Screening of *RET* Gene Mutations in Multiple Endocrine Neoplasia Type-2 Using Conformation Sensitive Gel Electrophoresis (CSGE)

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MARCELO A. C. G. DOS SANTOS
ELISANGELA PEREIRA DE S. QUEDAS
RODRIGO DE ALMEIDA TOLEDO
DELMAR M. LOURENÇO-JÚNIOR
SERGIO PEREIRA DE A. TOLEDO

Endocrine Genetics Unit (LIM-25), Endocrinology, Department of Internal Medicine, Hospital das Clínicas, and University of São Paulo School of Medicine, SP.

ABSTRACT

Multiple endocrine neoplasia type 2 (MEN2) is an autosomal dominant inherited tumor syndrome caused by RET proto-oncogene germline mutations (*RET*). Here we tested the Conformation Sensitive Gel Electrophoresis (CSGE) as a screening method for *RET* hot-spot mutations. Seven MEN2 families were studied by direct sequencing analysis, CSGE and Single Strand Conformational Polymorphism (SSCP). Using CSGE/SSCP, we were able to detect four out of five types of *RET* mutations verified by sequencing analysis: Cys620Arg, Cys634Arg, Cys634Tyr, and Met918Thr, furthermore a missense substitution at codon 648 (Val648Ile). *RET* polymorphisms 691 and 769 were also verified. Data obtained using CSGE/SSCP were fully concordant. We conclude that CSGE showed to be a sensitive, fast, low-cost, and simple procedure to detect *RET* mutations in codons which are reported as the most prevalent *RET* variants (~ 95%) in large MEN2 series. As to the Val804Met mutation, this method still needs to be optimized. **(Arq Bras Endocrinol Metab 2007;51/9:1468-1476)**

Keywords: Genetic screening; CSGE; SSCP; Genetic sequencing; MEN-2; RET proto-oncogene

RESUMO

Rastreamento de Mutações do Gene *RET* na Neoplasia Endócrina Múltipla Tipo 2 por Eletroforese em Gel Sensível à Conformação (CSGE).

A neoplasia endócrina múltipla tipo 2 (NEM2) é uma síndrome tumoral herdada por mutações germinativas no proto-oncogene RET (*RET*). Analisamos a aplicação do método Eletroforese em Gel Sensível à Conformação (CSGE) no rastreamento de mutações *hot spots* do *RET*. Sete famílias com NEM2 foram rastreadas pelo seqüenciamento gênico, CSGE e análise do Polimorfismo Conformacional de Cadeia Simples (SSCP). Usando ambas as metodologias de rastreamento, identificamos quatro dos cinco tipos de mutações verificadas pelo seqüenciamento: Cys620Arg, Cys634Arg, Cys634Tyr e Met918Thr, além da variação gênica Val648Ile. Das análises englobando mutações *hot spots* do *RET*, 90,6% concordaram com o seqüenciamento genético (incluindo a variação gênica Val648Ile). Polimorfismos nos códons 691 e 769 foram documentados. Os dados obtidos por CSGE/SSCP foram totalmente concordantes. Concluímos que o CSGE revelou ser metodologia sensível, rápida, de fácil execução e baixo custo no rastreamento de mutações nos códons associados à grande maioria (~ 95%) dos pacientes com NEM2. **(Arq Bras Endocrinol Metab 2007;51/9:1468-1476)**

Descritores: Rastreamento gênico; CSGE; SSCP; Seqüenciamento gênico; NEM2; Proto-oncogene RET

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Multiple endocrine neoplasia type 2 (MEN2) is an inherited tumor syndrome characterized by the presence of medullary thyroid carcinoma (MTC), primary hyperparathyroidism (HPT), and pheochromocytoma (PHEO). MEN2 is classified as MEN2A (MTC, PHEO, and HPT); MEN2B (MTC, PHEO, and mucosal neuromas); and familial MTC (FMTC) (1-5). The genetic basis of MEN2 is associated with germline activating mutations in the RET proto-oncogene (*RET*). RET gene contains 21 exons, which encode a tyrosine kinase receptor protein with an extracellular domain rich in cysteine residues and an intracellular domain enriched in tyrosine residues (1,6).

Several Brazilian studies on MEN2 have been recently published, in which clinical and genetic aspects of MEN2 were extensively reviewed (2-5,7-14). All clinical variants of MEN2 have a high penetrance rate for MTC and most (90%) *RET* mutation carriers will exhibit evidence for MTC during their lifetime (15). Briefly, MEN2A comprises 75% of MEN2 cases and most cases (98%) harbor a *RET* mutation in exons 10 or 11 (codons 609, 611, 618, 620, 630, and 634). *RET* variants in codon 634 cause 85% of MEN2A cases, mostly with the Cys634Arg and Cys634Tyr mutations (1,7). Furthermore, FMTC cases comprise 20% of MEN2 patients and most of them (85%) have *RET* mutations in codons 10 or 11 (1,8,15). Less frequent mutations in codons 609, 611, 768, and 804 are usually associated with FMTC, but rarely to MEN2A (1,7). Met918Thr (exon 16) mutation has been reported in 95% of MEN2B cases, which comprise 5% of MEN2 cases (1,15). Rarely, two *RET* variants have been reported as co-segregating in MEN2 cases (3,8). Thus, the vast majority (95%) of *RET* disease-causing mutations are associated to codons 620, 634, and 918 (15).

Molecular diagnosis of *RET* mutations has become a crucial tool for the management of MEN2 as it may (a) identify 1–7% of inherited MTC cases among MTC patients previously considered as "sporadic" cases; (b) identify *RET* mutation carriers in at-risk family members and eliminate the need for annual follow-up in relatives who do not carry *RET* mutations; and finally (c) it is the rational basis for indicating preventive total thyroidectomy, usually under 5 years of age, in all *RET* mutation carriers (4-6,15). Direct DNA sequencing is the gold-standard method for genetic studies in the detection of disease-causing mutations. However, several genetic screening techniques, such as denaturating gradient gel electrophoresis (DGGE), restriction enzymes, single strand conformational polymorphism (SSCP) and denaturing high performance liquid chromatography (DHPLC) have been applied to genetic screening of at-risk relatives in families with several inherited diseases (1-8,15,16).

Conformation Sensitive Gel Electrophoresis (CSGE) has been shown to be a useful method for mutation detection. CSGE detects the differences in electrophoresis migration patterns of homo- and heteroduplex formations. These diverse bands are caused by structural alterations in DNA double helix, which are favored by mild denaturing solvents (16-18). Interestingly, this method presents important advantages when compared to restriction enzymes, SSCP, DGGE (19), and DHPLC methodologies. CSGE may present higher sensitivity for 200–800bp PCR fragment sizes, when compared to SSCP sensitivity (16,20). Comparing to DGGE, CSGE is a feasible method to standardize and does not need a 50bp GC-clamp coupled to one of the sequences (16,20). Moreover, CSGE does not require an expensive wave DNA fragment analysis system, such as in DHPLC. Although CSGE has been applied to several inherited conditions, limited information is available on CSGE to identify *RET* mutations (19,20). As there is an increasing demand for *RET* genetic diagnosis in the endocrine practice, the optimization of *RET* mutations is a worthwhile effort. Thus, this study aimed at validating CSGE as genetic screening approach for *RET* hot-spot mutations in patients with classical MEN2 phenotype presentations.

MATERIALS AND METHODS

Patients

This study was approved by local ethic committees (CAPPesq — HC-FMUSP, protocol number 265/ 04). A written informed consent was obtained from all individuals involved in this project. In this study, seven previously identified (clinically and genetically) MEN2 families were included: two with MEN2A; one with MEN2A cosegregating with congenital megacolon; three with FMTC; and one MEN2B (3-5,8). A total of 64 individuals (7 index cases and 57 at-risk family members) were screened by CSGE and a subset group of 37 patients were compared to SSCP screening. Initially, mutation analysis of all index cases was performed by directly sequencing the *RET* hot-spots exons 10, 11, 13, 14, 15, and 16. Other at-risk family members were also submitted to DNA sequencing, although it was restricted to the specific exon in which the *RET* germline mutation and/or polymorphism were identified. The PCR products of index cases and at-risk relatives found to be mutated and/or polymorphic were then screened by CSGE, and a subset of them by SSCP. Samples from 27 at-risk family members identified

as healthy individuals were used as controls. All individuals have been followed at the Outpatient Service of the Division of Endocrinology, Hospital das Clínicas, University of Sao Paulo School of Medicine. Index cases were diagnosed by classical clinical, biochemical, and genetic parameters (2,3,8-12,15).

Methods

Genomic DNA extraction and PCR

Genomic DNA was extracted using the salting-out method (21). DNA concentration was measured by spectrophotometry (Pharmacia Biotech, Sweden). Exons 10, 11, 13, 14, 15, and 16 were amplified by PCR (Minicycler — MJ Research, Waterstone, MA, USA) (22), using previously reported primers (7,13,23). Other primers were designed specifically for CSGE and SSCP analyses, as shown in table 1. PCR protocols were optimized as follows: 10 mM Tris HCl pH 8.4, 50 mM KCl pH 8.4, 0.4 mM of dNTPs, 2.0U of Taq-polimerase, 5% of Dimethyl sulfoxide – DMSO. The $MgCl_2$ and primer concentrations were: 1.76 mM; 20 pmol for exon 10; 2.40 mM; 14 pmol for exon 11; 2.40 mM; 20 pmol for exons 13 and 15; 3 mM; 10 pmol for exon 14; and 3 mM; 20 pmol for exon 16. Genomic DNA concentration ranged from 100–200 ng. The optimized cycling programs used were: single cycle at 94ºC for 10 min. (exons 10, primer setting 11A, 11B, 15, and 16) and 3 min. (exons 13, primers settings 14A and 14B); 38 cycles at 94°C for 30 sec. (all exons), annealing for 30 sec. (exons 10 and 15) and 1 min. (primers settings 11A, 11B, 13, 14A, 14B, and 16), as indicated in table 1, and extension at 72°C for 1 min. (all exons), followed by an extension cycle at 72°C for 4 min. For exon 14, amplification with clamp primers (primer setting 14C) cycle temperatures were: single cycle at 95°C for 4 min.; 35 cycles at 94°C for 45 sec., annealing for 45 sec. and extension at 72°C for 10 min. PCR products were confirmed by electrophoresis at 70V for 1 h. (Power PAC 3000, Biorad, USA) in 2% agarose gel with TAE 1X buffer stained with ethydium bromide (0.5 mg/ml), where 5 ml of genomic amplified material were mixed to 1 ml of Loading Buffer 6 x and visualized in UV transiluminator (Foto Analyst Mini Visionary, Fotodyne, USA).

Sequencing analysis

RET direct sequencing was performed as follows: 2 ml of Big Dye terminator buffer (Applied Biosystems, Foster City, CA, USA), 6 ml of 0.25% buffer (Tris-HCl 0.5 M pH 9.0 with 25 mM of $MgCl_2$), 1.60 pm of primer and 5–10 ng of amplified DNA, in a total volume of 20 ml. Cycle sequencing conditions were: single cycle at 96ºC for 2 min.; 40 cycles at 96ºC for 10 sec., annealing at 60ºC for 20 sec. and extension at 60ºC for 4 min. After sequencing, reactions were purified with isopropanol/ethanol according to the manufacturer's instructions, and diluted in 2.5 ml of blue dextran buffer with deionized formamide (TSR, Applied Biosystems). Sequencing products were denaturated at 90ºC for 2 min. and immediately cooled before applied in an ABI 310 DNA sequencer. Electropherograms were analyzed by AB Navigator software (Applied Biosystems).

CSGE

The MDE gel was prepared as follows: MDE 0.5 X (Cambrex Bio Science, Rockland, ME, USA), 6% de TBE 10 X, 2.5% de glycerol, 220 ml of ammonium persulfate 10% and

Table 1. Primers sequences and annealing temperatures for RET hot-spot exons. (Adapted from refs. 7, 13, 23)

Exons	Primers sequences	Annealing (°C)	Bp
10	10F: 5′AggCTgAgTgggCTACgTCTg 3′ 10R: 5′gTTgAgACCTCTgTggggCT 3′	60	205
11A	11F: 5′ATgAggCAgAgCATACgCAgCC 3′ 11R: 5′CTTgAAggCATCCACggAgACC 3′	60	332
11B	11F: 5′ATgAggCAgAgCATACgCAgCC 3′ 11R: 5′TTgTgggCAAACTTgTggTA 3′	60	199
13	13F: 5′AACTTgggCAAggCgATgCA 3′ 13R: 5′AgAACAgggCTgTATggAgC 3′	62	276
14A	14F: 5′CCTggCTCCTggAAgACC 3′ 14R: 5′CATATgCACgCACCTTCATC 3′	62	298
14B	14F: 5′CCTggCTCCTggAAgACC 3′ 14R: 5′CCAggCAAATgAgATgAggT 3′	62	241
14C	14F: 5′gCgCCCCCCgCCCCgCCCgCCgCggCgCCg CCCAgggATAgggCCTgggCTTC 3′ 14R: 5′TAACCTCCACCCAAgAgAg 3′	65	395
15	15F: 5′CATggCCTgACgACTCgTgC 3′ 15R: 5′CCTgggAgCCCCgCCTCATC 3′	60	192
16	16F: 5′CTgAAAgCTCAgggATAggg 3′ 16R: 5′TAACCTCCACCCCAAgAgAg 3′	60	202

Table 2. Genotype-phenotype correlation for RET mutant carriers screened by CSGE.

Cases	Mutated exon	Phenotype	Genotype	Polymorphic exons	Polymorphisms
1	11	MEN2A	Cys634Arg		
2	11	MEN2A	Cys634Arg		
3	11	MEN2A	Cys634Arg	11	Gly691Ser
4	11	MEN2A	Cys634Arg	11	Gly691Ser
5	11	MEN2A	Cys634Arg	11	Gly691Ser
6	11	MEN2A	Cys634Arg		
7	11	MEN2A	Cys634Arg		
8	11	MEN2A	Cys634Arg		
9	11	MEN2A	Cys634Arg		
10	11	MEN2A	Cys634Arg		
11	11	MEN2A	Cys634Arg	11	Gly691Ser
12	11	MEN2A	Cys634Arg	11	Gly691Ser
13	10	MEN2A	Cys620Arg	11	Gly691Ser
14	10	MEN2A	Cys620Arg	11	Gly691Ser
15	10	MEN2A	Cys620Arg	11	Gly691Ser
16	10	MEN2A	Cys620Arg	11	Gly691Ser
17	10	MEN2A+HSCR	Cys620Arg	11	Gly691Ser
18	10	MEN2A+HSCR	Cys620Arg	11-13	Gly691Ser, Leu769Leu
19	10	MEN2A	Cys620Arg	11	Gly691Ser
20	10	FMTC	Cys620Arg		
21	10	FMTC	Cys620Arg		
22	10	FMTC	Cys620Arg		
23	10	FMTC	Cys620Arg		
24	11	FMTC	Cys634Tyr		
25	11	FMTC	Cys634Tyr		
26	11	FMTC	Cys634Tyr		
27	11	FMTC	Cys634Tyr		
28	11	FMTC	Cys634Tyr		
29	11	FMTC	Cys634Tyr		
30	11	FMTC	Cys634Tyr		
31	11	FMTC	Cys634Tyr		
32	16	MEN2B	Met918Thr		

Val804Met mutation and Ser904Ser polymorphism were not included in this table.

22 ml of N,N,N',N'-Tetramethylethylenediamine. Gel was applied into plates (coated with repel-silane and gamma-methacryloxypropyltrimethoxysilane) and left to polymerize for 2 h. The electrophoresis system was pre-run (Sequencing System Model S2, Life Technologies, Gibco/BRL, Gaithersburg, USA) for 45 min. at 8W with TBE 0.6 X buffer. Temperature conditions for heteroduplex formation were: single cycle at 94°C for 5 min. (exons 10, primer setting 11A, 13, and 16) or 10 min. for exon 11 (primer setting B), followed by annealing at room temperature for 40 min. (exons 10, primers settings 11A and 11B, 13 and 16). 5 ml of sample (2:1 of amplicon and Loading Buffer 1 X) (Triple Dye Loading Buffer 6 X, Cambrex Bio Science) were loaded in gel and electrophoresed for 1 h. at 15 W (exons 11 primer setting A, and exon 13). Subsequently, exon 16 was loaded in gel, and all exons were electrophoresed at 8 W for 11 h. Amplified genomic material from exons 10 and 11 (primer setting B) were electrophoresed for 11 h. at 2 W. CSGE gel was silver stained.

SSCP

SSCP gel and electrophoresis conditions were the same as used for CSGE. After denaturation (same temperatures used in CSGE), amplicons were rested in ice before loading in gel. A loading buffer (95% formamide, 10 mM NaOH, 0.025% Bromophenol Blue, 0.025% Xylene Cyanol) was used. SSCP gel was silver stained.

RESULTS

Figure 1 and table 3 summarize *RET* mutation findings when CSGE, SSCP, and sequencing methods were applied.

Sequencing analysis

RET hot-spots exons (10, 11, 13, 14, 15, and 16) of all index cases (7 subjects) and in one genetically non-

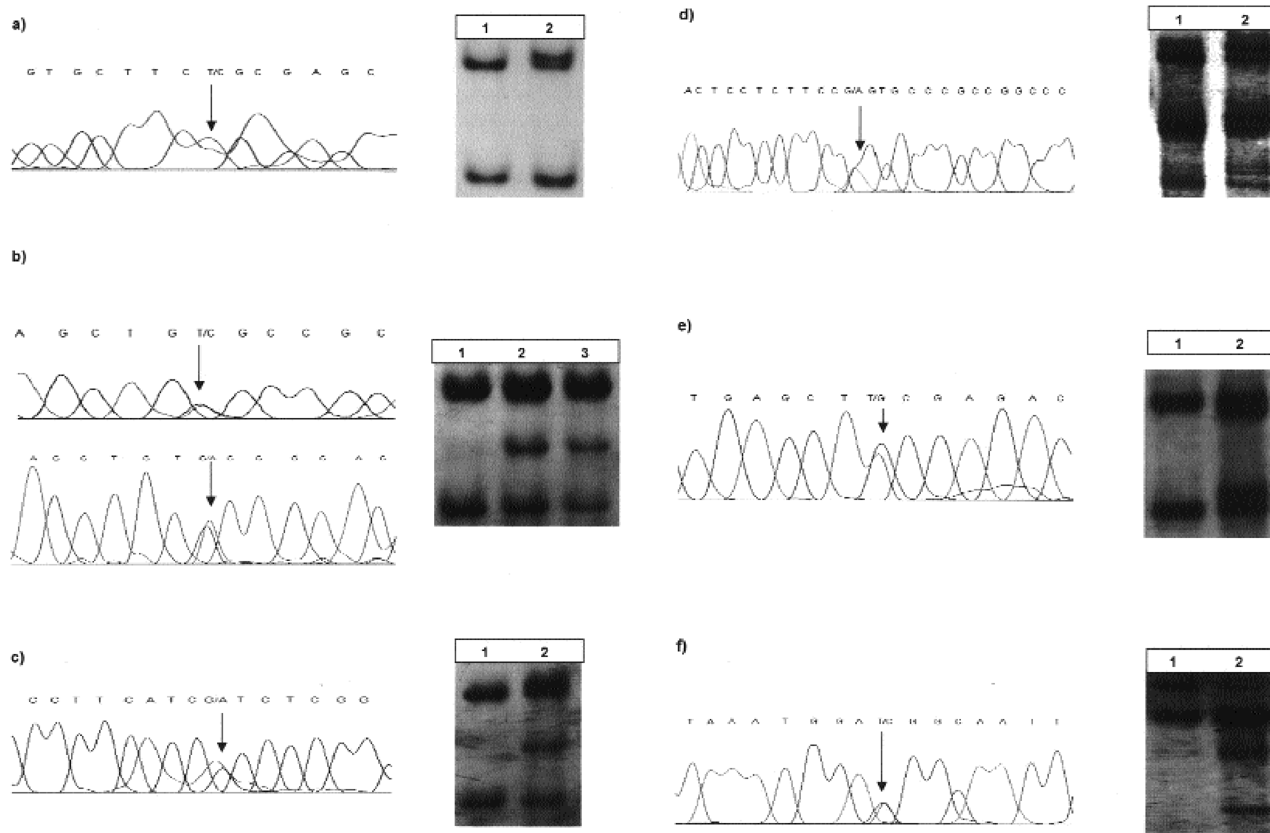

Figure 1. *RET* mutation analysis using direct sequencing and CSGE.
a) T to C substitution (TGC-CGC) in *RET* codon 620 (exon 10) leading to a Cys620Arg mutation (5). CSGE exon 10 differentiation patterns: lane 1, Cys620; and lane 2, Cys620Arg.
b) (Top) T to C substitution (TGC-CGC) in *RET* codon 634 (exon 11) leading to a Cys634Arg mutation (5); and, (Bottom) G to A substitution (TGC-TAC) in *RET* codon 634 (exon 11) leading to a Cys634Tyr mutation. CSGE exon 11 differentiation patterns (*primers 11B*): lane 1, Cys634; lane 2, Cys634Arg; and lane 3, Cys634Tyr.
c) G to A substitution (GTC-ATC) in *RET* codon 648 (exon 11) leading to a Val648Ile missense substitution (5). CSGE exon 11 differentiation patterns (*primers 11B*): lane 1, Cys648; and lane 2, Val648Ile.
d) G to A substitution (GGT-AGT) in *RET* codon 691 (exon 11) leading to a Gly691Ser polymorphism. CSGE exon 11 differentiation patterns (*primers 11A*): lane 1, codons 634 and/or 648 not differentiated electrophoretic patterns; and lane 2, Gly691Ser.
e) T to G substitution (CTT-CTG) in *RET* codon 769 (exon 13) leading to a Leu769Leu polymorphism. CSGE exon 13 differentiation patterns: lane 1, Leu769; and lane 2, Leu769Leu.
f) T to C substitution (ATG-ACG) in *RET* codon 918 (exon 16) leading to a Met918Thr mutation (5). CSGE exon 16 differentiation patterns: lane 1, Met918; and lane 2, Met918Thr.

affected at-risk member of each family (7 subjects), used as healthy normal controls, were performed. Other 50 at-risk family members were submitted to *RET* sequencing of the specific exons segregating mutations and/or polymorphisms. Using direct sequencing we totally performed 186 amplicon analyses, and were able to identify mutations at *RET* codons Cys620Arg (TGC-CGC) in 11 MEN2 cases; Cys634Arg (TGC-CGC) in 12 MEN2 cases; Cys634Tyr (TGC-TAC) in 8 MEN2 cases; Val804Met (GTG-ATG) in 3 MEN2 cases; and Met918Thr (ATG-ACG) in one MEN2 case. We also identified 2 other at-risk members from a MEN2A family, who presented Val648Ile (GTC-ATC) missense substitution, as previously described (2,8). Some of these *RET* mutation data had been partially published by us elsewhere (5) (figure 1). Polymorphisms at codons Gly691Ser (GGT-AGT), Leu769Leu (CTT-CTG), and Ser904Ser (TCC-TCG) were also identified. The *RET* Val804Met mutation and the Ser904Ser polymorphism could only be identified by direct sequencing. Of the seven MEN2 families here studied, 35 of 64 individuals (54.7%) were found to be *RET* mutant carriers (disease-causing mutations): 17 of them (48.6%) were MEN2A cases; 2 cases (5.7%) presented MEN2A disease associated with congenital megacolon; 15 of them (42.9%) were FMTC and 1 patient (2.8%) had MEN2B.

Table 3. Screening methods: comparative study in RET hot-spot exons.

Hot-spot exons	Direct sequencing	CSGE	SSCP	Screened Mutations Substitution	Screened Missense	Screened Polymorphisms
10	Sensitive	Sensitive	Sensitive	Cys620Arg		
11	Sensitive	Sensitive	Sensitive	Cys634Arg; Cys634Tyr	Val648Ile	Gly691Ser
13	Sensitive	Sensitive	Sensitive			Leu769Leu
14	Sensitive	Not sensitive	Not sensitive	Val804Met		
15	Sensitive	Not sensitive	Not sensitive			Ser904Ser
16	Sensitive	Sensitive	Sensitive	Met918Thr		

CSGE

Regarding CSGE screening mutation, the PCR products of the index cases (7 subjects) and at-risk relatives (57 family members) found to be mutated or polymorphic were screened by CSGE and compared to wild-type exons from 27 at-risk family members identified as healthy individuals. For CSGE-screened mutations, we analyzed 128 amplicons (encompassing *RET* hot-spot mutations and codon 648 missense substitution). 116 of 128 amplicons (90.6%) were found to be concordant with DNA sequencing (gold standard). In twelve amplicons (9.4%), all representing the Val804Met mutation (exon 14), the electrophoretic migration pattern could not be standardized even with alternative primer settings (14B and 14C) and electrophoresis conditions. In relation to the polymorphism analysis, we screened 168 amplicons: 100 of them (59.5%) were concordant regarding CSGE and DNA sequencing. In 68 (40.5%) of the analyzed amplicons (all representing the Ser904Ser polymorphism), CSGE analysis could not be standardized. Briefly, patients presented the following genotype-phenotype correlations when examined by CSGE: (a) *RET* exon 10 Cys620Arg (TGC-CGC) in 5 MEN2A, 2 cases of congenital megacolon and 4 FMTC cases; (b) *RET* exon 11 Cys634Arg (TGC-CGC) in 12 MEN2A cases; (c) *RET* exon 11 Cys634Tyr (TGC-TAC) in 8 FMTC carriers; (d) *RET* exon 11 Val648Ile (GTC-ATC) in 2 so far clinically non-affected MEN2A carriers; and (e) *RET* exon 16 Met918Thr (ATG-ACG) in a case presenting MEN2B phenotype. Polymorphisms Gly691Ser (GGT-AGT) and Leu769Leu (CTT-CTG) were found to occur in *RET* mutation carriers, codon 648 missense substitution-carriers and non-carriers (13, 1 and 7 subjects, respectively).

SSCP

For *RET* hot-spot screening analysis using SSCP, we selected a subset of 37 subjects comprising all mutation, missense substitution and polymorphism genotypes here studied: 4 subjects presenting Cys620Arg + Gly691Ser + Ser904Ser; 3 Cys634Arg; 3 Cys634Tyr; 2 Val648Ile; 3 Val804Met (one of them carrying Leu769Leu); 1 Met918Thr; 2 Leu769Leu and 19 wild-type subjects. Considering SSCP-screened mutations, 14 of 37 subjects were *RET* hot-spot mutants carriers for codons 620, 634, 804, and 918; 2 of 37 subjects were codon 648 missense substitution carriers and nineteen subjects were healthy controls. As it was observed when applying CSGE technology, the same 12 amplicons (17%) representing Val804Met mutation (exon 14), could not be standardized by SSCP, either. For SSCP polymorphism screening analysis, the subset group (37 subjects) comprised four subjects presenting codon 691 + 904 polymorphisms, three 769 polymorphism carriers and twelve healthy control subjects. In 16 (35%) of the analyzed polymorphic amplicons (Ser904Ser), electrophoretic migration patterns could not be standardized. Patients presented the same genotype-phenotype correlations when screened by either SSCP or CSGE. SSCP screening method showed the same migration patterns for all exons, when examining either *RET* mutations or polymorphisms. Data obtained by CSGE were fully concordant (100%) with those observed when SSCP was applied.

Comparing data encompassing *RET* hot-spot mutations and codon 648 missense substitution obtained from SSCP/CSGE assays with those from genetic sequencing, we documented that 58 analyses (83%) were concordant. Also, comparing polymorphism data obtained by SSCP/CSGE with those obtained with genetic sequencing, 30 analyses (65%) were concordant. Using CSGE and SSCP, we were able to detect four out of the five types (80%) of *RET* mutations verified by direct sequencing analysis in our MEN2 patients.

DISCUSSION AND CONCLUSION

Molecular diagnosis offers a specific and highly accurate indication for prophylactic total thyroidectomy in human *RET* mutation carriers, which may alter MEN2

natural course of disease (15,24). In our MEN2 cases, likely due to the smallness of our sample, Val804Met *RET* mutation was relatively over-represented, with 3 individuals out of 35 (8.6%), compared to its less than 3% prevalence in large MEN2 series (1,15).

In the present study, we reported the CSGE use as a *RET* mutation screening method in typical MEN2 families. It presented high specificity for hot-spot mutations and for polymorphism analyses, as no false-positive result was detected. As for sensitivity (false negatives), Val804Met mutation could not be detected by this method. Importantly, when using the CSGE method we were able to identify *RET* mutations (or polymorphisms) in exons 10, 11, 13, and 16, which have been reported to cause the majority of MEN2 cases (15). Val804Met (exon 14) is a rare genetic variation, representing less than 3% of *RET* disease-causing mutations reported in MEN2 cases so far (1,15,25). We have occasionally successfully screened Val804Met using DGGE (3,4,8).

Direct genetic sequencing is the "gold standard" in genetic mutation analysis. However, for extended genealogies or populations, the cost of this procedure may be significantly higher than other screening methods. For instance, CSGE has been successfully used in several genetic screenings and may result in six-fold lower costs than sequencing, considering only reagents. Furthermore, it has been shown that screening methods such as DHPLC (that uses a Wave system analyzer), DGGE, SSCP, and restriction enzymes are also adequate alternatives for direct DNA sequencing (4,8,16). Each of these methods has advantages and disadvantages, as follows: DHPLC uses an automatic system for reading heteroduplex bands and is a sensitive method (90–95%), although its specific wave analyzer costs must be considered (23). Restriction enzyme sensitivity and reproduction analysis may present some drawbacks, such as: (a) inadequate recognition of restriction site and a consequently non-defined band in agarose gel electrophoresis, and (b) incomplete discrimination of amplified sequences with similar base pair sizes (4,14,16,26). Despite these limitations, a previous study reported total correlation when comparing restriction enzymes to direct sequencing (14). SSCP sensitivity usually reaches 80% for PCR fragment sizes smaller than 300 bp, meaning that non-mutant electrophoresis migration patterns do not exclude the possibility of a mutation carrier (4,14,16,26). According to other studies, SSCP presents an overall sensitivity of 95–100% for *RET* hot-spots mutation analysis (27,28). Also, DGGE is a highly sensitive method (90–100%) and is a very useful screening approach for *RET* mutations, which has been used in our laboratory (3,4,8). DGGE has several advantages, such as: (a) sensitivity and reproducibility higher than SSCP; (b) a high correlation with direct sequencing; (c) non-radioactive protocols; (d) allows little operational manipulation; and (e) does not need to recognize restriction sites. On the other hand, DGGE has some disadvantages such as: (a) requires a 50 bp GC-clamp coupled to one of the primer sequences; (b) each analyzed exon melting temperature needs to be addressed by a special computer software or hard standardization; (c) initial investment at specific electrophoresis system; (d) difficult standardization for guanine-cytosine rich sequences; and (e) polymorphism analysis may yield false-positive results (3,4,8).

In the present study, we reported CSGE application as a *RET* screening method in 7 typical MEN2 families with a sensitivity of 90.6% for hot-spot mutations (encompassing codon 648 missense substitution) and 59.5% for polymorphism analyses. According to the MEN Consensus, the Val804Met mutation, which was not detected in our CSGE assay, is a rare genetic variation, representing less than 3% of *RET* disease-causing mutations (1,15,25).

Additionally, we were interested in testing CSGE for detecting *RET* polymorphisms as they may act as epigenetic factors interacting with *RET* mutations and possibly altering the clinical presentation, course, and morbidity of MEN2 disease (29-33).

In summary, our study has shown that CSGE (and SSCP) is a useful method for screening *RET* hot-spot mutations, considering the following points: (a) using CSGE, we have successfully screened 4 of 5 types of *RET* mutations among our cases; (b) these four mutations detected by CSGE have been reported as being responsible for most (95%) of the disease-causing mutations in different MEN2 samples; (c) there was a total correlation (100%) between CSGE and SSCP data; (d) CSGE is a low-cost, fast and feasible to standardize method; (e) it does not use a radioactive protocol; and (f) it allows little operational manipulation, which decreases the possible chance of error. Moreover, CSGE dispenses with some requirements, such as: (a) the recognition of restriction sites; (b) different gel concentrations for each studied exon and 50 bp GC-clamp coupled to one of the primers sequences; and finally, (c) this method does not require the use of specific software to analyze each exon melting temperature.

In conclusion, CSGE is an effective, rapid, and low-cost screening method for the most frequently

occurring *RET* mutations in MEN2. It is also worthwhile to note that our results reflect a retrospective study of a small number of MEN2 families (seven families) that need to be addressed to a prospective study from a greater number of families, where genetic sequencing and CSGE screening may be simultaneously applied.

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Address for correspondence:

Sergio P.A. Toledo
Unidade de Endocrinologia Genética,
Endocrinologia, Clínica Médica
Av. Dr. Arnaldo 455, 5º andar
01246-903 São Paulo, SP
Fax: (11) 3061-7252
E-mail: toldo@usp.br