ELECTRONIC LETTER

# *SHOX* mutations detected by FISH and direct sequencing in patients with short stature

**L Stuppia, G Calabrese, V Gatta, S Pintor, E Morizio, D Fantasia, P Guanciali Franchi, M M Rinaldi, G Scarano, D Concolino, A Giannotti, F Petreschi, M T Anzellotti, M Pomilio, F Chiarelli, S Tumini, G Palka**

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Height is the result of interactions of several factors including those of genetic origin. About 3% of people have short stature and in most of them the cause is unknown.[1] Recently, the *SHOX* gene (short stature homeobox containing gene), mapped on the pseudoautosomal region (PAR1) of the X and Y chromosomes, has been specifically associated with the short stature of patients with Turner syndrome or with Leri-Weill dyschondrosteosis (LWD).[2–7] Few data have been reported on the relationship between *SHOX* mutations and idiopathic short stature. Rao *et al*[2] reported one change among 91 patients with idiopathic short stature, and Binder *et al*[8] found a mutation in one out of 68 patients. The largest study was recently published by Rappold *et al*,[9] who found a frequency of 2.4% of *SHOX* mutations using fluorescence in situ hybridisation (FISH) on 150 patients and single strand conformational polymorphism analysis (SSCP) on 750 patients.

We report a study carried out on 56 patients with short stature of unknown origin detecting *SHOX* mutations in seven (12.5%) by using FISH and direct sequencing analyses.

**Key points**

- The *SHOX* gene was investigated in 56 patients with short stature of unknown origin using FISH analysis and direct sequencing.
- Four patients (7.1%) showed deletion of the *SHOX* gene, while three cases (5.3%) showed an identical point mutation consisting of a C-G transversion at nucleotide 548 (C548G) within exon 3 leading to an Arg-Gly change within the SHOX homeodomain.
- The prevalence of *SHOX* mutations detected in this study is higher than the ones previously published. This is probably because of the different number of patients investigated and the techniques used, in particular the use of direct sequencing which is more sensitive than SSCP analysis.

## MATERIALS AND METHODS

### Patients

Fifty-six patients, 33 females and 23 males, with a mean age of 12.2 years (range 5-18 years) entered this study. All patients were unrelated, coming from different regions of central and southern Italy. In order to exclude patients with dyschondrosteosis or other diseases associated with short stature, we used the following criteria: (1) height at or below the 3rd centile; (2) absence of obvious skeletal abnormalities on physical examination; (3) absence of other diseases on physical examination and routine analyses; (4) normal bone age; (5) normal hGH values using a polyclonal in house RIA (lower detection limit 0.1 ng/µl, mean intra-assay coefficient of variation 6.9%, and mean interassay coefficient 9.5%); and (6) normal karyotype in 16 metaphases studied by GTG banding at the 500 band level. One patient (patient 4) was at the 50th centile at the time of molecular analysis, but was included in the study since she had short stature at the time of her first clinical examination, before rhGH treatment. All patients had both parents in the normal range of height for the Italian population[10] except the mothers of patients 6 and 7 who were 148 and 150 cm tall, respectively (table 1). Fifty people with normal stature were used as controls.

### FISH analysis

FISH experiments were performed according to Calabrese *et al*[11] on metaphase spreads, and on nuclei from pepsin digested, ethanol fixed, peripheral blood smears stored at room temperature for one week or up to one year at –20°C before hybridisation. Cosmid probes 34F5 and F20 were used for *SHOX* gene analysis.[1] A YAC clone for the X chromosome (922B5, CEPH Mega YAC library) and a plasmid clone for the *SRY* gene on the Y chromosome (pHu14)[12] were also used as controls probes. For each patient, 16 metaphases and 100 nuclei were examined. Results were regarded as positive when at least 75% of cells showed three signals only, one corresponding to *SHOX* and two to the control probe.

### PCR and SSCP

For the study of point mutations, exons 2 to 6a of the *SHOX* gene were PCR amplified using specific primers.[2] For SSCP analysis, PCR products were denatured for five minutes at 95°C, then run for three to five hours at 500 V on 10% polyacrylamide or for 15 to 18 hours at 150 V on MDE (mutation detection enhancer) gels. After the run, gels were stained with ethidium bromide (EB) and observed through a Gel Doc 1000 Image Analyser (Bio-Rad Laboratories).

### Direct sequencing

In addition to SSCP analysis, all patients were also investigated by direct sequencing of exons 2-6a. For this analysis, PCR products were purified and submitted to direct sequencing using an ABI PRISM 310 Genetic Analyzer. Each PCR product was sequenced both in the forward and in the reverse strand. Detected mutations were confirmed by repeating the sequencing analysis on a new PCR product.

## RESULTS

FISH analysis on chromosome spreads and peripheral blood nuclei showed deletion of the *SHOX* gene in four out of 56 patients (patients 1-4) (fig 1). Direct sequencing showed the presence of a point mutation in three patients (patients 5-7). This change consisted of a single missense mutation with a C-G transition of nucleotide 548 (C548G) within exon 3 of the *SHOX* gene, leading to a Arg-Gly change in the amino acid residue 153 of the protein product (Arg153Gly) (fig 2). In all

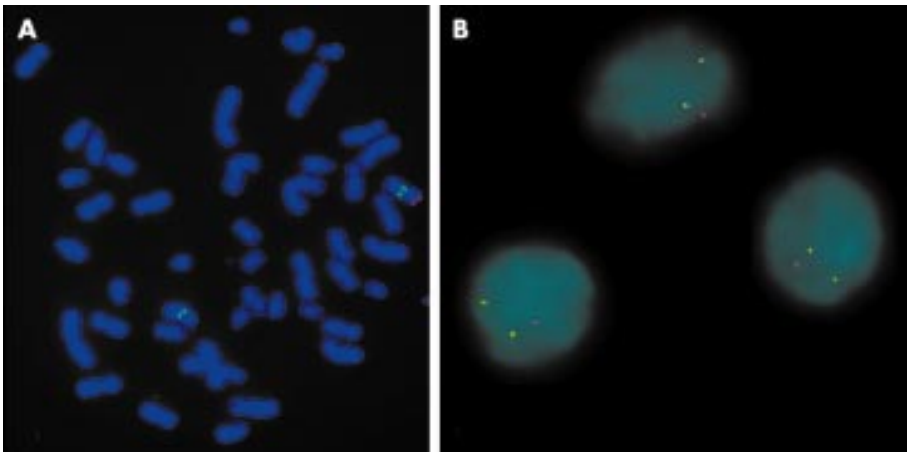


**Figure 1** (A) Dual colour FISH analysis in patient 2 showing the presence of one copy of the *SHOX* gene (red signals) on an X chromosome; YAC clone 922B5 (green signals) was used as a control probe to indicate both X chromosomes. (B) FISH analysis on peripheral blood nuclei from the same patient showing one red signal for *SHOX* and two control yellow signals for X chromosomes.

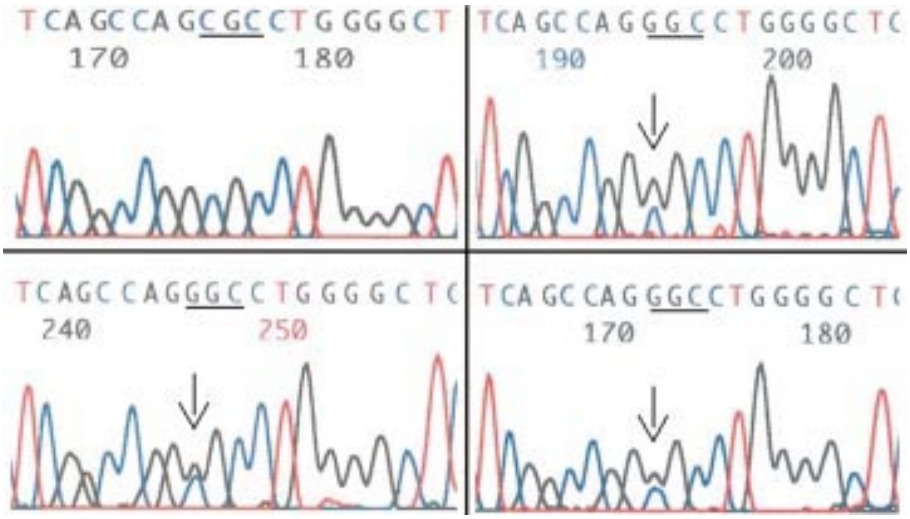


**Figure 2** Sequence of exon 3 of the *SHOX* gene: (A) wild type; (B-D) C548G change in patients 5, 6, and 7.

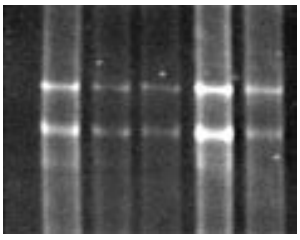

**Figure 3** SSCP analysis of exon 3 of the *SHOX* gene. Lanes 1-3 = patients 5-7, carriers of the C548G mutation; lanes 4 and 5= normal controls.

three cases, this mutation had not been detected by SSCP analysis, which did not show an abnormal band pattern in any patient (fig 3).

Data on the patients with rearrangements of the *SHOX* gene are reported in table 1.

## DISCUSSION

In the present study, we found seven rearrangements of the *SHOX* gene in 56 patients with idiopathic short stature (12.5%). They consisted of four deletions (7.1%) and three point mutations (5.3%) as shown by FISH and direct sequencing analyses, respectively. These results confirm that a number of patients with short stature of unknown origin display *SHOX* mutations and that deletions are more frequently found than intragenic point mutations.[7 9 13] The prevalence of *SHOX* deletions detected by FISH is different from those previously published. Musebeck *et al*[14] and Rappold *et al*[9] using FISH analysis detected, respectively, no deletion and three haploinsufficiencies (2%) in a cohort of 36 and 150 patients with unexplained short stature. No skeletal abnormalities were detected in the seven patients at the time of examination. Nevertheless, according to other reports, since these patients have lost one copy of the *SHOX* gene, bone anomalies may appear later.[7 9] For this reason, we suggest performing FISH analysis on peripheral blood cell nuclei in all patients with short stature.

Also, the prevalence of molecular rearrangements was higher than in other studies since Rao *et al*,[2] Binder *et al*,[8] and Rappold *et al*,[9] in a cohort of 91, 68, and 750 patients, found 1%, 1.5%, and 0.4% of point mutations of the *SHOX* gene, respectively. These discrepancies may be because of the different techniques used. In fact, the first time we performed SSCP analysis for the screening of point mutations, similarly to other authors[2 8 9] no point mutation was found. The detection rate of the SSCP analysis is considered to be about 80%; however, it has been recently shown that for some genes the actual detection rate of this approach is at or below 65%.[15 16] Moreover, previous reports have shown that the prevalence of *SHOX* mutations is higher than previously thought using direct sequencing.[17 18] For this reason, we reanalysed our samples with direct sequencing and detected a point mutation in

**Table 1** Clinical and molecular data of patients with *SHOX* rearrangements

| Patient No | Sex/ age* | Stature (SD)* | Bone *x* rays | GH value | *SHOX* mutation | Parental heights (cm) | |
|---|---|---|---|---|---|---|---|
| | | | | | | F | M |
| 1 | F/7 | 110 cm (–2.08) | Normal | Normal | Deletion | 174 | 164 |
| 2 | M/12 | 127 cm (–3.03) | Normal | Normal | Deletion | 163 | 159 |
| 3 | F/11 | 130 cm (–2.03) | Normal | Normal | Deletion | 172 | 165 |
| 4 | F/5 | 106 cm (+0.83) | Normal | Normal | Deletion | 174 | 167 |
| 5 | M/12 | 132 cm (–2.00) | Normal | Normal | C548G | 157 | 157 |
| 6 | F/18 | 148 cm (–2.48) | Normal | Normal | C548G | 163 | 148 |
| 7 | F/2 | 74 cm (–2.59) | Normal | Normal | C548G | 170 | 150 |

GH = growth hormone; F = father; M = mother.
*At the time of molecular investigation.

three cases. We believe that the C548G is a functional mutation since it was not found in 50 unrelated healthy subjects studied as controls, and it has been previously reported to segregate with the disease in an Italian family with LWD.[19] Moreover, this mutation falls within the homeodomain region of the SHOX protein, where missense mutations of this gene are clustered[20] and involves a codon previously found affected by another missense mutation in a LWD patient.[18] This mutation was detected also in the mother of patient 7, but not in the parents of patient 6, suggesting a different origin of the short stature in this patient and in her mother. The parents of the third patient carrier of this mutation were not available. The presence of the C548G mutation with such a high prevalence in our series would suggest the presence of a founder effect in the Italian population. However, this mutation had a de novo origin in patient 6. Further studies on Italian patients are required in order to clarify whether the high recurrence of this mutation is the result of a founder effect or the presence of a hot spot region at codon 153.

In conclusion, our results suggest the following: (1) FISH analysis on peripheral blood cells is recommended in all patients with unexplained short stature, since deletion is the most frequent mutation of the *SHOX* gene and FISH is an easy and reliable technique; (2) FISH and sequencing are also suggested in cases of familial short stature, since rearrangements of the *SHOX* gene are present in a number of these subjects.

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**Authors' affiliations**
**L Stuppia, G Calabrese, V Gatta, S Pintor, E Morizio, D Fantasia, P Guanciali Franchi, G Palka,** Dipartimento di Scienze Biomediche, Università "G D'Annunzio", Chieti, Italy
**L Stuppia,** Istituto di Citomorfologia Normale e Patologica CNR, Chieti, Italy
**G Calabrese, E Morizio, D Fantasia, P Guanciali Franchi, G Palka,** Servizio di Genetica Umana, Ospedale Civile di Pescara, Italy
**M M Rinaldi,** Servizio di Genetica Medica, Ospedale Cardarelli, Napoli, Italy
**G Scarano,** Servizio di Genetica Medica, Ospedale di Benevento, Italy
**D Concolino,** Servizio di Pediatria, Ospedale di Catanzaro, Italy
**A Giannotti, F Petreschi,** Servizio di Genetica, Ospedale Bambino Gesù, Roma, Italy
**M T Anzellotti, P Pomilio, F Chiarelli, S Tumini,** Dipartimento di Pediatria e Ginecologia, Università "G D'Annunzio", Chieti, Italy

Correspondence to: Dr G Palka, Dipartimento di Scienze Biomediche, Sezione di Genetica Medica, Via dei Vestini 35, 66013 Chieti, Italy; gdpalka@phobos.unich.it

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