specific sequence GTGACTCCGGGGCC. The NED-labelled reverse primer (circle) binds after the VNTR. **c** Products of the multiplex PCR amplification. A mixture of DNA fragments with different lengths is created. **d** Example of a result from the capillary electrophoresis analysis. Each FAM-coloured peak (Rep 3–13) in the spectrum represents an individual repeat within the VNTR. The NED-coloured peak (Total repeats) represents the total length of the VNTR

FAM-labelled reverse primer (CEL_VNTR_R1) hybridized to all repeats harbouring the exact sequence GTGACTCCGGGGCC, creating several FAM-labelled DNA fragments (Fig. 1b, c). The second reverse primer (CEL_VNTR_R2), which is NED-labelled, hybridized downstream of the repeats, amplifying a product covering the complete VNTR (Fig. 1b, c). For fragment analysis, 1.0 µl of the PCR products was added to a mixture of 8.8 µl HiDi formaldehyde and 0.2 µl Rhodamine MapMarker (50–1,000 bps) size standard (BioVentures Inc, Murfreesboro, TN, USA). The samples were analysed on an ABI 3100 Genetic Analyser using POP4 polymer (Applied Biosystems, Foster City, CA, USA). The fragments could also be separated on an ABI 3730 DNA

analyser using POP7 polymer. When using ABI 3730, the PCR products had to be diluted 1:100 in water and 1.0 µl of the diluted samples was added to a mixture of 8.9 µl HiDi formamide and 0.1 µl Rhodamine MapMarker size standard. In the spectrum resulting from capillary electrophoresis, the FAM-labelled DNA fragments were visible as several blue peaks; each peak representing the length between the forward primer and an internal VNTR-repeat recognized by the CEL_VNTR_R1 primer (Figs. 1d, 2a–d). The size of the NED-labelled DNA fragment(s) (black) corresponded to the total number of repeats in each allele (Figs. 1d, 3a–e). Applied Biosystems Genemapper software version 4.0 was used to analyse the data from the capillary sequencer. A DNA sample with a known single-

Fig. 2 Examples of spectra after DNA fragment analysis for deletions and insertions (FAM labelling). Each peak represents one repeat of the *CEL* VNTR, from repeat 4 (123) to repeat 8 (252). The size (bp) for each peak is shown underneath the peak. Single peaks, as shown in **a**, indicate that no insertions or deletions are present in either allele. Two neighbouring peaks as shown in **b** (deletion in repeat 1), **c** (deletion in repeat 4) and **d** (insertion in repeat 4) indicate heterozygosity for a 1 bp insertion or deletion. The total number of peaks seen varies depending on the combination of the VNTR segments in each patient. The peak marked by a star (*) is an unspecific reaction product which is seen in all reactions and does not interfere with the interpretation



base deletion in the first repeat and a normal sample were included in each run as a positive and negative control, respectively.

Statistics

Tests of VNTR-length-allele frequency differences between the populations were done with the UNPHASED software (version 3.0.10, <http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/>) (Dudbridge 2008). UNPHASED allows for both individual and global tests of multi-allelic markers using likelihood-based approaches. We applied a rare frequency threshold of 1% and set all other parameters to default values (similar results were obtained using a threshold of 5%, results not shown).

Sequencing *CEL* exon 11

In order to ensure specific amplification of *CEL* and to avoid interference from the closely located *CELP* pseudogene, we amplified a region that included the genomic sequence from *CEL* intron 7 to exon 11 (primers *CEL*-intron7F and *CEL*-exon11R; Table 1), creating a 3.9 kb product. The PCR reaction volume was 10.0 μ l, containing 1 \times GC buffer (TaKaRa), 1.6 μ M of each primer, 0.375 μ l ddH₂O, 1.0 M betaine solution (Sigma-Aldrich), 0.4 mM of each dNTP, 0.03 U LaTaq Polymerase (TaKaRa) and 5.0 μ g DNA template. Amplification started with a denaturation step at 94°C followed by 14 cycles of 94°C for

20 s and 60°C for 10 min; then 20 cycles of 94°C for 20 s and 62°C for 10 min, and a final elongation step of 72°C for 10 min followed by cooling to 4°C.

Before sequencing, the PCR-product was treated with ExoSAP (USB Corporation, Cleveland, OH, USA) as described by the manufacturer. Sanger sequencing was carried out on an ABI 3730 capillary sequencer using the primers *CEL*-exon11F and *CEL*-exon11R (Table 1). The reaction volume was 10.0 μ l containing 0.5 μ M primer, 1.0 M betaine solution (Sigma), 2.75 μ l ddH₂O, 1.0 μ l BigDye v1.1, 2.0 μ l Sequencing buffer (Applied Biosystems) and 2.0 μ l ExoSAP-treated PCR-product. Cycling conditions were as follows: 96°C for 10 min, 25 cycles of 96°C for 10 s and 58°C for 5 s and 60°C for 4 min, and cooling to 4°C.

Clinical samples and analyses

A total of 56 members of the two previously identified Norwegian families with mutations in the *CEL* VNTR were screened using the multiplex PCR method. Moreover, a total of 241 probands (95 from Denmark and 146 from UK) with diabetes who met the minimal diagnostic criteria for MODY (at least two generations affected and diagnosis before the age of 25) were analysed. All probands had tested negative for mutations in seven known MODY genes (Stride and Hattersley 2002) and had therefore been classified as MODYX. A total of 233 population-based controls with unknown diabetes status were also included. Moderate

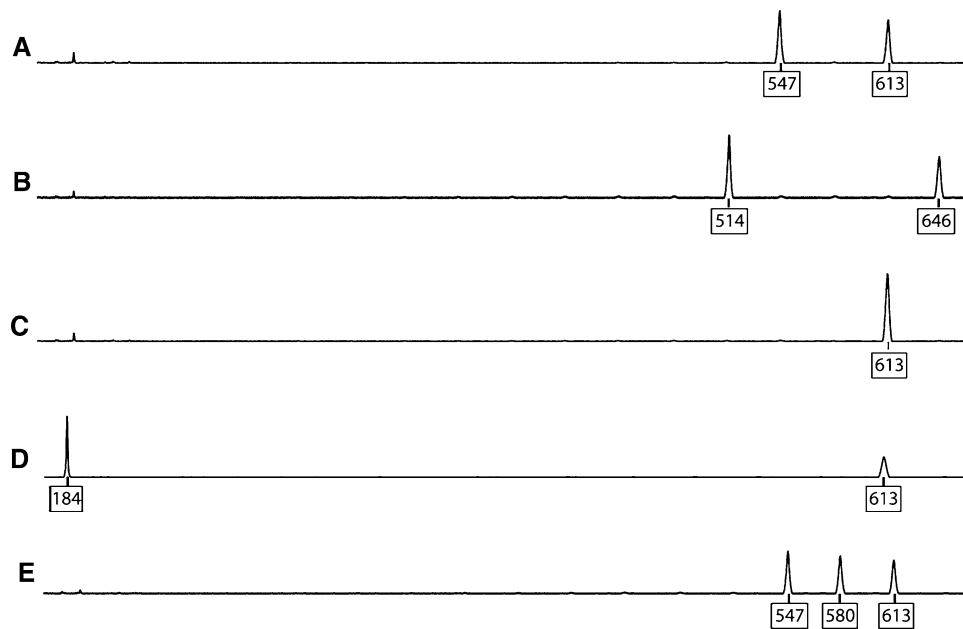


Fig. 3 Examples of spectra after DNA fragment analysis for determining the total length of the *CEL* VNTR (NED labelling). The peak sizes (shown underneath each peak) correspond to the total number of repeated segments in each allele. **a** A sample heterozygous for a 14- and 16-repeat allele (547 and 613 bp, respectively). **b** A sample heterozygous for a 13- and 17-repeat allele (514 and 646 bp,

respectively). **c** A sample homozygous for a 16-repeat allele (613 bp). **d** A sample from the Danish family with a 3-repeat VNTR allele. This subject is heterozygous for the 3-repeat (184 bp) and a 16-repeat allele (613 bp). **e** A sample with three copies of the *CEL* VNTR with 14 (547 bp), 15 (580 bp) and 16 (613 bp) repeats

and severe exocrine dysfunction was defined as faecal elastase-1 values below 200 and 100 $\mu\text{g/g}$, respectively. Oral glucose tolerance tests were performed by the standard method (75 g glucose) and World Health Organization criteria for diabetes were applied.

Results

Multiplex PCR and fragment analysis on patient samples

We initially re-analysed the two families with known mutations in the *CEL* VNTR (Raeder et al. 2006) in order to verify the sensitivity of the multiplex PCR method. The samples were run on an ABI 3100. All 23 individuals previously found to have single-base deletions in repeat 1 or repeat 4 were correctly identified. An individual harbouring a single-base insertion in repeat 4 was also detected by the method. All VNTR lengths found were in concordance with the lengths estimated previously by a different fragment analysis protocol (Raeder et al. 2006).

We then analysed the *CEL* VNTR in 95 MODYX patients from Denmark using the same approach. We did not find any cases with single-base insertions/deletions in

the first eight repeats of the *CEL* VNTR in this material. This result was confirmed by sequencing. However, one MODYX proband with a very short VNTR allele, consisting of only three repeats, was found (Fig. 3d). Further samples of his family were collected and six individuals carrying the 3-repeat VNTR allele were identified. Of the seven mutation carriers, four had diabetes, one had impaired fasting glycemia and one had impaired glucose tolerance. The pedigree and clinical characteristics are presented in Fig. 4 and Table 2, respectively. Family members with normal VNTR lengths were all normoglycemic. Mean BMI of the short VNTR allele carriers was not different from that of the normal allele carriers (26.5 vs. 25.7 kg/m^2 , respectively). Stool samples were available from four of the family members with the 3-repeat allele; two of them had moderate faecal elastase deficiency, whereas the two others had normal faecal elastase values. To rule out the possibility of a rare mutation linked to the 3-repeat allele, we sequenced all exons and exon-intron boundaries of *CEL* in the Danish proband. No unknown sequence variants were detected. The 3-repeat allele was not found in 233 controls genotyped in this study, and was not present in the 377 subjects sequenced and described in Raeder et al. (2006).

The UK MODYX material ($n = 146$) was tested using the same multiplex PCR method, but run on an ABI 3730

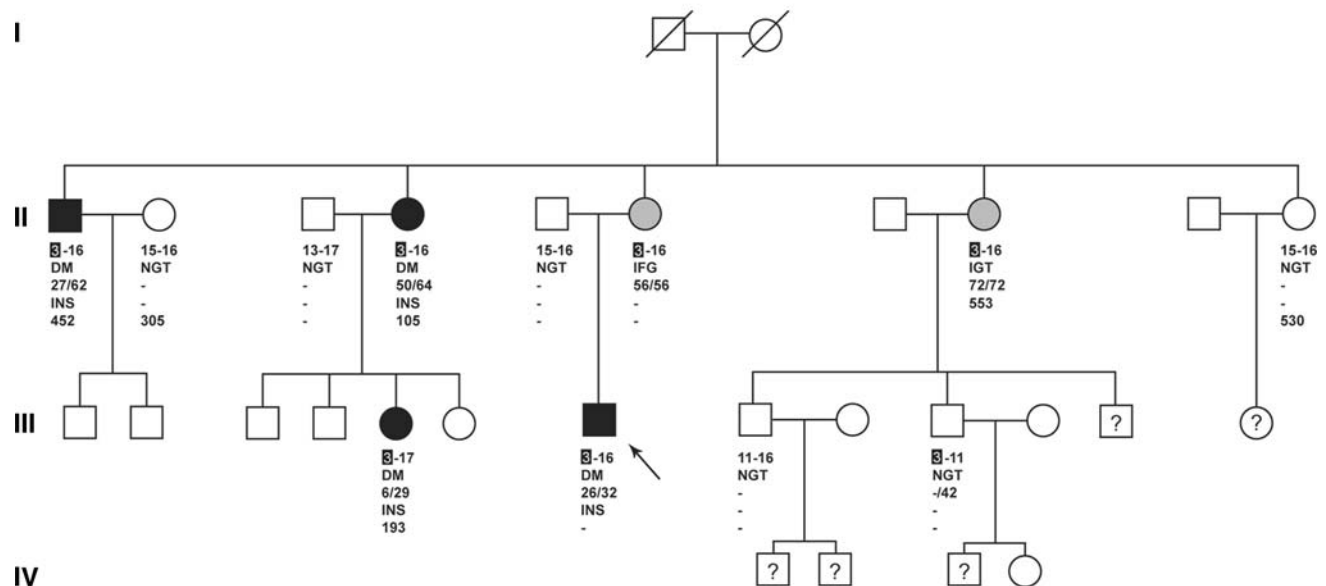


Fig. 4 The pedigree of the Danish family carrying the 3-repeat *CEL* VNTR allele. *Filled black symbols* represent patients with diabetes, *filled grey symbols* represent patients with IFG or IGT. Genotype (i.e. the number of VNTR repeats), diabetes status, age of onset/age of examination, treatment and pancreatic exocrine function as defined in

“Materials and methods” are shown underneath each symbol. An *arrow* indicates the proband. *DM* Diabetes mellitus, *IFG* impaired fasting glycemia, *IGT* impaired glucose tolerance, *NGT* normal glucose tolerance, *INS* insulin

Table 2 Clinical characteristics of the Danish family with a 3-repeat *CEL* VNTR

Characteristics (normal range)	3-repeat allele carriers		Normal allele carriers (Mean ± SD)
	Diabetic/impaired glucose tolerance/impaired fasting glucose subjects (Mean ± SD)	Healthy subjects Value	
Total number of subjects/number of males	6/2	1/1	6/4
Present age, years	52 ± 19	37	59 ± 8
Body mass index, kg/m ²	26.5 ± 1.2	25.0	25.7 ± 2.8
Endocrine pancreas dysfunction			
Mean age at diagnosis of diabetes, years	27 ± 18		
HbA1C, % (4.0–6.4)	8.2 ± 1.9	5.6	5.9 ± 0.4
Fasting plasma glucose, mmol/l (3.9–6.0)	10.5 ± 5.1	4.9	5.5 ± 1.0
Exocrine pancreas function			
Faecal elastase-1, µg/g	326 ± 211		418 ± 159

DNA Analyzer. In this material, we did not detect insertions or deletions in the first eight repeats of the *CEL* VNTR. The 3-repeat allele was not observed.

The overall VNTR-length allele frequency distribution between populations (Table 3) was not significantly different between the UK and DK MODYX materials ($P_{diff} = 0.72$) as assessed by the global likelihood ratio test UNPHASED (Dudbridge 2008). The Norwegian control material had a slightly lower frequency of the 14-repeat allele than both the Danish and UK samples ($P_{nominal} = 0.01$ and 0.02, respectively), and higher 16-repeat

frequency than the UK sample ($P_{nominal} = 0.05$), but the overall distribution was not significantly different.

Structural genetic rearrangements of *CEL*

During the fragment analysis, we observed some MODYX patients with three NED-labelled peaks instead of the expected one or two peaks (Fig. 3e). This would suggest that some individuals harbour three copies of a complete *CEL* VNTR, indicating that *CEL* alleles which have undergone major structural rearrangements exist. In the

Table 3 Overview of *CEL* VNTR-length allele frequencies for the different populations

Number of repeats	Frequency (counts)		
	Danish MODYX (<i>n</i> = 95)	UK MODYX (<i>n</i> = 146)	Norwegian controls (<i>n</i> = 233)
3	0.005 (1)	–	–
4	–	–	0.002 (1)
6	–	–	0.002 (1)
7	–	–	0.002 (1)
10	–	0.003 (1)	0.004 (2)
11	–	0.003 (1)	0.006 (3)
12	–	0.010 (3)	0.006 (3)
13	0.063 (12)	0.084 (25)	0.075 (36)
14	0.135 (26)	0.125 (37)	0.077 (37)
15	0.130 (25)	0.159 (47)	0.140 (67)
16	0.630 (121)	0.581 (172)	0.646 (309)
17	0.031 (6)	0.027 (8)	0.038 (18)
18	–	0.003 (1)	–
21	–	0.003 (1)	–
23	0.005 (1)	–	–

combined MODYX material, there were six individuals (2.5%) with three NED-labelled peaks (two probands from the Danish material, four from UK material). Among the 233 controls, three NED-labelled peaks were detected in 12 individuals (5%).

Discussion

Methodological considerations

Multiplex PCR is a method that enables simultaneous amplification of several loci by using more than one primer set in one reaction tube. We used this principle to develop an assay that detects insertions and deletions in the VNTR of *CEL* with 1-bp resolution (Fig. 2). The method allowed determination of the total number of repeated segments in the VNTR as well (Fig. 3). This approach was considerably faster than standard sequencing followed by manual reading, and control experiments showed that the new method had the same sensitivity in detecting 1-bp deletions as sequencing.

The Norwegian and Danish patient materials were run on an ABI 3100 Genetic Analyser with POP4 polymer. Running several samples with known single-base insertions and deletions showed that the double peak patterns could consistently be detected up to repeat 8 of the VNTR, corresponding to a DNA fragment length of 283 bp (Fig. 1). Resolution decreased with increasing DNA fragment length, and peaks after repeat 8 were less defined and the size-calling was more imprecise. In addition, shorter PCR

products were amplified more effectively than larger ones, causing the peak heights to decrease rapidly after the 283 bp peak. Single base insertions and deletions in repeats 9, 10, 11 and 12 are known polymorphisms (with a combined frequency of 0.08 in healthy controls) and they are unlikely to have a strong impact on the development of exocrine dysfunction or diabetes (Raeder et al. 2006). We therefore considered an analysis of the first eight repeats of the *CEL* VNTR combined with determination of total repeat number sufficient for the current study. We emphasize that, compared with standard sequencing, the multiplex PCR method presented here yields less information about the DNA sequence. In particular, missense and non-sense mutations will be missed. However, the only disease-causing mutations reported for exon 11 of *CEL* so far are single-base deletions in the VNTR (Raeder et al. 2006). These deletions lead to a frame shift and a predicted new amino acid composition of the protein C-terminus, although the pathogenic mechanism is not clear. In summary, the current method should be well suited as a rapid screening method for variants of the *CEL* VNTR.

The UK material was run on an ABI 3730 with 48 capillaries and POP7 polymer. Compared to the ABI 3100 with POP4 polymer, the intensity of the peaks from ABI 3730 were considerably higher. Too strong signals can compromise the interpretation of the results, so we had to dilute the product from the PCR reactions 1:100 before running the samples on the instrument. The sensitivity of peak detection was also increased on ABI 3730, resulting in double peaks for normal samples and triple peaks for samples with heterozygous single-base deletions or

insertions. The reason for the extra peak is most likely the tendency of Taq-polymerases to add adenosine (A) at the 3'-end of PCR products (Smith et al. 1995). However, the software handled this problem and called the right peaks in all cases.

Screening of patient materials

A total of 241 diabetic patients were screened using the multiplex PCR method. As an internal control, exon 11 in the 95 MODYX samples from Denmark was sequenced. The sequencing confirmed the results from the multiplex PCR and fragment analysis, both regarding the structure of the proximal VNTR repeats and the total length of the VNTR. The UK material was not sequenced. We did not detect any insertions or deletions in the *CEL* VNTR in the examined MODYX patients from Denmark and UK. The most obvious reason is that such mutations are a rare cause of MODY. Patients with diabetes suspected to have a monogenic origin might not be the right patient group to analyse, as diabetes is likely to be secondary to exocrine pancreatic deficiency in the two families with *CEL* mutations (Raeder et al. 2006). Patients with tropical pancreatitis and chronic pancreatitis could be more relevant materials when it comes to searching for pathogenic *CEL* VNTR variations.

Identification of a family with a 3-repeat *CEL* VNTR

In the Danish material, we identified a proband with a novel VNTR allele consisting of only three repeated segments. To exclude the possibility of a rare causal mutation in *CEL* linked to the 3-repeat allele, the complete gene was sequenced in the proband. No undescribed changes in nucleotide sequence were found. When the family was analysed, six of seven members harbouring the 3-repeat allele had diabetes, impaired fasting glycemia or impaired glucose tolerance. Thus, the segregation pattern could suggest that the short variant predisposes to diabetes, albeit with lower penetrance than the two previously described *CEL*-MODY families. There was, however, no evidence of co-segregation with exocrine deficiency in the Danish family with the 3-repeat allele, as measured by elastase testing. This is in contrast to the two previously described families where mutation carriers had very low levels of elastase already in childhood. Furthermore, the deletion mutations in the previously published families give rise to a frameshift and predicted novel C-terminal part of the protein, whereas the *CEL* variant in the Danish family is expected to result in a very short, but—importantly—normal C-terminal tail. This strongly suggests that the intrinsic properties of the mutant proteins are dissimilar. Hence, if there is a link to diabetes for the short 3-repeat

allele, the mechanism would most likely be different than in the previously described families. It should be mentioned, though, that we cannot rule out that the mutation is not aetiological. The apparent co-segregation could be due to imperfect linkage to another risk variant outside the *CEL* gene, although we find it relatively unlikely since the *CEL* VNTR has been linked to diabetes in two previously described Norwegian families (Raeder et al. 2006). Another possibility is that the apparent co-segregation has occurred by chance. The short 3-repeat allele has not been found in a population-based control material, but one individual with four VNTR repeats was identified (Table 3). However, the controls have not been screened for the presence of diabetes so it remains unclear if very short VNTRs are part of the normal variation of the *CEL* gene. But a link between very short VNTRs and predisposition to diabetes as suggested by the Danish family described here is intriguing and needs further attention.

Rearrangements of the *CEL* locus?

Our screening method revealed the presence of a third NED-labelled peak in some individuals, suggesting three copies of a complete VNTR. Duplications and deletions involving the *CEL* locus on chromosome 9 have been reported previously by three independent studies (de Smith et al. 2007; Kidd et al. 2008; McCarroll et al. 2008). Kidd et al. found an area including exon 1–4 of *CEL* duplicated. The study by de Smith et al. identified a duplication affecting *CEL* exon 8–11, while the study by McCarroll et al. found both duplications and deletions of a similar region. In the two latter studies, the subjects with duplications had three copies of the genomic *CEL* region, which are in concordance with our findings, whereas subjects with deletions had one allele deleted.

The organization of the *CEL* locus could explain the mechanism that creates the copy number variations (CNVs). *CEL* is located in tandem with its pseudogene *CELP* separated by an 11 kb intergenic region. Exons 2–7 are not present in *CELP*, and the remaining exons 1, 8, 9, 10 and 11 share 97% sequence homology with *CEL* (Lidberg et al. 1992; Madeyski et al. 1998). The high degree of identity in the *CEL* and *CELP* sequences together with the head to tail orientation of the genes increase the risk of misalignment during chromosomal replication (Metzenberg et al. 1991). We propose that the VNTR CNV is the result of a homologous unequal recombination event, resulting in one chromosome with two VNTR copies and a reciprocal deletion of the VNTR on the other chromosome. Furthermore, it should be noted that the presence of a third VNTR copy can be detected by our method only when the three VNTRs are of different lengths. For example, a genetic constitution of two VNTRs of 16 and 14 repeats on

one chromosome and one VNTR of 16 repeats on the other will be detected as two peaks in the assay. The true number of individuals with an extra *CEL* VNTR copy is therefore likely to be higher than the frequencies observed here. The precise breakpoints of the CNVs involving *CEL* have to be identified in order to further explore the functional consequences of the rearrangements and their possible involvement in disease.

Conclusion

We have presented a new and simple multiplex PCR method which robustly detects small deletions and insertions in the *CEL* VNTR, simultaneously determining the total number of repeats within the VNTR. By employing the method, we found that mutations in the *CEL* VNTR apparently are a very rare cause of MODY. However, a new *CEL* variant with only three repeats within the VNTR was discovered. Our data also support the existence of additional *CEL* variants, possibly involving duplication of the whole VNTR. *CEL* is a highly polymorphic gene and its role in diabetes and other diseases needs further evaluation.

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References

- Bengtsson-Ellmark SH, Nilsson J, Orho-Melander M, Dahlenborg K, Groop L, Bjursell G (2004) Association between a polymorphism in the carboxyl ester lipase gene and serum cholesterol profile. *Eur J Hum Genet* 12:627–632
- Blackberg L, Angquist KA, Hernell O (1987) Bile-salt-stimulated lipase in human milk: evidence for its synthesis in the lactating mammary gland. *FEBS Lett* 217:37–41
- Bruneau N, Nganga A, Fisher EA, Lombardo D (1997) O-Glycosylation of C-terminal tandem-repeated sequences regulates the secretion of rat pancreatic bile salt-dependent lipase. *J Biol Chem* 272:27353–27361
- Bruneau N, Bendayan M, Gingras D, Ghitescu L, Levy E, Lombardo D (2003a) Circulating bile salt-dependent lipase originates from the pancreas via intestinal transcytosis. *Gastroenterology* 124:470–480
- Bruneau N, Richard S, Silvy F, Verine A, Lombardo D (2003b) Lectin-like Ox-LDL receptor is expressed in human INT-407 intestinal cells: involvement in the transcytosis of pancreatic bile salt-dependent lipase. *Mol Biol Cell* 14:2861–2875
- Caillol N, Pasqualini E, Mas E, Valette A, Verine A, Lombardo D (1997) Pancreatic bile salt-dependent lipase activity in serum of normolipidemic patients. *Lipids* 32:1147–1153
- de Smith AJ, Tsalenko A, Sampas N, Scheffer A, Yamada NA, Tsang P, Ben-Dor A, Yakhini Z, Ellis RJ, Bruhn L, Laderman S, Froguel P, Blakemore AI (2007) Array CGH analysis of copy number variation identifies 1284 new genes variant in healthy white males: implications for association studies of complex diseases. *Hum Mol Genet* 16:2783–2794
- Downs D, Xu YY, Tang J, Wang CS (1994) Proline-rich domain and glycosylation are not essential for the enzymic activity of bile salt-activated lipase. Kinetic studies of T-BAL, a truncated form of the enzyme, expressed in *Escherichia coli*. *Biochemistry* 33:7979–7985
- Dudbridge F (2008) Likelihood-based association analysis for nuclear families and unrelated subjects with missing genotype data. *Hum Hered* 66:87–98
- Hansson L, Blackberg L, Edlund M, Lundberg L, Stromqvist M, Hernell O (1993) Recombinant human milk bile salt-stimulated lipase. Catalytic activity is retained in the absence of glycosylation and the unique proline-rich repeats. *J Biol Chem* 268:26692–26698
- Higuchi S, Nakamura Y, Saito S (2002) Characterization of a VNTR polymorphism in the coding region of the *CEL* gene. *J Hum Genet* 47:213–215
- Kidd JM, Cooper GM, Donahue WF, Hayden HS, Sampas N, Graves T, Hansen N, Teague B, Alkan C, Antonacci F, Haugen E, Zerr T, Yamada NA, Tsang P, Newman TL, Tuzun E, Cheng Z, Ebling HM, Tusneem N, David R, Gillett W, Phelps KA, Weaver M, Saranga D, Brand A, Tao W, Gustafson E, McKernan K, Chen L, Malig M, Smith JD, Korn JM, McCarroll SA, Althuler DA, Peiffer DA, Dorschner M, Stamatoyannopoulos J, Schwartz D, Nickerson DA, Mullikin JC, Wilson RK, Bruhn L, Olson MV, Kaul R, Smith DR, Eichler EE (2008) Mapping and sequencing of structural variation from eight human genomes. *Nature* 453:56–64
- Kodwala A, Ghering AB, Davidson WS, Hui DY (2005) Carboxyl ester lipase expression in macrophages increases cholesterol ester accumulation and promotes atherosclerosis. *J Biol Chem* 280:38592–38598
- Li F, Hui DY (1998) Synthesis and secretion of the pancreatic-type carboxyl ester lipase by human endothelial cells. *Biochem J* 329(Pt 3):675–679
- Lidberg U, Nilsson J, Stromberg K, Stenman G, Sahlin P, Enerback S, Bjursell G (1992) Genomic organization, sequence analysis, and chromosomal localization of the human carboxyl ester lipase (*CEL*) gene and a *CEL*-like (*CELL*) gene. *Genomics* 13:630–640
- Lindquist S, Blackberg L, Hernell O (2002) Human bile salt-stimulated lipase has a high frequency of size variation due to a hypervariable region in exon 11. *Eur J Biochem* 269:759–767
- Lombardo D (2001) Bile salt-dependent lipase: its pathophysiological implications. *Biochim Biophys Acta* 1533:1–28
- Lombardo D, Guy O (1980) Studies on the substrate specificity of a carboxyl ester hydrolase from human pancreatic juice. II. Action on cholesterol esters and lipid-soluble vitamin esters. *Biochim Biophys Acta* 611:147–155
- Madeyski K, Lidberg U, Bjursell G, Nilsson J (1998) Structure and organization of the human carboxyl ester lipase locus. *Mamm Genome* 9:334–338
- McCarroll SA, Kuruvilla FG, Korn JM, Cawley S, Nemes J, Wysoker A, Shaper MH, de Bakker PI, Maller JB, Kirby A, Elliott AL, Parkin M, Hubbell E, Webster T, Mei R, Veitch J, Collins PJ, Handsaker R, Lincoln S, Nizzari M, Blume J, Jones KW, Rava R, Daly MJ, Gabriel SB, Althuler D (2008) Integrated detection and population-genetic analysis of SNPs and copy number variation. *Nat Genet* 40:1166–1174
- Metzenberg AB, Wurzer G, Huisman TH, Smithies O (1991) Homology requirements for unequal crossing over in humans. *Genetics* 128:143–161
- Raeder H, Johansson S, Holm PI, Haldorsen IS, Mas E, Sbarra V, Nermoen I, Eide SA, Grevle L, Bjorkhaug L, Sagen JV, Aksnes

- L, Sovik O, Lombardo D, Molven A, Njolstad PR (2006) Mutations in the CEL VNTR cause a syndrome of diabetes and pancreatic exocrine dysfunction. *Nat Genet* 38:54–62
- Raeder H, Haldorsen IS, Erslund L, Gruner R, Taxt T, Sovik O, Molven A, Njolstad PR (2007) Pancreatic lipomatosis is a structural marker in nondiabetic children with mutations in carboxyl-ester lipase. *Diabetes* 56:444–449
- Roudani S, Miralles F, Margotat A, Escibano MJ, Lombardo D (1995) Bile salt-dependent lipase transcripts in human fetal tissues. *Biochim Biophys Acta* 1264:141–150
- Smith JR, Carpten JD, Brownstein MJ, Ghosh S, Magnuson VL, Gilbert DA, Trent JM, Collins FS (1995) Approach to genotyping errors caused by nontemplated nucleotide addition by Taq DNA polymerase. *Genome Res* 5:312–317
- Stride A, Hattersley AT (2002) Different genes, different diabetes: lessons from maturity-onset diabetes of the young. *Ann Med* 34:207–216
- Vesterhus M, Raeder H, Aurlien H, Gjesdal CG, Bredrup C, Holm PI, Molven A, Bindoff L, Berstad A, Njolstad PR (2008) Neurological features and enzyme therapy in patients with endocrine and exocrine pancreas dysfunction due to CEL mutations. *Diabetes Care* 31:1738–1740
- Wang CS, Dashti A, Jackson KW, Yeh JC, Cummings RD, Tang J (1995) Isolation and characterization of human milk bile salt-activated lipase C-tail fragment. *Biochemistry* 34:10639–10644