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FOXE1 gene mutation screening by multiplex PCR/DHPLC in CHARGE syndrome and syndromic and non-syndromic cleft palate

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Abstract

Denaturing high-performance liquid chromatography (DHPLC) has established itself as one of the most powerful tools for DNA variation screening. *FOXE1*, a highly GC-rich gene involved in syndromic cleft palate, is under investigation in thyroid dysgenesis, nonsyndromic cleft palate and squamous cell carcinoma. A technique for fast and simultaneous detection of sequence variants in the entire coding region of the *FOXEl* gene based on multiplex PCR/DHPLC is presented here. Given its characteristics of high sensitivity and rapidity, the testing strategy developed by us appears to be a reliable approach for *FOXE1* analysis in the screening of a large population at risk.

Keywords: *FOXE1* gene; Multiplex PCR/DHPLC; Sequence variant analysis; Oral clefting

1. Introduction

The detection of DNA sequence variations is one of the key steps in genomic analysis. Since direct nucleotide sequence analysis is technically demanding, costly and time-consuming, alternative screening techniques are required. Single-strand conformation polymorphism (SSCP), dideoxy fingerprinting assay (DDF), protein truncation test (PTT), conformational-sensitive gel electrophoresis (CSGE), denaturing gradient gel electrophoresis (DGGE) and enzyme mutation detection (EMD) are commonly used. However, these methods suffer from various disadvantages. SSCP (one of the most widely used mutation scanning methods), although technically simple, is not completely satisfying, for its low sensitivity, which ranges from 60 to 90% [1–3]. DDF (a combination of a Sanger sequencing reaction with multiple-fragment SSCP) is more sensitive than SSCP analysis, but it is still labour intensive [4]. PTT can detect only sequence alterations leading to a truncated protein [5], while DGGE and CSGE, despite their improved sensitivity over SSCP, are technically challenging and, for this reason, have not been widely adopted [6,7]. EMD methods for mutation scanning still lack the sensitivity and specificity of the chemical cleavage of the mismatch method and are difficult to automate [8].

More recently, denaturing high-performance liquid chromatography (DHPLC) has emerged as the most powerful tool for DNA variation screening, owing to its high sensitivity, high degree of automation and low cost of operation [3,9–13]. Under conditions of partial heat denaturation within a linear acetonitrile gradient, heteroduplexes having internal sequence variation display a reduced column retention time and abnormal elution patterns relative to their homoduplex counterparts. The technique has been shown to be superior even relative to direct sequencing analysis in the detection of mutant alleles present at low levels [14]. This technology has been further improved with the simultaneous analysis of two or more DNA fragments, but, unfortunately, this latter attractive approach has been until now limited to the study of a few genes [15–19].

In this paper, we investigated the usefulness of multiplex PCR/DHPLC as a method to simultaneously detect sequence alterations in the entire coding region of the Forkhead Box El

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(*FOXE1*) gene, also referred as Thyroid Transcription Factor-2 (TTF-2) (OMIM number: #602617).

The *FOXE1* gene, until now implicated in two syndromic cleft palate conditions [20,21], is currently under investigation for its involvement in non-syndromic cleft palate [22,23], in thyroid disgenesis [22,24,25] and in the pathogenesis of squamous cell carcinoma [26]. Although it is a single-exon gene, its high content in GC bp_s has caused difficulty for the amplification of the entire coding region [27] and fast scanning [21,22].

In view of the increasing awareness of the role of *FOXE1* in the aetiology of several pathologies and the lack of population-association studies, the availability of an efficient, rapid and reliable method, such as multiplex PCR/DHPLC, may be of great scientific and clinical interest.

2. Experimental

2.1. Control DNA specimens

Genomic DNA samples, harbouring specific *FOXE1* genotypes, as previously identified by automatic sequencing (see below), were anonymized by label-stripping and used as controls for the development of the diagnostic test. Forty healthy people, 20 patients affected by nonsyndromic cleft palate (CP), 20 nonsyndromic cleft lip-palate (CLP patients), 12 CP patients suffering from CHARGE association (coloboma of the eye, heart defects, choanal atresia, retardation of growth and development delay, genitalia and ear abnormalities) and 1 patient affected by a nosographic condition characterized by thyroid agenesis, spiky hair and cleft palate (DNA kindly provided by Dr. Polak, Pediatric Endocrinology Unit, Paris, France) were enrolled.

Written informed consent for the use of DNA in this study had been obtained prior to the initial testing.

2.2. DNA extraction

Genomic DNA was isolated from 200 μl of human whole blood drawn by venipuncture using the peripheral blood lymphocytes, using a QIAamp DNA Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All the DNA samples were adjusted to a final concentration of 50 ng/μl for assay development.

2.3. Sequence analysis

PCR products were amplified according to Castanet et al. [21] and purified with a Qiagen PCR product purification kit (Qiagen, Hilden, Germany). The purified products (1 μl) were electrophoresed in a 2% agarose gel containing 10 mg/ml ethidium bromide for 30 min with a DNA mass ladder (Life Technologies, CA) to determine the yield and quality of each amplification product. Each product was then sequenced in forward and reverse directions with the CEQ DTCS Quick Start Kit (Beckman Coulter S.p.A., Milan, Italy), with the same primers as those used for the PCR amplification. Following the removal of primers and unincorporated dNTPs, the products were analysed in an automated sequencer (Beckman Coulter CEQ 2000 Analysis System). Allelic frequencies of FOXE1 sequence variants are reported in Table 1.

2.4. Multiplex-polymerase chain reaction (PCR)

The sequence of the *FOXE1* gene was obtained from GeneBank at the publicly available NCBI website http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene. Accession number NM_004473. Three pairs of primers were carefully designed to co-amplify in a single reaction the three segments in which the entire FOXE1 coding-region was divided. Amplification primers were as follows:

Fragment 1F: 5′-CAGCCCGCGACGATCCCCTGAGCTCT-3′; 1R: 5′-TGCCGTCTCGCCGCGCTCTTCCTTCA-3′ (PCR product size: 204 bp_s).
Fragment 2F: 5′-GCCGGAGGTGCTGGCTACCGTGAAGG-3′; 2R: 5′-TGTAAGCCGGGTAGGTGGAGAGGTCCG-3′ (PCR product size: 449 bp_s).
Fragment 3F: 5′-AAGCGCTCGGACCTCTCCACCTACCC-G-3′; 3R: 5′-CGCTGGCTCACATGGCGGACACGAACC-3′ (PCR product size: 682 bp_s).

To validate the robustness of the method in the case of the long amplicon size of fragment 3, a further multiplex for DHPLC analysis was performed. To this end, two pairs of primers encompassing this region were designed as follows:

Fragment 3aF: 5′-AGCGCTCGGACCTCTCCACCTACCCG-G-3′; 3aR: 5′-CTGGGCTGAGCGGCCGCTCAGGAACCA-3′ (PCR product size: 255 bp_s).

Table 1
FOXE1 sequence variations detected by automatic sequencing[a]

Nucleotide[b]	Amino acid	Allelic frequency				
		Control	Nonsyndromic CP	Nonsyndromic CPL	CHARGE association/CP	Syndromic CP
c.169G > A	S57N	0/80	0/40	0/40	0/24	2/2
c.387T > C	L129L	45/80	22/40	18/40	10/24	2/2
c.714C > T	P238P	42/80	24/40	18/40	8/24	0/2
c.825C > T	S275S	40/80	16/40	15/40	9/24	0/2
c.532–537del	AA178, 179del	60/80	26/40	22/40	12/24	2/2

[a] DNA sequence analysis was conducted in an automated sequencer (Beckman Coulter CEQ 2000 Analysis System).
[b] Position of sequence variants is according to NCBI ref: GeneBank accession no. NM_004473.

Fragment 3bF: 5′-TTCCTGAGCGGCCGCTCAGCCCAGAGC-3′; 3bR: 5′-CGTCCCTACGCTGGCTCACATGGCGGA-3′ (PCR product size: 458 bp_s).

Multiplex PCR was conducted in an Applied Biosystem thermocycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA) in 50 μl reactions containing 80–100 ng of genomic DNA, 200 μM of each dNTP, 12.5 pmol of each forward and reverse oligodeoxynucleotide primer, 1X Optimase buffer (Transgenomic Inc., San Jose, CA, USA), 1.5 mM of $MgSO_4$ and 2.5 units of *Optimase* (Transgenomic). Q Solution (Qiagen, Hilden, Germany) was added to aid in the amplification of GC-rich regions.

The cycling profile was: one cycle at 95 °C for 3 min, 35 cycles at 94 °C for 40 s, 68 °C for 50 s and 72 °C for 1 min, and one cycle at 72 °C for 7 min. PCR products were fractionated by electrophoresis in 2% agarose gels and visualized using staining with ethidium bromide and ultraviolet light.

2.5. Multiplex-DHPLC analysis

PCR products were analysed either without or following addition of wild-type (wt) DNA to determine whether homozygous samples were wt or mutant. In this latter step, after mixing 10 μl of the patient PCR product with 10 μl of PCR-amplified wt DNA, a denaturation for 5 min at 95 °C was carried out. The mixture was then re-equilibrated at room temperature and analysed using the Transgenomic Wave DNA fragment analysis system (Wave System 3500, Transgenomic) by DNASep cartridge technology (Transgenomic), High Precision Peltier temperature control (precision: ±0.1 °C; reproducibility: ±0.1 °C; linearity over full temperature range: ±0.1 °C) and UV absorbance monitoring at 260 nm. Performance of the instruments was checked routinely using the three mutation standards commercially available from Transgenomic (Wave Low Range Mutation Standard: 56 °C; Wave Mid Range Mutation Standard: 64 °C; Wave High Range Mutation Standard: 70 °C). The multiplex-PCR amplified products relative to fragments 1–3 were run either at non-denaturing (50 °C) or partially denaturing conditions to search for insertions/deletions and point substitutions, respectively. Because of the presence of multiple melting domains, seven different temperatures (61–67 °C) were employed in order to obtain a partial denaturation of the amplicons over the entire screened regions. The two amplicons named 3a and 3b were run at the partially denaturing temperatures of 65, 66 and 67 °C.

The gradient mobile phase consisted of buffer A (0.1 M triethylammonium acetate, pH 7.0) (Transgenomic) and buffer B (0.1 M triethylammonium acetate, pH 7.0 and 25% acetonitrile) (Transgenomic). These buffers were mixed to produce a linear gradient with increases of %B from 35 to 65 in 10 min. Analysis of each sample took approximately 15 min, including regeneration and re-equilibration to the starting conditions.

We used an empirical approach to find optimum fragment separation conditions by DHPLC. Three variables were evaluated to optimise gradient separation: the initial percentage of organic solvent (% B), the final percentage of organic solvent, and the gradient time. In order to maintain optimum run times, we first defined the conditions that allowed adequate peak separation. We then tried to reduce the gradient time while maintaining a constant gradient retention factor (selectivity was kept constant). Further adjustments were made to ensure a time window of 2.5 min between the injection peak and the first eluted peak to avoid variations in retention time for the first eluted peak. An increase or decrease in the flow rate did not markedly improve separation and it was therefore kept at 0.9 ml/min.

3. Results

The DNA samples from subjects reported in Table 1 were previously genotyped by automatic sequencing and then blindly screened by multiplex PCR/DHPLC. Results obtained showed 100% concordance with data from the sequencing analysis without any false positives or false negatives.

Fig. 1 shows the chromatograms obtained at the non-denaturing temperature of 50 °C from a control (Fig. 1A) and from a non-syndromic CLP patient (Fig. 1B) heterozygous

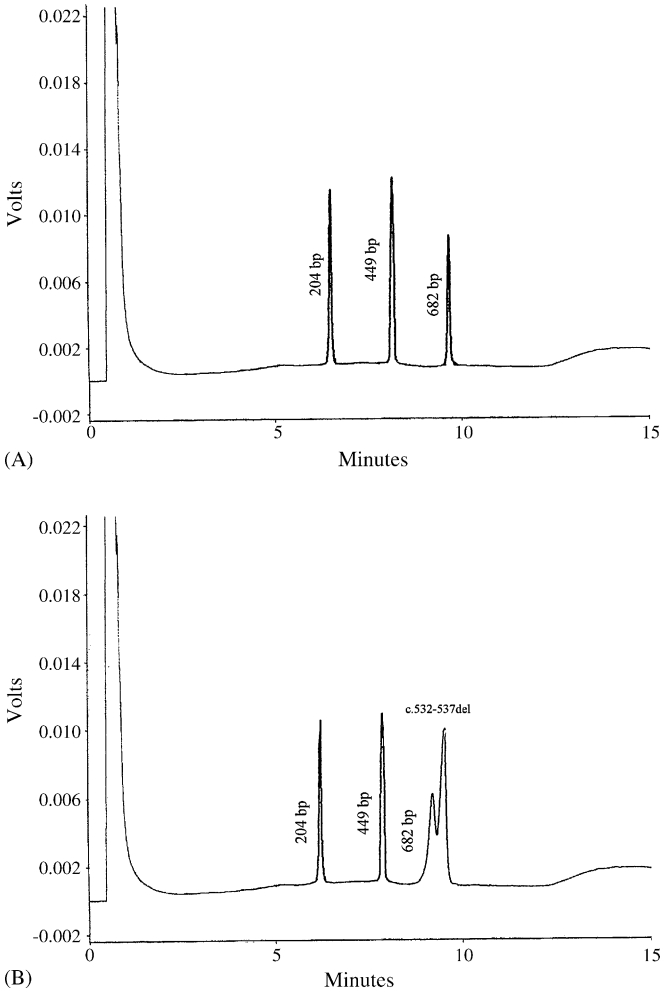

Fig. 1. Analysis of the multiplex PCR-amplified products under nondenaturing conditions (50 °C) from a wild-type individual (section A) and from a nonsyndromic CP patient, heterozygous for a deletion of 6 bps in the third fragment (c.532–537del) (section B).

for the 6-bp deletion c.532–537del, which results in the loss of two alanine residues in the poly-alanine stretch of the FOXE1 protein (AA178.179del). The chromatogram in Fig. 1A shows three PCR product peaks that eluted at 6.213, 7.625 and 8.988 min, respectively, corresponding to the first, second and third fragment of the FOXE1 coding-region. On the contrary, the chromatogram in Fig. 1B clearly shows an additional peak at 8.415 min, which represents a heteroduplex formed between

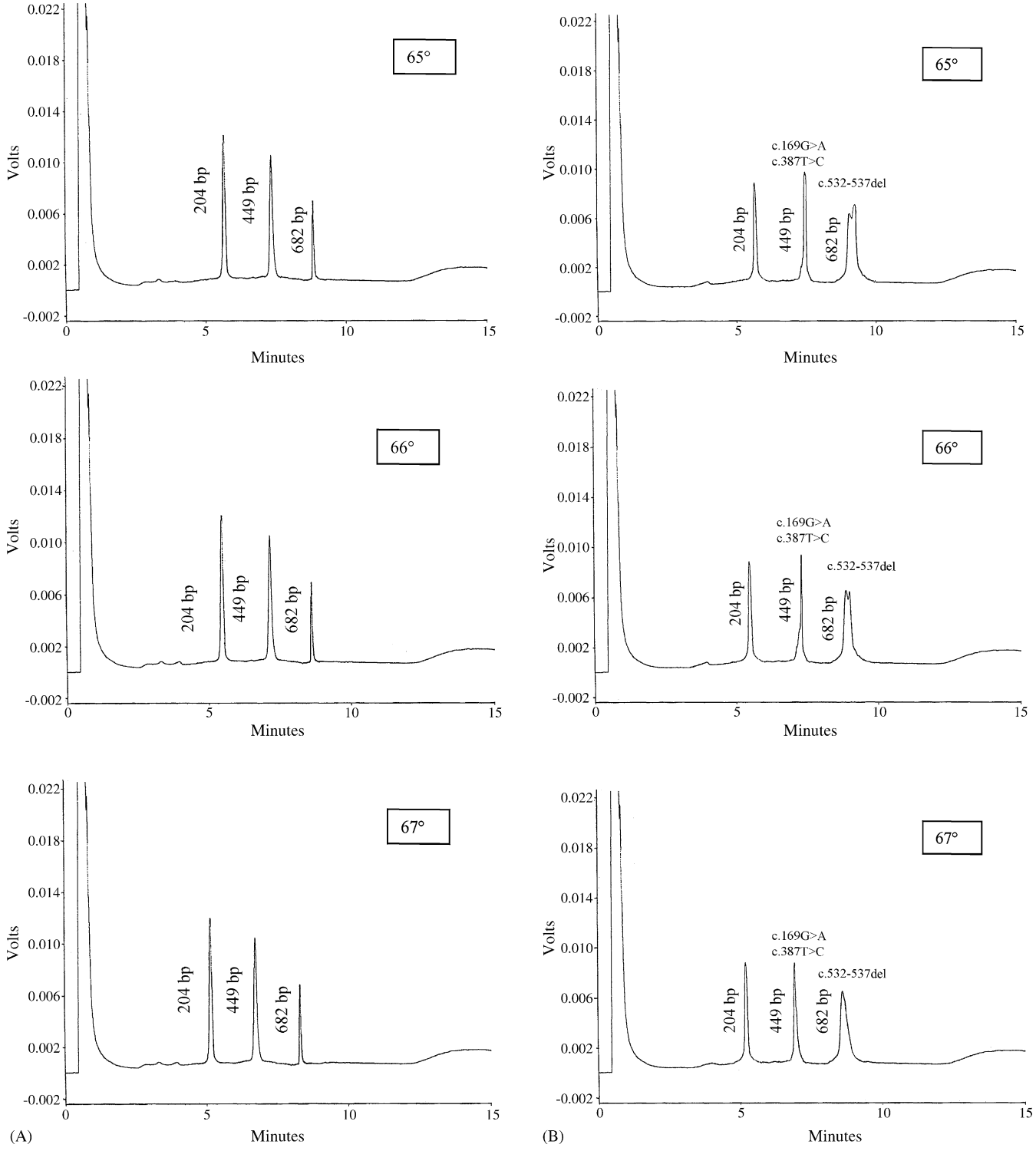

Fig. 2. Multiplex PCR/DHPLC analysis of FOXE1 gene coding-region at 65–67 °C. (A) Elution of the multiplex PCR products amplified from a normal individual. (B) Elution of the PCR products amplified from a patient suffering from syndromic CP (c.169G > A; c.387T > C; c.532–537del) (*patient 1*). (C) Elution of the PCR products amplified from a patient affected by CHARGE association (c.532–537del) (*patient 2*). (D) Elution of the PCR products amplified from a patient affected by nonsyndromic CP (c.387T > C; c.714C > T) (*patient 3*). (E) Elution of the PCR products amplified from a patient affected by nonsyndromic CLP (c.825C > T) (*patient 4*). Samples were run on a DNASep column at 66 °C.

the wt and the deleted allele. The retention time reproducibility was calculated from at least 20 analyses, and the coefficient of variation (CV) was less than 3%.

Fig. 2 shows the chromatograms from five individuals, each representative of a clinical condition, obtained at 65, 66 and 67 °C. These temperatures, gave the best duplex resolution among the seven tested (61–67 °C). The chromatograms of section A show three symmetric and sharp peaks corresponding to the three wt fragments. The elution profiles of the chromatograms in sections B–E are quite different and easily

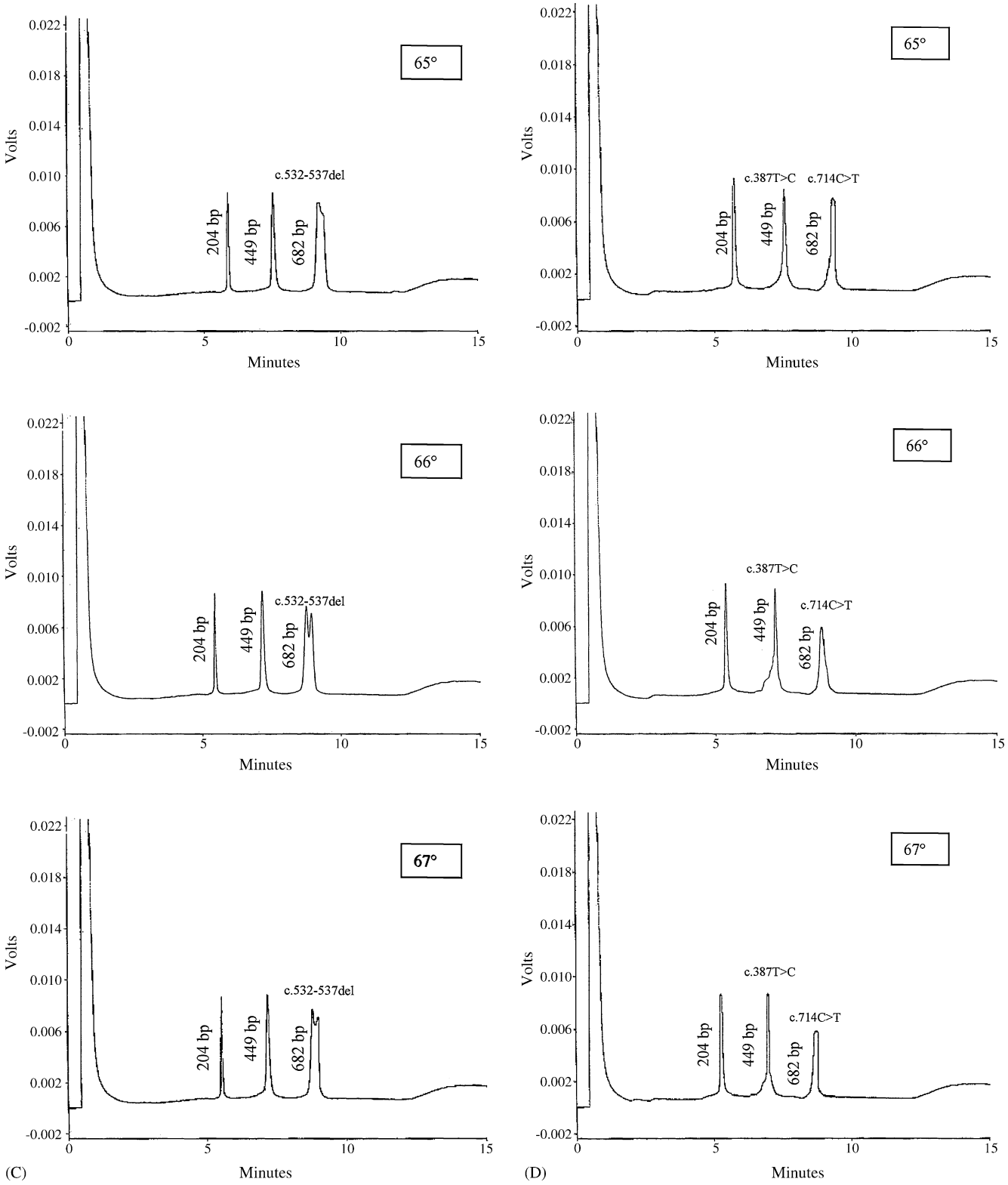

Fig. 2. (*Continued*).

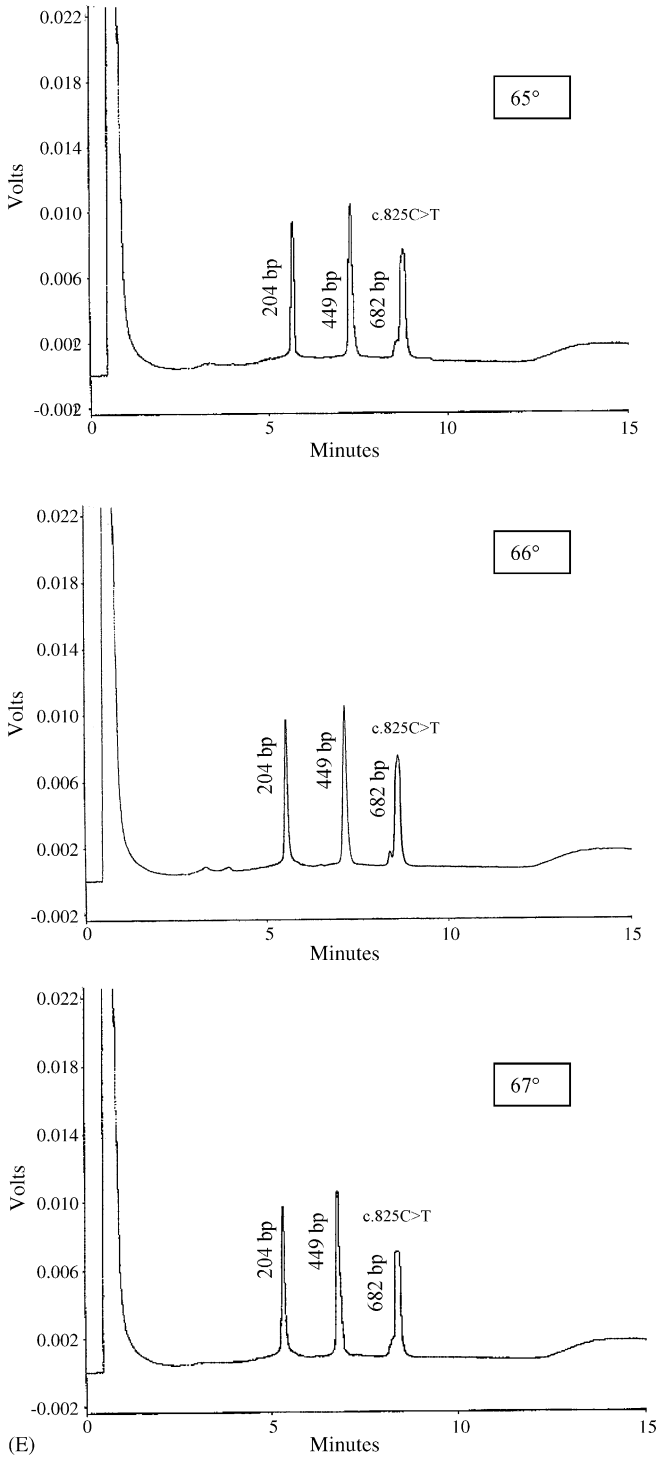

Fig. 2. (*Continued*).

distinguishable owing to evident abnormal patterns. In particular, the chromatograms of sections B and D show altered elution profiles of peaks 2 and 3, which appear asymmetric and broader than those reported in section A. The modifications are indicative of the following sequence variations: c.169G > A, c.387T > C (fragment 2) and c.532–537del (fragment 3) found in the patients with thyroid agenesis, spiky hair and cleft palate (*patient 1*), and c.387T > C (fragment 2) and c.714C > *T* (fragment 3) from a non-syndromic CP subject (*patient 3*), respectively. Sections C and E show anomalies in the third peak due to c.532–537del in one of the patients affected by the CHARGE syndrome (*patient 2*) and to c.825C > *T* polymorphism in a subject with nonsyndromic CLP (*patient 4*).

Therefore, all the chromatograms of sections B–E provide evidence of the sequence variations in the *FOXE1* gene detected by automatic sequencing.

Fig. 3 shows the chromatograms of the two amplicons encompassing fragment 3 relative to the subjects reported in Fig. 2. Section A shows a chromatogram with two symmetric and sharp peaks eluted at 6.395 and 7.782 min and corresponding to the wt fragments. Sections B (*patient 1*) and C (*patient 2*) show altered profiles of peak 1 due to c.532–537del, while sections D (*patient 3*) and E (*patient 4*) show anomalous patterns of peak 2 for the presence of c.714C > *T* and c.825C > *T* polymorphisms, respectively.

Since the profiles shown in Fig. 2B and C, as well as in Fig. 3B and C, appeared slightly different from each other even in the presence of the same mutation, we repeated the sequencing analysis five times. No mutations other than the c.532–537del were found.

4. Discussion

In this paper, we describe a multiplex PCR/DHPLC-based method that enables sequence variation screening of the entire coding-region of the *FOXE1* gene in a single DHPLC run. The method yielded results consistent with direct sequence analysis. The multiplex PCR protocol comprised three primer pairs of similar length and annealing temperature, yielding overlapping amplicons of 204, 449, and 682 base pairs in length, respectively. The results showed that no reciprocal interference occurred. The three amplicons obtained had compatible melting characteristics and were therefore detectable with equal sensitivity during DHPLC analysis. An optimal balance between time of analysis and resolution of duplexes was reached, since the analysis took less than 15 min per sample, including regeneration and re-equilibration back to the starting conditions, and the peaks appeared well defined and highly resolved. To ensure a very accurate and complete mutation detection, all multiplex-PCR-amplified products were run in DHPLC at seven partially denaturing temperatures (61–67 °C). The results obtained clearly showed that the temperatures ranging from 65 to 67 °C can suffice to detect all the mutations in the entire coding region. The robustness of the method was assured by the results obtained from an additional multiplex PCR-DHPLC analysis of two shorter amplicons encompassing the entire fragment 3. Data reported here showed that the experimental conditions settled on by us avoid the possibility to obtain both false negative and false positive results, even for the longer fragment 3.

The results were equally reliable either for deletion or point substitution discovery, as shown by Figs. 1–3. The lack of false negatives shows the high sensitivity of our method, while the absence of false positives is an index of high specificity.

As until now no variants have been reported in the first fragment of the *FOXE1* coding region, we were unable to validate

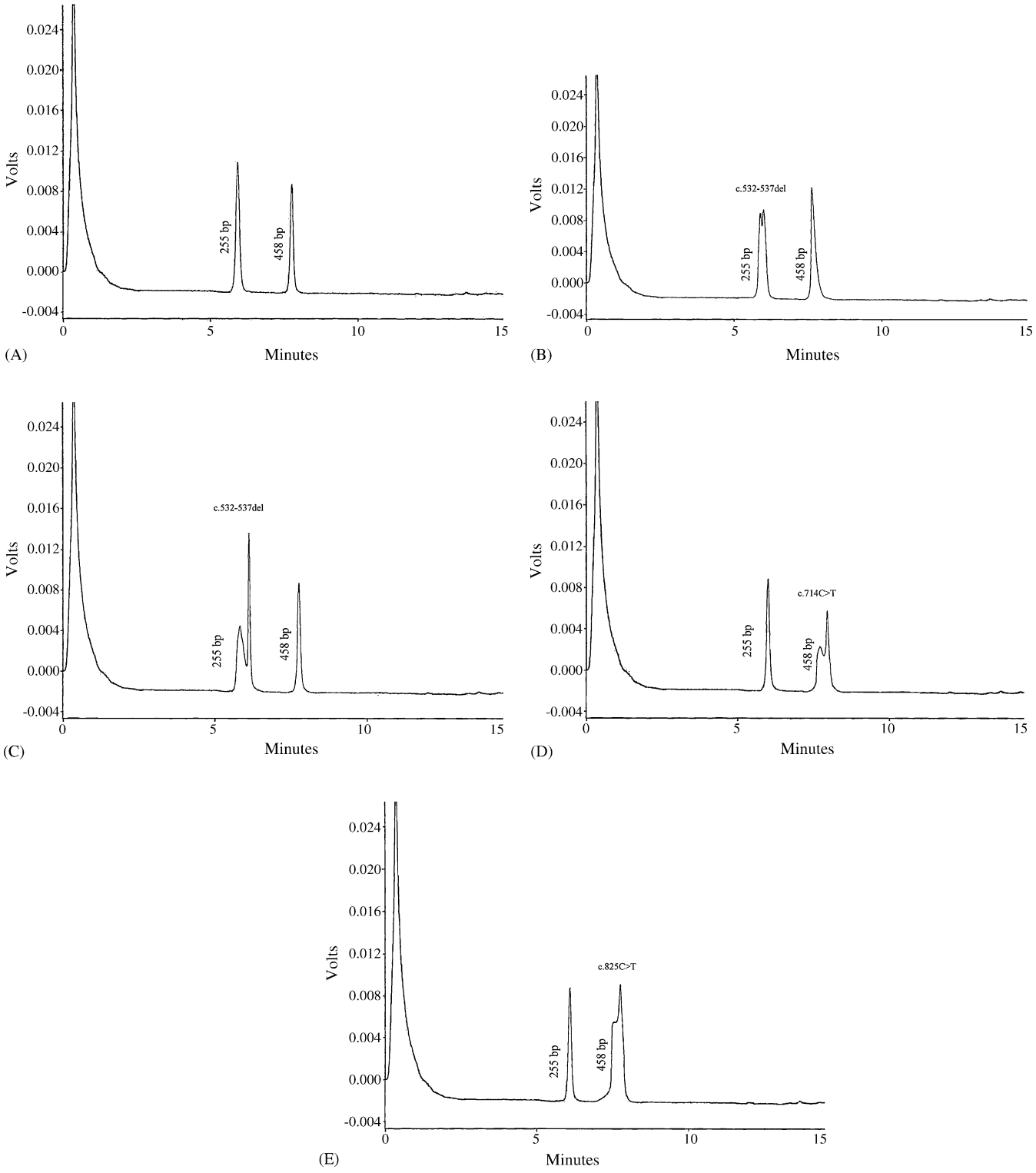

Fig. 3. Multiplex PCR/DHPLC analysis of the amplicons encompassing fragment 3. (A) Elution of the multiplex PCR products amplified from a normal individual. (B) Elution of the PCR products amplified from the person reported as *patient 1* in Fig. 2. (C) Elution of the PCR products amplified from the person reported as *patient 2* in Fig. 2. (D) Elution of the PCR products amplified from the person reported as *patient 3* in Fig. 2. (E) Elution of the PCR products amplified from the person reported as *patient 4* in Fig. 2. Samples were run on a DNASep column at 66 °C.

the DHPLC conditions for this region. However, because we applied the same criteria to the design of each fragment and optimisation of DHPLC analysis, we expect that this technique might provide the same performance.

The results obtained are of interest because they highlight the reliability and the very high sensitivity of the strategy developed and validated by us as a scanning method for detection of mutation in the *FOXE1* gene.

This new application of multiplex PCR-DHPLC to *FOXE1* is particularly relevant because of the characteristics of this gene. In fact, *FOXE1* is a GC-rich gene and this feature makes it ill-suited to be analysed by DGGE technique that prevents the complete strand separation by employing GC clamps [28]. Moreover, the variations found in *FOXE1* are mainly point substitutions or deletions which might be undetectable by SSCP, a technique highly sensitive to detect frame shift mutations [28–32].

Therefore, in view of the increasing involvement of the *FOXE1* gene in several ontogenic and neoplastic diseases [20–26], the method proposed here may be useful in the screening of a large population at risk, in view of its characteristics of rapidity, sensitivity and specificity.

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