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**Arancha Cebrián · Sergio Ruíz-Llorente · Alberto Cascón
Ana Osorio · Beatriz Martínez-Delgado · Javier Benítez
Mercedes Robledo**

# A rapid and easy method for multiple endocrine neoplasia type 1 mutation detection using conformation-sensitive gel electrophoresis

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**Abstract** Until now, the study of the multiple endocrine neoplasia type 1 (*MEN1*) gene in patients suspected of having the disease was expensive and laborious due to the large size of the gene. We have optimized the conformation-sensitive gel electrophoresis (CSGE) technique to analyze by four rather simple multiplex PCR reactions, and a single electrophoresis run, the entire coding region of the *MEN1* gene, plus the exon–intron boundaries. This improvement of the CSGE technique was confirmed as an effective procedure for screening for the *MEN1* gene by detecting ten previously known *MEN1* gene mutations and four polymorphisms. The *MEN1* gene of 12 patients with unknown mutations was then screened, and an abnormal CSGE profile was identified in 10/12 cases. Subsequent DNA sequencing demonstrated 3 of them to be novel mutations (E45K, 4479delACAG, 6073insC) and 7 to have been previously reported; in the remaining 2 patients, we confirmed the absence of any alteration of the coding sequence of *MEN1*. Mutation screening of the *MEN1* gene using CSGE was demonstrated to be a fast, simple, and inexpensive method to study patients suspected of having MEN1 disease.

**Key words** *MEN1* · Mutation screening · CSGE · Multiplex PCR · Efficient · Easy · Inexpensive method

## Introduction

Multiple endocrine neoplasia type 1 (MEN1) is a single-gene autososomal dominant disease (OMIM *131100) characterized by the presence of tumors of the parathyroid, endocrine pancreas, and anterior pituitary glands. Affected individuals may also show other complex lesions such as neuroendocrine tumors (carcinoids), secreting or non-secreting adrenal tumors, paraganglioma, thyroid adenoma, or lipoma (Lips et al. 1984; Raue and Zink 1992; Trump et al. 1996). MEN1 is known to have very high penetrance, about 98.8% by the age of 53 (Trump et al. 1996). The *MEN1* gene is a tumor-suppressor gene that contains ten exons (with the first exon untranslated) and extends across 9 kb. It encodes for a product of 610 amino acids named menin (Chandrasekharappa et al. 1997) that has been identified as a nuclear protein, suggesting possible roles as a component in transcriptional regulation, DNA replication, or cell cycle control (Guru et al. 1998). So far, more than 200 different germline mutations have been described in MEN1 families. These mutations are scattered throughout the coding sequence of the gene, without obvious hotspots suggestive of functional domains and with no apparent genotype–phenotype correlation (Chandrasekharappa et al. 1997; Agarwal et al. 1997; Debelenko et al. 1997; European Consortium on MEN1 1997; Bassett et al. 1998; Cebrián et al. 1999; Karges et al. 2000). For this reason, different mutation-screening techniques have been used to detect alterations, such as single-strand conformation polymorphism (SSCP) (Bassett et al. 1998; Martín-Campos et al. 1999; Wenbin et al. 1999), denaturant gradient gel electrophoresis (DGGE) (Morelli et al. 2000), dideoxyfingerprinting (ddF) (Goebel et al. 2000) and nucleotide sequencing (Cebrián et al. 1999). All these commonly used methods are effective, but are either labor intensive or expensive, or both. Conformation-sensitive gel electrophoresis (CSGE) is a simple, inexpensive, and efficient mutation-screening technique that has been used as a practical procedure for the detection of mutations within other complex genes, such as collagen genes, the *BRCA1* gene, and the *FIX* gene (Körkkö et al. 1998; Markoff et al. 1998; Hinks et al. 1999). The basic principle of CSGE is that it enhances the conformational differences produced by single-base mismatches in double-stranded DNA, and thereby increases the differential migration in electrophoretic gels of heteroduplexes and homoduplexes (Ganguly et al. 1993).

A. Cebrián (✉) · S. Ruíz-Llorente · A. Cascón · A. Osorio ·
B. Martínez-Delgado · J. Benítez · M. Robledo
Department of Human Genetics, Molecular Pathology Program,
Centro Nacional de Investigaciones Oncológicas
C/ Melchor Fernández Almagro, 3, 28029 Madrid, Spain
Tel. +34-91-2246947; Fax +34-91-2246923
e-mail: acebrian@cnio.es

In the present study, CSGE was applied for the first time to the mutation analysis of 23 Spanish patients suspected of having MEN1 disease. Eleven of those patients had been already tested by our group by SSCP and/or sequencing analysis, and the results of some of these tests were previously published (Cebrián et al. 1999). The remaining group of patients was referred to our center in order to confirm the suspected clinical diagnosis and to perform a carrier diagnosis in all families.

## Subjects and methods

### Patients

A first panel of 11 cases in which the causative mutation was know was used to validate this mutation-screening method. In this group was included one patient who presented only the polymorphism A541T. Subsequently, three cases exhibiting common polymorphisms (R171Q, D418D, and S145S) were added to the study.

In addition, 12 unrelated patients suspected of having MEN1 disease and whose genetic status was unknown were studied. MEN1 patients were selected following criteria previously described (Thakker et al. 1989). Informed consent was obtained from all the patients.

### DNA amplification

The nine coding exons of the *MEN1* gene were amplified with oligonucleotide primers under conditions previously described (European Consortium on MEN1 1997; Cebrián et al. 1999). We optimized four multiplex amplifications to hasten the CSGE analysis: exon 2 + exon 5-6, exon 3 + exon 10, exon 7 + exon 8, and exon 9 + exon 4. The primers used and the amplified region are shown in Table 1. The components of the reaction were as follows: in 50 μl of a mixture containing 1 × polymerase chain reaction (PCR) buffer (Roche Diagnostics, Mannheim, Germany), 200 μM dNTP, 5–20 pmol of each primer, 200 ng of genomic DNA, and 1 U *Taq* polymerase (Roche Diagnostics). PCR conditions were 10 cycles of 30 s at 94°C, 30 s at 68°C, and 30 s at 72°C, followed by 20 cycles of 30 s at 94°C, 30 s at 62°C or 63°C, 1 min at 72°C, with a 5-min final extension at 72°C.

### Gel composition

The gel composition used had 1 × MDE gel solution (BMA, Rockland, ME, USA), 15% formamide (Amersham Pharmacia Biotech, Barcelona, Spain), 10% ethylene glycol, and 0.6 × tris borate + ethylenediaminetetraacetate (TBE) composition.

### CSGE analysis

Fifty–100 ng of amplified DNA was subjected to denaturation at 94°C for 3 min and then incubated at 68°C for 1 h to generate heteroduplexes. Samples were allowed to cool down slowly to room temperature and 3–5 μl of the PCR products were mixed with 1 μl of triple dye loading buffer 6 × (FMC, Rockland, ME, USA). PCR products were then electrophoresed under two conditions: (1) 1-mm-thick gel with 16 × 16 cm glass plates, in 0.6 × TBE buffer at a constant voltage of 150 V for 20 h at room temperature (Fig. 1); and (2) 0.4-mm-thick gel with 21 × 40 cm glass plates, in 0.6 × TBE buffer at a constant power of 7 W for 17–20 h according to the size of the PCR products. After the electrophoresis, the gel was silver-stained and dried for documentation.

### Nucleotide sequencing of PCR products

The PCR products were purified using columns (E.Z.N.A cycle pure kit, OMEGA Biotech, Doraville, GA, USA) and

**Table 1.** Polymerase chain reaction primers used for multiplex amplification of the multiple endocrine neoplasia type 1 (*MEN1*) gene

| Primer | Primer sequence (5′ to 3′) | Size (bp) | Region amplified | |
|---|---|---|---|---|
| Exon 2 f | aaccttagcggaccctgggaggag | 574 | 2191 to 2765 | |
| Exon 2 r | ccagtaggtgggaatcttatccatg | | | 1st multiplex |
| Exon 5 f | cctgttccgtggctcataactc | 297 | 5138 to 5435 | |
| Exon 6 r | ggagacc ctaacagtgg ctgag | 347 | 5138 to 5485 | |
| Novel exon 6 r | agaac tttgtgtgtt ggggg | | | |
| Exon 3 f | gcacagaggaccctctttcattac | 305 | 4258 to 4563 | 2nd multiplex |
| Exon 3 r | actgtagtagcccaagccaccca | | | |
| Exon 10 f | tcaccttgctctccccactg | 537 | 7552 to 8089 | |
| Exon 10 r | ggacttcggaccgcttgtggg | | | |
| Exon 7 f | ggctgcctccctgaggatc | 250 | 5985 to 6235 | 3rd multiplex |
| Exon 7 r | ccaaccaccctcgtccag | | | |
| Exon 8 f | gtgagacccctcagaccctac | 217 | 6579 to 6796 | |
| Exon 8 r | cctgtgtccagc ctccca | | | |
| Exon 9 f | ggtgagtaag agactgatct gtgc | 245 | 7150 to 7395 | 4th multiplex |
| Exon 9 r | cacaga ggtctgggca ctaca | | | |
| Exon 4 f | gggccatcatgagacataatg | 191 | 4677 to 4868 | |
| Exon 4 r | ctgagccaatggggcag | | | |

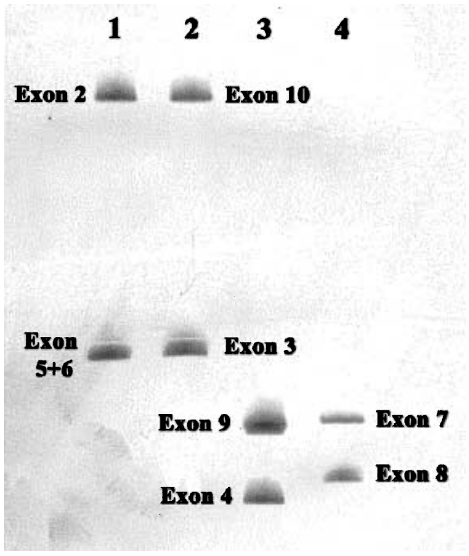


**Fig. 1.** Appearance of all four multiplex polymerase chain reactions on a single conformation-sensitive gel electrophorosis (CSGE) from the current analysis of the multiple endocrine neoplasia type 1 (*MEN1*) gene. Details of the primers used and the size of the fragment are presented in Table 1. *Lane 1* corresponds to *exons 2* and *5 + 6*; *lane 2* shows *exons 3* and *10*; *lane 3* shows *exons 4* and *9*; and *lane 4* shows *exons 7* and *8*

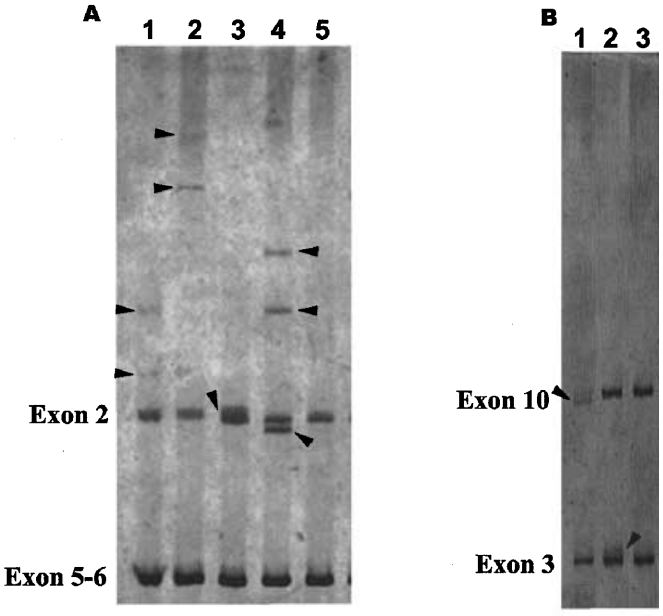


**Fig. 2.** Detection of previously known mutations. *Arrowheads* indicate abnormal bands. **A** CSGE gel from the amplification of *exon 2* and *exons 5 + 6*. *Lanes 1–4* show abnormal patterns corresponding to 2556delAT, 2535delTGTC, 2530insC, and 2416delCGT, respectively. *Lane 5* is a negative control. **B** CSGE gel from the amplification of *exons 3* and *10*. *Lanes 1* and *2* correspond to mutations 7736del25bp and Q209X, respectively. *Lane 3* is a negative control

bidirectionally sequenced with a BigDye terminator cycle sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Sequences were determined by using a ABI3700 automated sequencer, and sequence traces were manually analyzed for the presence of mutations. Changes were confirmed by the sequencing of independent PCR products.

## Results

### The CSGE method identifies all mutations in a previously characterized panel of patients

Heteroduplex detection by CSGE was initially carried out in 11 patients with previously detected *MEN1* gene alterations (Fig. 2). Nine of them had been reported by Cebrián et al. (1999): 5 deletions in exons 2 and 10; 1 insertion in exon 2; 1 insertion/deletion in exon 4; 2 nonsense mutations in exons 3 and 7. The remaining two patients had previously described alterations: a polymorphism in codon 541 (Chandrasekharappa et al. 1997; Agarwal et al. 1997; Sato et al. 1998; Tanaka et al. 1998) and a nucleotide substitution in the donor site of a splice in intron 6 (Mutch et al. 1999).

Nine of these 11 alterations were confirmed in the first single assay by using CSGE. Only the polymorphic variant in codon 541 (A541T) and the nucleotide substitution in intron 6 were not detected. As we had mainly frameshift mutations available, which are easier to detect, three patients with common polymorphisms — R171Q, D418D and S145S — were added to the study to assess the sensitivity of the technique. All of them were easily detected in the first single assay.

### Improved conditions for the detection of mutations by CSGE

The initial data suggested that alterations within the first or last 50 bp can be missed by the heteroduplex analysis (Ganguly et al. 1993; Körkkö et al. 1998; Markoff et al. 1998), and other studies have found that alterations were readily detected by reducing the size of the PCR product to less than 300 bp . We designed a new primer that hybridized further downstream from the donor site in intron 6, and with this primer the mutation could finally be detected by the CSGE method. With regard to the polymorphism A541T, we reduced the PCR product size from 540 bp to 340 bp, but the variant was not detected. We explored the effect of varying the electrophoretic conditions in both the product containing this polymorphism and in the other variants. We found that the polymorphism was not observed, but the rest of the mutations were more readily detected when the electrophoretic conditions were changed from 150 V for 20 h to 7 W for 17–20 h (see Subjects and methods).

Analysis by CSGE in 12 previously unstudied patients with *MEN1*

All exons and exon/intron boundaries of the *MEN1* gene were analyzed by CSGE in a panel of 12 newly diagnosed patients with MEN1 who had not participated in any previous molecular study. With the improved electrophoretic conditions, an abnormal CSGE profile, indicating a heteroduplex, was observed in 10/12 patients (Fig. 3). Each PCR fragment showing an abnormal CSGE profile was then sequenced. Ten different alterations were characterized: 7 of them had been previously reported and 3 had not been previously described. One of these latter alterations, Glu45Lys, identified in patient 00S47, is probably a missense mutation rather than a common benign polymorphism because it was not detected in 220 independent alleles from a control population. These data are summarized in Table 2.

In 2/12 patients, we did not detect abnormal profiles by CSGE, and we confirmed by sequencing analysis the absence of any alteration in the coding sequence in these patients.

## Discussion

CSGE analysis has been previously reported as being a very efficient technique for detecting mutations in different genes (Ganguly et al. 1993; Körkkö et al. 1998; Markoff

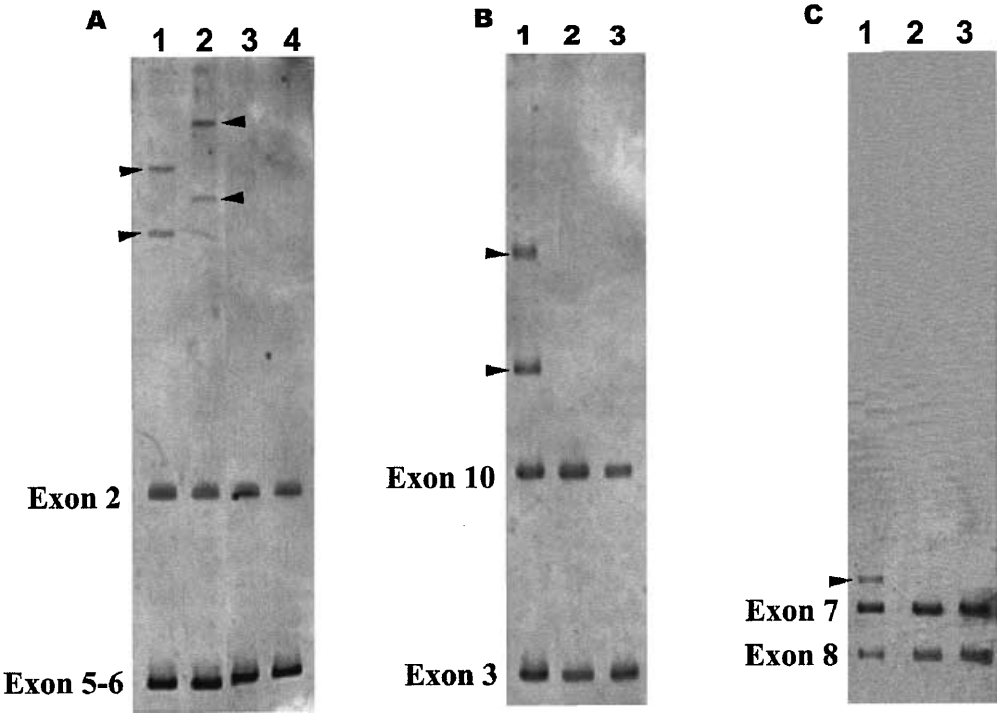


**Fig. 3A–C.** CSGE gels showing the detection of new mutations. *Arrowheads* indicate abnormal bands. **A** Amplification of *exon 2* and *exons 5 + 6*. *Lanes 1* and *2* show mutations 2534delCTGT and 2489insGCCCC. **B** Amplification of *exons 3* and *10*. *Lane 1* shows mutation 7897delAGA. **C** Amplification of *exons 7* and *8*. *Lane 1* shows mutation W341X. *Lanes 3* and *4* (**A**), *2* and *3* (**B**), and *2* and *3* (**C**) correspond to two individuals who were suspected of having MEN1 disease but yielded a normal CSGE band pattern. The samples from these individuals were sequenced to confirm the absence of mutations

**Table 2.** *MEN1* gene analysis in 12 patients with MEN1 and their clinical characteristics

| Patient ID | Age of onset | HPT | Pancreatic tumors | Pituitary tumors | Location in *MEN1* gene | Nucleotide change[b] | Amino acid change |
|---|---|---|---|---|---|---|---|
| 98/958 | 26 y. | yes | None | ACTH secreting | Exon 2 | C402T | Arg 98 Ter |
| 99/242 | 30 | yes | Gastrinoma | None | Exon 7 | G1132A | Trp 341 Ter |
| 99/615 | 40 | yes | Nonfunctioning | Prolactinoma | Exon 2 | 357 del CTGT | Leu 83 Fs |
| 99/1129 | 34 | yes | Glucagonoma | None | Exon 4 | C891T | Gln 261 Ter |
| 00S2 | 25 | yes | Insulinoma | None | Exon 10 | 1780 del AGA | Lys 557 Fs |
| 00S49 | 41 | yes | Gastrinoma | Prolactinoma | Exon 2 | 312 ins GCCCC | Ala 68 Fs |
| 00S47 | 18 | yes | None | Prolactinoma | Exon 2 | G243A[a] | Glu 45 Lys |
| 00S110 | 35 | yes | Insulinoma | Nonsecreting | Exon 2 | 357 del CTGT | Leu 83 Fs |
| 00/214 | 16 | yes | None | Prolactinoma | Exon 3 | 738 del ACAG[a] | Thr 210 Fs |
| 99/442 | 30 | yes | Gastrinoma | None | Exon 7 | 1071 ins C[a] | Tyr 321 Fs |
| 99/1155 | 60 | yes | None | GH secreting | — | — | — |
| 00/154 | 54 | yes | None | Prolactinoma | — | — | — |

HPT, hyperparathyroidism; ACTH, adrenocorticotropic hormone; GH, growth hormone
[a] Novel mutation, not previously reported
[b] cDNA nomenclature

et al. 1998; Hinks et al. 1999) without the risk of obtaining false positive results (Hinks et al. 1999). Importantly, almost 100% of sequence variants can be detected under a single set of electrophoretic conditions (Ganguly et al. 1993).

With these antecedents, we tested this method for MEN1 disease, because to date the techniques used have been effective but labor intensive or expensive. In the present study we detected mutations and 3/4 common polymorphisms (except A541T) by CSGE in 20/22 unrelated patients. In the two patients in whom we did not detect any change, the absence of mutations was confirmed by sequence analysis. These results indicate that CSGE is able to detect 96% of alterations using a single set of electrophoretic conditions.

We did not detect the nucleotide substitution in the donor site of the splice of intron 6, located in the terminal 50 bp of the PCR fragment, in the first single CSGE assay. However, by designing another primer located further away from the mutation site, it was possible to detect the alteration. This result therefore confirmed that one of the limitations of CSGE, as reported by other authors (Ganguly et al. 1993; Körkkö et al. 1998; Markoff et al. 1998), is that this technique does not reliably detect mutations in the terminal 50 bp of each PCR fragment. To overcome this limitation, and since this technique allows us to work with large fragments, a simple solution would be to design primers that are at least 50 bp away from the splicing site.

As mentioned above, we could not detect the A541T polymorphism, which was not located within 50 bp of our amplified product. To solve this problem, we reduced the size of the fragment and changed the electrophoresis conditions. These electrophoresis improvements made the detection of all the mutations easier and more accurate, except for in the case of the A541T polymorphism, which remained undetectable.

To date, the study of the *MEN1* gene in patients suspected of having the disease has been hampered by the large size of the gene, requiring the use of screening techniques that are either expensive, labor intensive (sequencing and DGGE), or of relatively low sensitivity (SSCP). We have optimized the CSGE anolysis technique by the relatively simple process of performing four multiplex PCR reactions, followed by their discrimination in a single electrophoresis run (Fig. 1). Employing this improvement of the CSGE technique, we have detected all the mutations in our group of patients by a rapid, simple, and inexpensive experiment.

We propose the general use of CSGE of multiplex PCR for mutation screening because of its simplicity and sensitivity, which in our series was 96%, and its great capacity for detecting mutations in relatively large DNA segments at least 500 bp in length. Only the PCR products with abnormal CSGE profiles need be sequenced to discriminate the type of alteration involved (frameshift mutation, missense mutation, or polymorphism). Although further evaluation of this method is required to establish its sensitivity accurately, we believe that the CSGE technique is an efficient mutation screening method for the analysis of the *MEN1* gene that is easier to apply and less expensive than the techniques currently in use.

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