Improved Molecular Diagnostics of Idiopathic Short Stature and Allied Disorders: Quantitative Polymerase Chain Reaction-Based Copy Number Profiling of *SHOX* and Pseudoautosomal Region 1

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Context: Short stature has an incidence of three in 100 in children. Reliable molecular genetic testing may be crucial in the context of beneficial disease management. Deletions spanning or surrounding the *SHOX* gene account for a significant proportion of patients with idiopathic short stature (ISS) and allied disorders, such as Leri-Weill dyschondrosteosis.

Objective: Several shortcomings of current strategies for copy number profiling of the *SHOX* region prompted us to develop an improved test for molecular diagnostics of the *SHOX* region.

Design and Results: We introduced a quantitative PCR (qPCR)-based copy number profiling test, consisting of 11 amplicons targeting clinically relevant regions, *i.e.* the *SHOX* gene and regulatory regions. To ensure an optimal sensitivity and specificity, this test was validated in 32 controls and 18 probands with previously identified copy number changes. In addition, 152 probands with *SHOX*-associated phenotypes were screened, revealing 10 novel copy number changes.

Conclusion: This highly validated qPCR test supersedes other approaches for copy number screening of the *SHOX* region in terms of reliability, accuracy, and cost efficiency. In addition, another strong point is the fact that it can be easily implemented in any standard equipped molecular laboratory. Our qPCR-based test is highly recommended for molecular diagnostics of idiopathic short stature and allied disorders. **(*J Clin Endocrinol Metab* 95: 3010–3018, 2010)**

Short stature, with an incidence of three in 100 in children, may have a significant impact on the quality of life. Patients not only experience physical burdens but also have to cope with social, educational, and professional impact (1). The latter highlights the need for a multidisciplinary approach for these patients, including reliable molecular genetic testing.

Deletions spanning or surrounding the *SHOX* gene account for a significant proportion of patients with idiopathic short stature (ISS) and allied disorders, such as Leri-Weill dyschondrosteosis (LWD). Recently, it has been shown that patients with a molecular defect of *SHOX* benefit from GH therapy (2, 3). An early molecular diagnosis is essential for GH therapy because

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Abbreviations: gDNA, Genomic DNA; ISS, idiopathic short stature; LWD, Leri-Weill dyschondrosteosis; MLPA, multiplex ligation-dependent probe amplification; NRQ, normalized relative quantity; PAR1, pseudoautosomal region 1; qPCR, quantitative PCR; SRO, shortest region of overlap.

there is a time-sensitive window for treatment. In addition, it is expensive and time-consuming and implies medicalization. In light of this, an efficient diagnostic screening method to detect copy number changes spanning *SHOX* and/or the surrounding pseudoautosomal region 1 (PAR1) is thus of utmost importance.

The PAR1 on the distal end of the X and Y chromosomes spans approximately 2.7 Mb and is characterized by an extraordinarily high recombination frequency. The 24 known genes within this region escape X-inactivation, and their inheritance mimics an autosomal dominant inheritance pattern, referred to as pseudoautosomal inheritance (4). The short stature homeobox-containing gene (*SHOX*), encoding a transcription factor, represents the single known disease gene within the PAR1. Functional loss of SHOX has been associated with a reduced body height in different clinical entities, including ISS (OMIM300582), LWD (OMIM127300), and Langer mesomelic dysplasia (OMIM249700) (5–7). Haploinsufficiency of *SHOX* due to heterozygous intragenic mutations, total gene deletions, or microdeletions downstream of the transcription unit, accounts for at least 5% of the cases with ISS and for 50–100% of the cases with LWD depending on the cohort studied (8–11). Interestingly, deletions account for the major proportion of identified defects (10, 12). Several techniques have been applied to identify these abundant deletions, including fluorescence *in situ* hybridization, microsatellite analysis, quantitative PCR (qPCR) with amplicons restricted to the *SHOX* gene and more recently multiplex ligation-dependent probe amplification (MLPA) (10, 13–16). Fluorescence *in situ* hybridization analysis for the *SHOX* region has been used in a routine setting for many years but requires metaphase or interphase chromosomes and is limited in resolution. Microsatellite and single-nucleotide polymorphism analysis with a confined set of markers has been shown to be an effective alternative, but the major disadvantage is the need for parental DNA (14). MLPA overcomes this and has been demonstrated to be a sensitive and cost-effective alternative; however, these regularly changing probe mixes are intended for research purposes, requiring extensive in-house validation after each small modification of the MLPA probe mix (16, 17). Other drawbacks include the need for highly purified DNA, the long ligation step, and the multiple post-PCR handling steps. To overcome these shortcomings, we designed and validated an in-house developed qPCR test for *SHOX* and its surrounding 5′ and 3′ regulatory regions. We demonstrate that our new test is an appealing strategy for the identification of genomic rearrangements in the *SHOX* region in patients with ISS, LWD, or other phenotypes.

Patients and Methods

Patient samples

Overall, 213 consenting probands were enrolled in this study. The cohort consisted of 207 diagnostic referrals for ISS (*e.g.* short stature with unknown etiology) derived predominantly from pediatric endocrinology clinics during the period 2005–2008. Stature was recorded and SD scores were determined according to a matched population for age and gender. Inclusion criteria for LWD were presence of Madelung deformity and shortening of the limbs in the proband or a direct family member. In addition, six patients with other clinical indications were referred to our laboratory to confirm copy number changes in the PAR1, which were initially detected using other techniques such as subtelomeric MLPA. The study was conducted following the tenets of Helsinki.

Genomic DNA (gDNA) was purified from EDTA blood samples (1–10 ml) using the Gentra Systems (Minneapolis, MN) Puregene DNA purification method and subsequently stored at 4 C. DNA concentration and purity (A260/A280) were assessed using a Nanodrop ND-1000 (Thermo Scientific, Waltham, MA).

MLPA

From 2005 to 2007, MLPA was performed for 115 probands as described in the protocol provided by the manufacturer (MRC Holland, Amsterdam, The Netherlands). During this period, three generations of MLPA mixes for the *SHOX* region were released (P018, P018B, and P018C; MRC Holland). Each identified copy number change was confirmed by repeating the MLPA analysis. In the context of validation, 72 of 115 MLPA-prescreened samples were subsequently analyzed using our newly developed qPCR test. The remaining 43 MLPA-screened samples were not analyzed with qPCR.

All copy number changes newly identified using our qPCR test were confirmed using the MLPA mix P018D.

qPCR

A part of the human PAR1 sequence (chrX: 1–1,000,000) was retrieved from the UCSC Genome browser (Human March 2006 Assembly, hg18). A dedicated selection of qPCR assays (amplicons) was based upon 1) the coding region of *SHOX* and 2) the shortest region of overlap (SRO) of previously reported regulatory deletions 3′ to *SHOX*. Subsequently, a comparison was made with the location of the MLPA probes. Thirteen qPCR primer pairs were designed according to guidelines described previously (18) using PrimerQuest (Integrated DNA Technologies, Coralville, IA). The BLAST program from the NCBI browser (http://www.ncbi.nlm.nih.gov/BLAST) was used for *in silico* specificity analysis (primer sequences submitted in RTPrimerDB; primer IDs in Table 1). Subsequently, single-nucleotide polymorphisms and copy number polymorphisms were excluded at the annealing sites using the corresponding tracks from the UCSC browser (http://genome.ucsc.edu). In addition, the absence of secondary structures in the region in which the primers anneal was verified using MFOLD (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/dna-form1.cgi) (19). Primers were synthesized without special purification (Biolegio, Nijmegen, The Netherlands). The *in silico* validation was followed by an extensive empirical validation. First, amplification efficiencies were calculated based upon the generation of standard curves using gDNA dilution series. Assays with amplification efficiencies between 85 and 115% were considered as ac-

TABLE 1. qPCR primers and validation

	Amplicon length (bp)	Location on chromosome X	Primer (5′–3′)	RTprimerDB ID[a]	Standard curve				Linear dynamic range (ng)	SD[b]
					Slope	E (%)	SE (E) (%)	r^2		
1	131	249963–250093	GCTTTGGGTGACGGGCTCTTTA AAGCCAGCCGCCTGTTCATTA	7865	−3.606	89	0.6	0.998	0.125–64	0.12
2	143	501091–501233	TTGGGAAGCAAAGTGTGCCTCTTC AGGACGCACCGGCTTAAGAAAT	7864	−3.519	92	0.7	0.998	0.125–64	0.11
3	127	511341–511467	ACGGACGCCAAACAGTGATGAA GCTGCCTGTCTGGTGCAATTACT		−3.865	81	0.4	0.998		
4	125	515403–515527	ACGGGCAGACCAAGCTGAAA CTGAGCTCCTCGCGCATGAA	7863	−3.482	93	0.2	1	0.0624–64	0.10
5	107	521445–521551	AGCTCCCAGAGGTGCAAAGT GGGAGAAGCAGAGAGAGATGTGAAGA	7866	−3.426	96	1.0	0.993	0.0624–64	0.09
6	126	521781–521906	CCTACGTCAACATGGGAGCCTTA TGTCAGGATGCGGCAGCAAATA	7867	−3.689	87	0.5	0.998	0.0624–64	0.06
7	94	525674–525767	TGCGTCTTCCTCTGCTATACCCTATG AGCTCCGGGATCTTTGCAGTTT	7868	−3.602	90	0.3	0.999	0.0624–64	0.11
8	110	544828–544937	AGCAGCGTTCGGCTTCACATTT CCATGACCCGCCACAACATTCTAA	7869	−3.614	90	0.6	0.997	0.0624–64	0.20
9	129	618901–619029	GCATTGTGGCATTCAGCGTGTA GCCACAAGGCACTGATATGCACTTA		−3.462	94	0.5	0.998		
10	138	693629–693766	TTCACTGTTGGGCCGCTCTATTA GCAGGGACAAGACACAGGAGAATA	7870	−3.266	102	0.7	0.997	0.125–64	0.08
11	144	738263–738406	GCGCTCTGGAATGCAAAGCTGTAA ACCACCCAAGCACAGTTGAAGA	7871	−3.698	87	0.7	0.997	0.0624–64	0.09
12	106	755247–755352	GTCAGAAATCAAATGTCGTAAAT TCTATGCAAACGTTCAGTTAT	7872	−3.404	97	0.8	0.996	0.0624–64	0.16
13	140	770528–770667	GAAGCCTCCCAGTCTGTGGTATTT TTGGTCTTTGCCCTGCACCTAT	7873	−3.544	92	0.3	0.999	0.0624–64	0.05

E, Efficiency.

[a] http://medgen.ugent.be/rtprimerdb/.

[b] The SD of the $\log_2$-transformed NRQs from 32 control samples.

ceptable (Table 1). The specificity of each amplicon was tested based upon melting curve analysis and microchip electrophoresis, using the DNA-500 kit (MultiNA; Shimadzu, Kyoto, Japan). qPCR was carried out in white 384-well plates (Roche, Basel, Switzerland) using the qPCR Core kit for SYBR Green I (Eurogentec, Seraing, Belgium) on the LightCycler 480 (Roche). The final volume of 7.5 μl contained 0.375 μl of each primer (working solution 5 μM), 3.75 μl master mix, and 10 ng gDNA. Each PCR was carried out in duplicate with the following cycling conditions: 10 min at 95 C; 40 cycles of 15 sec at 95 C, 45 sec at 60 C, and 1 sec at 72 C; and finally a dissociation run from 60–95 C (melting curve analysis). Quantification cycle values were extracted with the Lightcycler 480 software (version 1.5.0 SP3) using the second-derivative maximum algorithm. qbase^{PLUS} software (Biogazelle, Ghent, Belgium) was used for quality control and calculation of relative quantities using *ZNF80* and *GPR15* for normalization (20). Samples that did not pass quality control because of excessive variance between replicates (>0.5 cycle) were excluded from further analysis (21). Next, the normalized relative quantities (NRQs) were exported to Excel and transformed using a rescaling factor based on reference samples with known copy number. Where possible, the rescaling factor was calculated from multiple reference samples, *e.g.* two normal control samples and a sample with a known deletion.

Screening of 32 normal control samples was performed to assess the normal variation. The mean and the SD of the $\log_2$-transformed NRQs were calculated for each amplicon. These values were subsequently used to deduce amplicon-specific confidence intervals for samples with a normal copy, with a heterozygous deletion, and with a heterozygous duplication.

The qPCR assays were successfully performed either using an automated laboratory liquid handling system (Freedom Evo 75; Tecan, Männedorf, Switzerland) or by manual pipetting for 170 probands. In general, one manually performed run consisted of nine samples of interest, three reference samples, and one no-template control. Complete runs or single samples that did not pass quality control were repeated. The optimal cutoff values to define deletions and duplications, without generating false negatives or many false positives, were assessed. First, amplicon-specific 95% confidence intervals were deduced for samples with a normal copy [$\log_2(2) \pm 2.04 \times$ SD], with a heterozygous deletion [$\log_2(1) \pm 2.04 \times$ SD], and with a heterozygous duplication [$\log_2(3) \pm 2.04 \times$ SD]. Subsequently, cutoff values based upon the generated confidence intervals were evaluated (similar to array comparative genomic hybridization and MLPA). Each identified copy number change was confirmed by repeating the qPCR analysis.

An RDML file of the entire study was created using qbase^{PLUS} (available upon request) (22).

This study is written according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines to ensure its technical qualities and to allow for correct interpretation and repeatability (23). A checklist is available (Supplemental data published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

Results

In this study, we developed a qPCR-based molecular test consisting of 11 assays. Our main criterion for the design consisted of the capacity to identify clinically relevant copy number changes of the PAR1, more specifically of the *SHOX* coding region and the SRO of previously reported regulatory deletions 3′ to *SHOX*.

MLPA data used for method comparison

From 2005–2007, MLPA was successfully applied for copy number screening of *SHOX* and the downstream PAR1 in 115 probands using different probe mixes P018, P018B, and P018C (Fig. 1). The description of these MLPA data is added for the method comparison of the qPCR data.

MLPA using the P018 probe mix was used in 44 probands, revealing seven heterozygous deletions of the *SHOX* gene and one heterozygous deletion downstream of *SHOX*. In addition, two heterozygous duplications of *SHOX* were found, one of which was identified in a patient with a clinical diagnosis of ISS. This first-generation MLPA mix comprised only a few probes downstream of *SHOX* (Fig. 1), which may have hampered the identification of deletions outside the *SHOX* coding region. To overcome this, additional probes were added by the manufacturer, resulting in the second generation probe mix P018B. Indeed, reanalysis of nine patients initially screened with P018 led to the identification of one new extragenic deletion. Forty-nine new probands were analyzed using P018B, revealing three new deletions. Two of three deletions comprised *SHOX*, whereas one deletion was located downstream of *SHOX*. For the region downstream of *SHOX*, only copy number changes spanning several probes were regarded as true positive mainly because of the presence of less reliable probes as indicated by the manufacturer. Later on, some of these unreliable probes were excluded from the mix, resulting in the P018C probe mix. The MLPA P018C probe mix consisted of eight probes for the *SHOX* coding region, two for the region 5′ to *SHOX* and 12 for the region 3′ to *SHOX*. Four patients prescreened with P018 and/or P018B were reanalyzed, and 22 new probands were screened. The included extragenic deletion was identified, and one new *SHOX* duplication was detected.

In-house developed qPCR-based molecular test

Eleven of the 13 *in silico* validated qPCR primer pairs met our quality control criteria based upon the generation of standard curves using gDNA dilution series and microchip electrophoresis (Fig. 2 and Table 1). Amplicons 3 and 9 were excluded due to inefficient amplification and nonspecific PCR products, respectively. To explore the normal copy number variability of remaining amplicons, the qPCR assays were performed on 32 normal control samples. Four included no-template control samples passed for all 11 targets, and the reference target stability value was high ($M < 0.5$). Each PCR amplicon was analyzed separately, and nine sample-target combinations that did not pass quality control because of excessive variance between replicates (>0.5 cycle) were excluded from the analysis. No copy number changes

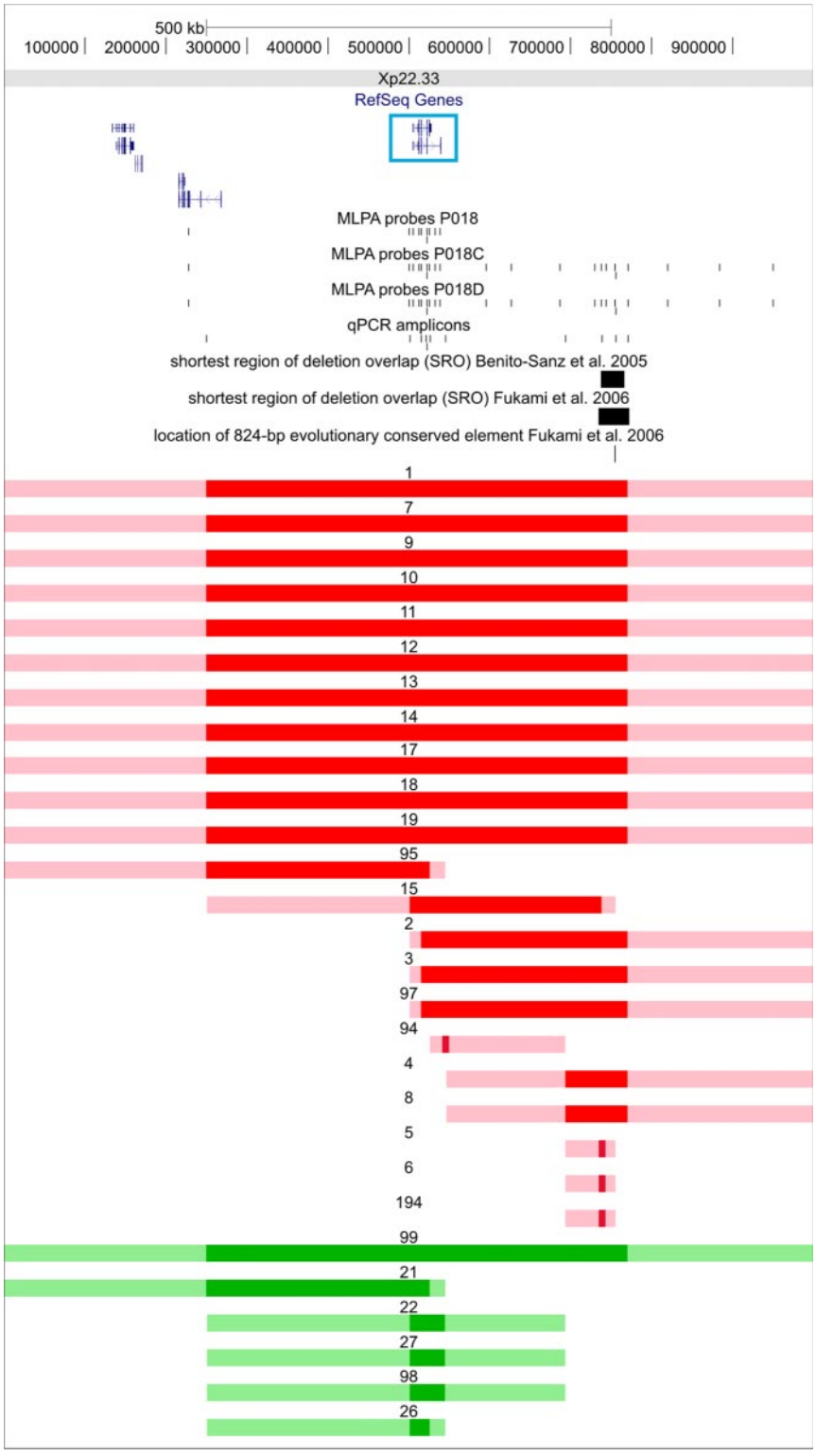

FIG. 1. Human Genome Browser view of the *SHOX* region. The *SHOX* region (chrX: 0–1,000,000) with custom tracks showing the MLPA probes (P018, P018C, and P018D), the qPCR amplicons used in the study, the SRO described by Benito-Sanz *et al.* (14), the SRO reported by Fukami *et al.* (26), and the location of an 824-bp evolutionarily conserved element described by Fukami *et al.* (27). The *horizontal bars* represent the different copy number changes identified by qPCR. Locations and sizes of the deletions/duplications based upon qPCR results are indicated by *horizontal red/green bars*, respectively. The *red/dark green bars* indicate the minimal deleted/duplicated regions, and the *pink/light green bars* indicate the regions harboring the breakpoints. The extent of the copy number changes represented in this figure is based upon the qPCR results for the different samples. However, it is important to notice that all copy number changes were confirmed using MLPA (different mixes). The numbers correspond with the patient codes used in the RDML files. In addition, the RefSeq Genes track and the Vertebrate Multiz Alignment and PhastCons Conservation (28 Species) track are included. The location of the *SHOX* gene is marked by the *blue rectangle*. The figure was drawn according to the UCSC Human Genome Browser, March 2006.

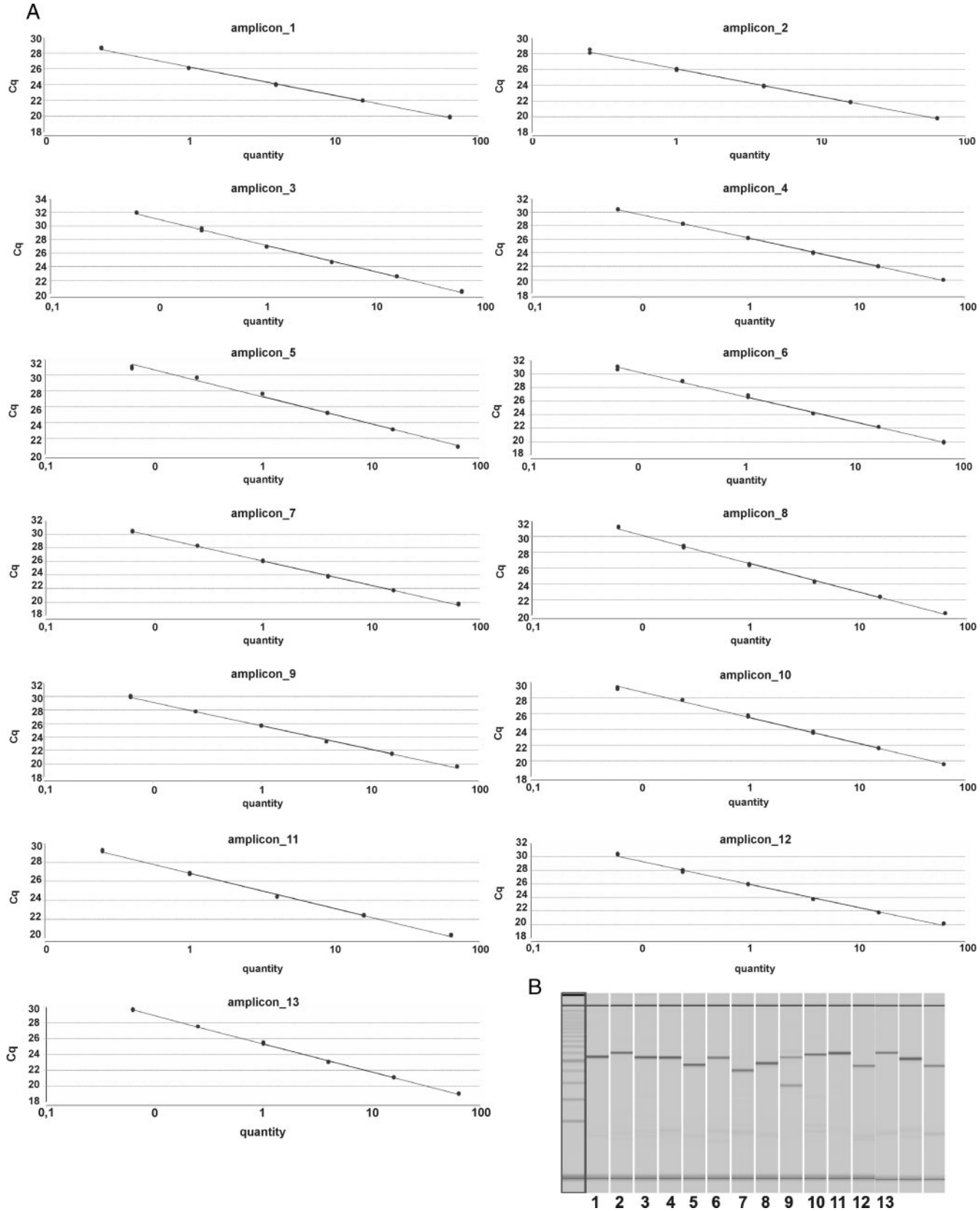

FIG. 2. Validation of 13 *in silico*-designed primer pairs. A, Standard curves for each of the 13 *in silico*-designed amplicons generated using gDNA dilution series. Failing replicates (>0.5 cycle difference) were excluded. The standard curves were extracted from qbase^{PLUS} (Biogazelle). All amplification efficiencies are more than 85%, except for amplicon 3. Cq, Quantification cycle. B, Results from microchip electrophoresis (MultiNA; Shimadzu). Shown from *left* to *right* areDNA-500 ladder, amplicons 1–13, *ZNF80*, and *GPR15*. All bands correspond with the correct size, except for amplicon 9 that shows two bands.

were detected in the control samples. The mean and SD of the $\log_2$-transformed NRQs was calculated for each amplicon. These values were subsequently used to deduce amplicon-specific 95% confidence intervals (Table 2). The confidence intervals for duplications and normal alleles show overlap for only two qPCR assays (*i.e.* amplicons 8 and 12). In general, duplications are more difficult to detect than deletions, because their ratio (3:2) is closer to the normal ratio (2:2). Therefore, an upper cutoff value of 1.29 was chosen, and duplications were regarded as true positives only if more than one adjacent qPCR assay showed values above 1.29. Confidence intervals for deletions and normal alleles do not overlap. Based upon the 95% confidence intervals, 0.5 was chosen as the lower cutoff value.

Overall, reliable qPCR results were obtained for 170 samples from unrelated patients, including 18 samples with known copy number changes (14 from our MLPA screenings and four subtelomere-positive patients), 58 negative samples previously screened by means of MLPA, and 94 new samples. The reasons for including 18 samples with a known copy number change (deletions and duplications) in the PAR1 were 3-fold: 1) to evaluate the cutoff values, 2) to assess the sensitivity of the qPCR-based test, and 3) to streamline the results generated using MLPA with different probe mixes (Fig. 1). All known copy number changes could be detected using the defined cutoff values, indicating a high sensitivity of the test. In addition, it was used to analyze 58 samples for which MLPA screening was negative, resulting in the detection of one new deletion downstream of *SHOX* (sample 8 in Fig. 1). The latter can be explained by the fact that this sample was screened with the first-generation MLPA mix (P018) not containing probes in the downstream regulatory region. However, we were able to demonstrate that the recent P018D MLPA mix also allows the identification of this deletion. In addition, 94 new samples were screened, revealing nine new copy number changes, including eight *SHOX* deletions (samples 1, 7, 9, 10, 11, 95, 97, and 94) and one regulatory deletion (sample 194 in Fig. 1). All newly identified copy number changes were confirmed using the P018D MLPA mix.

Phenotypic information of the mutation-positive patients

Of 213 probands, 207 were initially referred for molecular diagnosis of ISS. Reinspection of the clinical records after the identification of a copy number change in the PAR1 (17 *SHOX* deletions, one *SHOX* duplication, and five regulatory deletions) revealed that 15 were diagnosed with LWD and that six were diagnosed with ISS. In addition, two patients appeared to have Turner syndrome.

Apart from the latter group, six patients were referred to our laboratory for screening in the context of other clinical

TABLE 2. Theoretical values and amplicon-specific confidence intervals ($\log_2$ scale)

	Amplicon 1	Amplicon 2	Amplicon 4	Amplicon 5	Amplicon 6	Amplicon 7	Amplicon 8	Amplicon 10	Amplicon 11	Amplicon 12	Amplicon 13
Duplication											
Upper limit	1.83	1.80	1.78	1.76	1.71	1.81	1.98	1.74	1.76	1.91	1.69
Theoretical value	1.58	1.58	1.58	1.58	1.58	1.58	1.58	1.58	1.58	1.58	1.58
Lower limit	1.33	1.36	1.38	1.40	1.45	1.35	1.18	1.42	1.40	1.25	1.47
Normal											
Upper limit	1.25	1.22	1.20	1.18	1.13	1.23	1.40	1.16	1.18	1.33	1.11
Theoretical value	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Lower limit	0.75	0.78	0.80	0.82	0.87	0.77	0.60	0.84	0.82	0.67	0.89
Deletion											
Upper limit	0.25	0.22	0.20	0.18	0.13	0.23	0.40	0.16	0.18	0.33	0.11
Theoretical value	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Lower limit	−0.25	−0.22	−0.20	−0.18	−0.13	−0.23	−0.40	−0.16	−0.18	−0.33	−0.11

The first part of the table represents the amplicon-specific 95% confidence intervals that were deduced for samples with a normal copy [$\log_2(2) \pm 2.04 \times$ SD], with a heterozygous deletion [$\log_2(1) \pm 2.04 \times$ SD], and with a heterozygous duplication [$\log_2(3) \pm 2.04 \times$ SD]. For each amplicon, the upper and lower value of the intervals are indicated and the theoretical value for a duplication, a normal copy number, or a deletion.

indications to confirm duplications in the PAR1, previously found using subtelomeric MLPA. This group included three patients with *SHOX* duplications associated with congenital anomalies, more specifically gastrointestinal malformations (de Jong, E. M., T.-S. Barakat, B. H. Eussen, B. D'haene, E. De Baere, P. P. Poddighe, R.-J. Galjaard, J. Gribnau, A. S. Brooks, D. Tibboel, and A. de Klein in preparation). The three other patients presented (mild) mental retardation. There were no specific growth abnormalities reported in any of these six patients carrying *SHOX* duplications.

Discussion

MLPA with a commercially available mix has been shown to be instrumental for detecting copy number changes of the *SHOX* region, but we experienced the disadvantages of thorough in-house validation after each small adaptation to the probe mix. To overcome these and other shortcomings, we aimed to set up a robust and extensively validated molecular genetic test for ISS and LWD. We developed a qPCR-based test with 11 *in silico* and empirically validated amplicons located in the *SHOX* coding region and in the SRO of previously identified PAR1 deletions downstream of *SHOX*. Unlike the previously reported qPCR test for the *SHOX* coding region (13), our assays underwent dedicated design and validation described in detail here. The qPCR test presented by Tan and Loke (13) contained only targets for the *SHOX* gene itself and was validated on only nine patient samples and 12 controls. For our study, we followed the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines to ensure the relevance, accuracy, correct interpretation, and repeatability of our method (23). We screened 115 patients for copy number variations of the *SHOX* region using MLPA, resulting in the identification of a heterozygous copy number change in 15 probands, including nine *SHOX* deletions, three *SHOX* duplications, and three downstream regulatory deletions. Seventy-two of the 115 samples screened by different MLPA mixes were analyzed with our newly developed automated qPCR-based molecular genetic test. In addition, we analyzed four samples positive for subtemoleric MLPA screening. All included copy number changes previously identified by MLPA were also detected with the qPCR assay, demonstrating its sensitivity. In addition, we identified one new deletion downstream of *SHOX* that was missed by MLPA mix P018. Subsequently, this deletion could be confirmed with the P018D probe mix. Since the implementation of our novel test, we screened 94 new patients revealing eight *SHOX* deletions and one regulatory deletion. Although our molecular genetic test targets fewer regions than the MLPA probe mixes, it proved to reveal all copy number changes previously identified using MLPA, thus meeting our criteria. This can be explained by the dedicated selection of the targets based upon clinical relevance and by the validated primer pairs. We can conclude that our qPCR test is an appealing alternative to MLPA for the identification of copy number changes in the *SHOX* and downstream regulatory region.

Previously, it was suggested that *SHOX* gene duplications might be responsible for overgrowth (24). Recently, two studies were published reporting patients with heterozygous *SHOX* duplications (25, 26). The effect of the identified duplications appeared to be variable, with only a slightly elevated height in some patients. Here, we found a *SHOX* duplication in at least one patient diagnosed with ISS. Furthermore, other duplications were found in patients with clinical diagnoses other than ISS or LWD, in whom *SHOX* testing was requested to confirm a duplication previously identified by subtelomeric MLPA (de Jong, E. M., T.-S. Barakat, B. H. Eussen, B. D'haene, E. De Baere, P. P. Poddighe, R.-J. Galjaard, J. Gribnau, A. S. Brooks, D. Tibboel, and A. de Klein, in preparation).

High-density oligonucleotide arrays or array comparative genomic hybridization might be regarded as another alternative to MLPA and qPCR, but the high GC content of the PAR1 and the abundance of *Alu* repeats leading to an extremely high recombination rate hamper the design of targeted arrays for this region. Moreover, these arrays are still relatively expensive and require more hands-on time. It should be noted that the most recent array technologies possess an extremely high resolution (up to a few base pairs) either for a targeted region or genome wide, whereas the resolution a qPCR test depends on the number of assays and their location. A higher resolution requires additional amplicons and hence additional reactions. Therefore, a higher resolution will result in higher consumable costs. In general, qPCR is not convenient for genome-wide applications, in contrast to microarray-based copy number screening. Interestingly, advantages of our qPCR test are the short hands-on time and low average costs: pipetting, PCR, and data analysis takes less than 4 h for nine patients, and the cost per sample is relatively low, facilitating its implementation as a molecular genetic test. Without taking into account staffing and equipment costs, we calculated that our qPCR assays costs approximately 11 Euros per sample, whereas MLPA costs approximately 19 Euros per sample. We did not take into account personnel costs because these are similar for both techniques, and although equipment costs have not been taken into account, it should be noted that these are more expensive for MLPA (thermocycler, capillary electrophoresis analyzer, type ABI3730XL or ABI3130) than for qPCR (Lightcycler; Roche). Moreover, it can be fully automated using a liquid

handling system and, if required, new amplicons can be easily added without the need for additional validation of the existing amplicons, demonstrating its flexibility. In conclusion, our novel molecular test is a reliable and cheap alternative strategy for the identification of copy number changes in the *SHOX* region in patients with ISS, LWD, and allied phenotypes. It can be applied both in a research as well as in a routine setting, which is highly relevant because the incidence of *SHOX* rearrangements is high and because a correct clinical and molecular diagnosis of growth retardation is important with respect to proper management and beneficial therapy.

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