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Development of an Allele-Specific Real-Time PCR Assay for Discrimination and Quantification of *p63 R279H* Mutation in EEC Syndrome

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The ectrodactyly-ectodermal dysplasia–clefting syndrome is a rare autosomal dominant disorder caused by heterozygous mutations in the *p63* gene, a transcription factor belonging to the p53 family. The majority of cases of ectrodactyly-ectodermal dysplasia syndrome are caused by *de novo* mutations and are therefore sporadic in approximately 60% of patients. The substitution of arginine to histidine *(R279H)*, due to a c.836G>A mutation in exon 7 of the *p63* gene, represents 55% of the identified mutations and is considered a mutational hot spot. A quantitative and sensitive real-time PCR was performed to quantify both wild-type and *R279H* alleles in DNA extracted from peripheral blood and RNA from cultured epithelial cells. Standard curves were constructed for both wild-type and mutant probes. The sensitivity of the assay was determined by generating serial dilutions of the DNA isolated from heterozygous patients (50% of alleles mutated) with wild-type DNA, thus obtaining decreasing percentages of *p63 R279H* mutant allele (50%, 37.5%, 25%, 12.5%, 10%, 7.5%, 5%, 2.5%, and 0.0%). The assay detected up to 1% of the mutant *p63*. The high sensitivity of the assay is of particular relevance to prenatal diagnosis and counseling and to detect therapeutic effects of drug treatment or gene therapy aimed at reducing the amount of mutated *p63*. *(J Mol Diagn 2012, 14:38–45; DOI: 10.1016/j.jmoldx.2011.07.008)*

Ectrodactyly-ectodermal dysplasia–clefting (EEC) syndrome (MIM ID No. 604292; Online Mendelian Inheritance of Man; *http://www.ncbi.nlm.nih.gov/omim*, last accessed June 9, 2011) is one of more than 150 different ectodermal dysplasia syndromes. It is characterized by deformities of the hands and feet (ectrodactyly); abnormalities of the skin, hair, and nails (ectodermal dysplasia); and cleft lip or cleft palate, or both (clefting). Other features include dental, eye, skin, and kidney abnormalities.

The diagnosis of EEC syndrome can be challenging because of the phenotypic overlap with other ectodermal dysplasia syndromes and significant inter- and intrafamilial variability. The diagnosis of EEC syndrome is essentially clinical after physical examination, imaging (limb radiographs and renal ultrasonography), and occasionally skin biopsy. Furthermore, some clinical features are not apparent until affected children mature further.

The identification of the genetic basis of EEC syndrome has facilitated molecular genetic diagnosis. Heterozygous mutations in *p63* result in EEC syndrome and at least 5 other syndromes such as Rapp-Hodgkin syndrome (RHS; MIM ID No. 129400), Hay-Wells syndrome or ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC; MIM ID No. 106260), limb-mammary syndrome (LMS; MIM ID No. 603543), acrodermato-ungual-lacrimal-tooth (ADULT) syndrome (MIM ID No. 103285), and split-hand/foot malformation type 4 (SHFM4; MIM ID No. 605289).[1]

Mutations in *p63* account for 98% of patients with EEC characteristics. To date approximately 40 different pathogenic *p63* mutations have been identified in EEC syndrome.[2] Apart from a frameshift mutation in exon 13, all the other EEC mutations are heterozygous missense mutations located in the DNA binding domain[3] and predominantly involve codons 204, 227, 280, 304, and 279. These five mutations account for almost 90% of all patients with EEC syndrome.[3–5] Of these cases, ≥60% are *de novo* mutations, with alterations in *p63* being present in the affected patient but not in the parents or healthy siblings.[3]

We have recently investigated a cohort of 19 families (23 patients) affected with EEC syndrome from the UK, Ireland, and Italy and our results add to the database of common mutations in which all patients had *de novo*

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missense mutations in Arg304 and Arg279, thus providing new evidence that these are the two most frequently mutated amino acids in this disorder, representing 26.0% (6/23) and 30.4% (7/23) of the identified mutations respectively.[6] In the Italian cohort (n = 11), Arg279His (836G>A) mutation in exon 7 of the *p63* gene was observed more frequently than has been previously reported, accounting for almost 55% (6/11) of the patients so far analyzed.[6] Most of the cases (10/11) were sporadic and related to *de novo* mutations, with just 1 familial case.

Given the high frequency of the *R279H* mutation and the clinical need for a highly sensitive assay able to detect *de novo* mutations, it is of crucial importance that a simple, fast, reliable, and highly sensitive method be developed to detect this mutation. This assay could indeed represent a potential tool for prenatal diagnosis. In this study, therefore, we have developed a novel quantitative allele-specific real-time PCR assay able to detect the *p63 R279H* mutation in patients with EEC syndrome. In addition, this assay could also be used to quantify *p63* mutational load when drugs or gene therapy are evaluated as potential treatments to decrease the amount of mutated *p63* molecules. The development of such an assay able to quantify the *p63* mutational load as well as detect the presence of the mutations could be a breakthrough for clinical purposes.

Materials and Methods

Human Subjects

Patients with EEC syndrome were recruited from the Ectodermal Dysplasia Society (Gloucestershire, UK) and two Italian EEC societies—Associazione Italiana Sindrome EEC (Rieti, Italy) and Syndrome EEC International (Padova, Italy)—by circulating letters or references from clinicians. All applicable institutional and government regulations concerning the ethical involvement of human volunteers were strictly followed during this research. Recruitment of patients was approved by the Liverpool Research and Ethics Committee (UK), the Multiregional Research and Ethics Committee (North-West, UK) and the Venetian Clinical Research Ethics Committee (Venice, Italy).

After obtaining the consent, a full medical and ocular history was taken, and a complete family history was recorded to document the pedigree. To be recruited, EEC families or isolated cases had to show at least two of the three main clinical features of the syndrome.[3,5,7] These features include i) an ectodermal dysplasia affecting the skin, hair, nails, teeth, sweat glands, lacrimal ducts, or mammary glands; ii) hand or foot abnormalities consistent with the split-hand/foot spectrum; and iii) cleft lip with or without cleft palate. Of all of the subjects recruited, one patient carrying the *R279H p63* mutation (EEC014-1) gave the consent to donate both blood samples (for DNA isolation) and epithelial cells (for RNA isolation) derived from an oral mucosa biopsy specimen that became available after a surgical intervention unrelated to the study described in this article.

Human Samples

To set up the allele-specific real-time PCR for diagnostic purposes, 10 mL of venous blood was extracted from one patient with EEC syndrome carrying the *R279H* mutation and from one healthy control subject by standard sterile venipuncture and preserved in EDTA. Genomic DNA was isolated from peripheral blood leukocytes using standard protocols (Wizard DNA purification kit; Promega, Madison, WI). To set up the allele-specific real-time PCR assays for evaluation of mutated *p63* gene expression (eg to detect effects of therapeutic treatments), total RNA was extracted from cultured oral mucosa epithelial cells of one patient with EEC syndrome who carried the *R279H* mutation and of one healthy control subject (RNeasy Micro kit; Qiagen, Valencia, CA).

Mutational Analysis of p63 Gene

To identify whether *p63* was mutated or not, genomic DNA from both the patient with EEC syndrome and the healthy subject were used as templates for amplification of *p63* DNA-binding domains (exons 4–8), as previously described.[3,6,8] Amplified DNA fragments were sequenced using both forward and reverse primers and were analyzed on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). Identified sequence variants were described according to the guidelines published by the Human Genome Variation Society (*http://www.hgvs.org/mutnomen*, last accessed June 9, 2011). The position of the mutation was given according to the original published TAp63α sequence (GenBank accession no. AF075430), which does not include the 39 additional amino acids at the N-terminus, as instead reported by GenBank (accession no. AF091627; gi:3695081).[9]

Allele-Specific Real-Time PCR Development

p63 R279H *Detection Methods*

DNA samples obtained from patients with EEC syndrome were tested for the presence of *p63 R279H* mutation by using two real-time PCR platforms, the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems) and the Biorad iCycler iQ Real-Time PCR System (Bio-Rad Laboratories Inc., Hercules, CA), and by direct sequencing through the ABI 3730xl DNA Analyzer (Applied Biosystems). The aim was to design two TaqMan probes able to hybridize specifically either with the wild-type (G) or the mutated (A) *p63* allele, thus allowing a competitive analysis. These probes were labeled with two different fluorescent dyes: FAM for the wild-type *p63* allele and HEX for the mutated allele.

A single set of primers was devised and used so that mutation discrimination was achieved by using probes that differed only at the position of the *R279H p63* mutation. Such an approach has the advantage of detecting a *p63* mutation independently from the amplification efficiency. The labeling of the probes with different fluorophores for the wild-type and mutated alleles allows i) detection of two alleles in a single-tube analysis, ii)

Table 1. Sequences of Primers and Probes Used for Real-Time PCR Assay

Gene	Sequence	Primers/probes
p63 Primer	5′-GGGAAGAACTGAGAAGGAATCCCCAAC-3′	*p63*-DNA forward
	5′-CAAAGTGAAATCGTGGCTG-3′	*p63*-DNA reverse
	5′-CACCCCAGGTTGGCACTG-3′	*p63*-RNA-forward
	5′-GACTTGCCCATCTCTGGTTTCCC-3′	*p63*-RNA-reverse
	5′-(FAM)AATTGGACGGCGGTTCATCC(BHQ-1)-3′	*p63* wild-type probe
	5′-(HEX)AATTGGACGGTGGTTCATCCC(BHQ-1)-3′	*p63* Mutated probe
p63 Taqman probe	5′-AGAAGATGACCCAGATCATGTTTG-3′	Actin-RNA forward
	5′-CGTCGGTGAGGATCTTCA-3′	Actin-RNA reverse
β-Actin primers/probe	5′-(HEX)CCTGCGTCTGGACCTGGCT(BHQ-1)-3′	Actin-probe
	5′-GGGAAGAACTGAGAAGGAACAAC-3′	*p63*-DNA forward
	5′-CAAAGTGAAATCGTGGCTG-3′	*p63*-DNA reverse

use of the wild-type sequences as an internal control for the quality of genetic material and amplification process, and iii) quantification of the mutational load by calculating the mutant/wild-type allele ratio. Addition of the β-actin housekeeping quantitative RNA amplification during *p63* RNA analysis allowed the detection of possible undesired/unspecific down-regulation of the wild-type *p63* gene.

Primers and Probes Design

p63 R279H mutation-specific primers and probes were designed using freeware FastPCR and Oligo analyzer programs. Sequences were obtained from the NCBI database (*http://www.ncbi.nlm.nih.gov/gene?term=*NG-007550, last accessed June 9, 2011). TaqMan probes were custom synthesized by Metabion International AG (Metabion International AG, Martinsried, Germany). Sequences of primers and probes are provided in Table 1.

Reverse Transcription Mix for p63 *Expression Analysis*

The reverse transcription protocol was performed following the manufacturer's instruction manual (M-MLV Re-

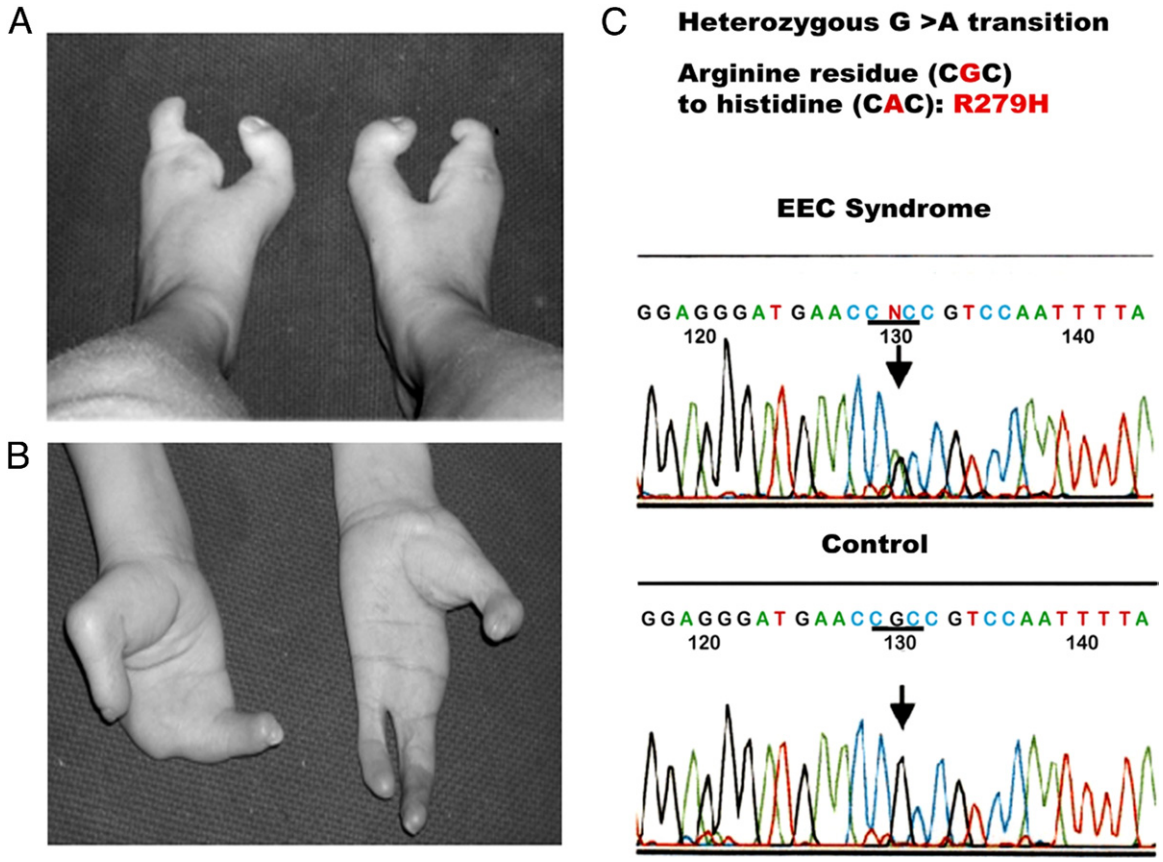


Figure 1. Bilateral ectrodactyly of the feet (**A**) and hands (**B**) in a EEC syndrome patient. **C:** Sequence analysis of the *R279H* mutation in exon 7 of the *p63* gene: a G to A transition at position 836 (G836A) leads to an arginine-to-histidine change at position 279 (*R279H*). **Arrows** indicate the position of mutation site.

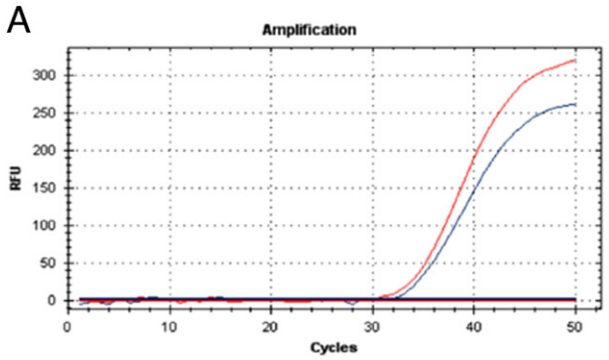
verse Transcriptase, Invitrogen Life Technologies, Milan, Italy).

Real-Time PCR Amplification Mixture

PCR reaction mixtures contained 2.5 μL of 10× reaction buffer, 2 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleotide triphosphates, hot-start Taq polymerase (1.25 U), 0.2 pmol/μL of each probe, 0.4 pmol/μL of each primer, 2% deionized formamide, and 50 ng DNA (or cDNA) in a final 25-μL volume. The same mixture was used with three different combinations of primers/probes: i) Mix 1 for mutated/wild-type DNA allele analysis: *p63*-DNA-For, *p63*-DNA-Rev, *p63*-probe–wild type, *p63*-probe–mutated; ii) Mix 2 for mutated/wild-type allele expression: *p63*-RNA-For, *p63*-RNA-Rev, *p63*-probe–wild type, *p63*-probe–mutated; iii) Mix 3 for *p63* gene expression analysis: *p63*-RNA-For, *p63*-RNA-Rev, *p63*-probe–wild type, actin-RNA–For, Actin-RNA–Rev, Actin-probe.

Thermal Profile

One PCR thermal profile was developed and optimized to allow the best compromise between the results from Mix 1, Mix 2, and Mix 3 using different real-time PCR platforms (Applied Biosystems 7300, 7900, and Biorad iCy-



D

	0.0% mut	12.5% mut	25.0% mut	37.5% mut	50.0% mut
HEX/FAM RATIO	0,16	0,28	0,39	0,45	0,67
	0,12	0,27	0,37	0,48	0,64
	0,13	0,30	0,37	0,49	0,63
	0,15	0,29	0,38	0,43	0,66
	0,15	0,32	0,35	0,47	0,59
	0,14	0,30	0,38	0,46	0,61
	0,13	0,26	0,33	0,41	0,66
	0,16	0,29	0,36	0,48	0,67
	0,14	0,29	0,36	0,49	0,65
	0,11	0,24	0,39	0,48	0,65
	0,16	0,26	0,37	0,50	0,59
	0,18	0,27	0,39	0,51	0,62
	0,17	0,26	0,36	0,47	0,61
	0,16	0,27	0,37	0,49	0,66
	0,14	0,27	0,40	0,50	0,58
AVERAGE HEX/FAM RATIO	0,15	0,28	0,37	0,47	0,63
ST. DEV. HEX/FAM RATIO	0,0191	0,0204	0,0181	0,0272	0,0308
ST. DEV. % HEX/FAM RATIO	13,06%	7,35%	4,87%	5,74%	4,87%
P. value	7,84E-12	3,87E-09	1,30E-10	2,84E-09	-

E

	0.0% mut	2.5% mut	5.0% mut	7.5% mut	10.0% mut
HEX/FAM RATIO	0,17	0,15	0,18	0,19	0,21
	0,14	0,19	0,14	0,21	0,24
	0,15	0,14	0,17	0,20	0,23
	0,17	0,17	0,16	0,24	0,25
	0,10	0,17	0,17	0,22	0,26
	0,15	0,15	0,19	0,18	0,24
	0,13	0,19	0,16	0,18	0,24
	0,13	0,14	0,16	0,16	0,25
	0,15	0,16	0,19	0,19	0,23
	0,12	0,16	0,19	0,17	0,26
	0,13	0,14	0,15	0,19	0,22
	0,14	0,13	0,18	0,21	0,24
	0,14	0,14	0,19	0,23	0,24
	0,16	0,13	0,16	0,20	0,20
	0,13	0,17	0,18	0,21	0,21
AVERAGE HEX/FAM RATIO	0,14	0,16	0,17	0,20	0,23
ST. DEV. HEX/FAM RATIO	0,0187	0,0196	0,0160	0,0220	0,0181
ST. DEV. % HEX/FAM RATIO	13,29%	12,61%	9,32%	11,07%	7,70%
P. value	0,0405	0,0236	0,0011	0,0001	-

Figure 2. Allele-specific real-time PCR assay for discrimination and quantification of the *p63 R279H* mutation. **A:** Amplification plots of wild-type and mutated *R279H* alleles of *p63* from a heterozygote sample. Amplification curves show the signals from FAM (blue, wild-type *p63* allele) and HEX label (red, mutated *p63* allele). Alleles have similar cycle thresholds, but slightly different fluorescence intensities as measured at the end-point analysis when the PCR cycles have finished. **B** and **C:** Construction of standard curves obtained by progressively diluting mutant DNA with wild-type DNA. The sensitivity of the assay was determined by generating serial dilutions of mutant DNA (from the heterozygous patient, with 50% alleles mutated) with wild-type DNA (no mutated alleles) to generate samples with different percentages of *p63 R279H* mutant DNA (50%, 37.5%, 25%, 12.5%, and 0%) (**B**). The same experimental scheme was adopted to detect the sensitivity of the test in a second-level analysis using lower mutated/wild-type allele ratios (10%, 7.5%, 5%, 2.5%, and 0.0%) (**C**). Efficiency of amplification was unaffected by the genotype, and there was a high correlation between input DNA and crossing points [r^2 values of 0.975 (wild-type) and 0.950 (mutant)]. Analysis of each individual value used to obtain the standard curve (**D**) showed that 0.0160 was the minimum SD observed and 0.0308 the maximum. This indicates that the test was highly reproducible. **E:** Significant differences were seen between 0% and 2.5% mutated/wild-type allele ratios ($P < 0.05$) and between 5% and 7.5% ($P < 0.01$).

cler). In all cases, the thermal profile used was the following: 15 minutes at 95°C, followed by 50 cycles of denaturation for 20 seconds at 95°C, annealing for 20 seconds at 64°C, and extension for 20 seconds at 72°C. During the latter, signals from FAM and HEX fluorescent dyes were detected.

Assay Assessment

The assays using Mix 1 and Mix 2 were validated for accuracy tests by constructing standard curves and analyzing DNA containing a fixed amount of normal allele and a different fraction of mutated allele. We tested five conditions (50%, 37.5%, 25%, 12.5%, 0% mutant/wild-type ratios). Each condition was set up for five times (intra-assay test). The experiments were replicated and performed on 2 different days (interassay test). Total data interpolation allowed the calculation of the SE and the curve linearity. The same experimental scheme (quadruplicate reactions in intra- and interassays) was adopted for sensitivity tests in a second-level analysis using five further conditions (0%, 2.5%, 5%, 7.5%, and 10% mutant/wild-type ratios).

Assay Analysis

The relative analysis between mutated/wild-type alleles was performed from Mix 1 and Mix 2 results. The data were interpolated starting from the fluorescent signal at the end of PCR (end-point analysis). The total *p63* gene expression was analyzed by comparing β-actin and *p63* transcripts. The relative differences were determined by the cycle threshold obtained after real-time PCR.

Results

Test Results for DNA and RNA Assays

The samples from one patient with EEC syndrome (Figure 1, A and B) carrying the *R279H p63* mutation (Figure 1C) and one healthy control subject were processed in a single-analysis session using both DNA and cDNA as templates. The aim of these experiments was to determine whether the mutant and wild-type alleles have the same expression (relative expression) and whether the total amount of *p63* gene transcript was different between unaffected and mutated samples. As shown in Figure 2A, real-time PCR results showed the same ratio of fluorescence intensity between FAM (quantitatively related to the wild-type allele) and HEX (quantitatively related to the mutated allele) labels. These results do indicate that both alleles have a similar cycle threshold but slightly different fluorescence intensities as measured at the end point analysis when the PCR cycles have finished. Similar results were observed when cDNAs (using RNA extracted from oral mucosa epithelial cells) were analyzed (data not shown).

Validation of the Relative Allele Expression of DNA and RNA Assays

The results from the experimental conditions obtained by mixing known proportions of the mutated DNA (or cDNA) with wild-type DNA are shown in Figure 2, B and C.

Since the primers used to amplify wild-type and mutated alleles were the same, this would suggest that the efficiency of amplification was unaffected by the geno-

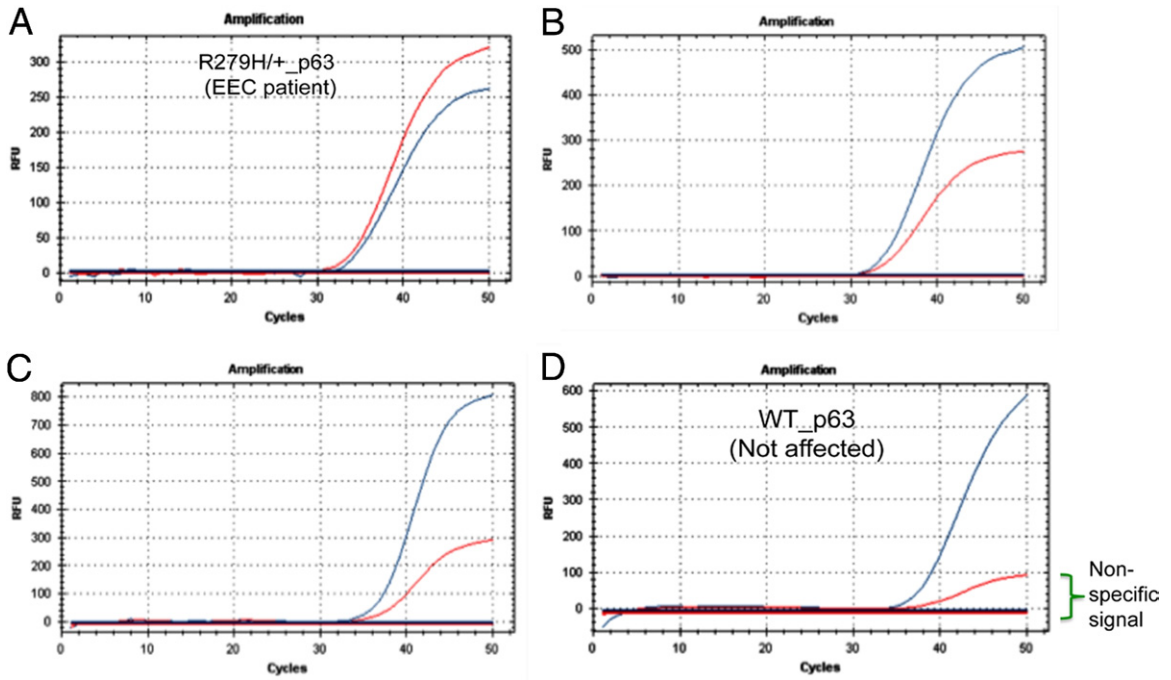


Figure 3. Amplification of four different cDNAs with *p63 R279H* mutant mRNA present at 50.0% [as in the patient with heterozygous EEC syndrome (**A**)], 37.5% (**B**), 12.5% (**C**), and 0.0% [as in the healthy wild-type control subject (**D**)]. Note the decreasing HEX relative fluorescence intensity due to diminishing mutant cDNA percentages. A small nonspecific signal from the mutant probe binding to wild-type sequences has been observed (**D**).

type and a high correlation between input DNA, and crossing point [(r^2 values of 0.975 (wild-type) and 0.950 (mutant)] was observed. The sensitivity of the assay was determined by mixing mutant DNA (obtained from the heterozygous patient with EEC syndrome and therefore having 50% of alleles with the mutation) with wild-type DNA, thus allowing generation of serial dilutions of the *p63 R279H* mutant allele (50.0%, 37.5%, 25.0%, 12.5%, and 0.0%; Figure 2B).

When individual values to obtain the standard curve were analyzed (Figure 2D), 0.0181 was the minimum SD observed, with 0.0308 being the maximum and thus indicating that the assay was highly reproducible. A further refinement of the test sensitivity was obtained by analyzing mutated/wild-type allele ratios <10% (10.0%, 7.5%, 5.0%, 2.5%, 0.0%; Figure 2C). Significant differences were seen between 0% and 2.5% mutated/wild-type allele ratios ($P < 0.05$) and between 5% and 7.5% ($P < 0.01$) (Figure 2E).

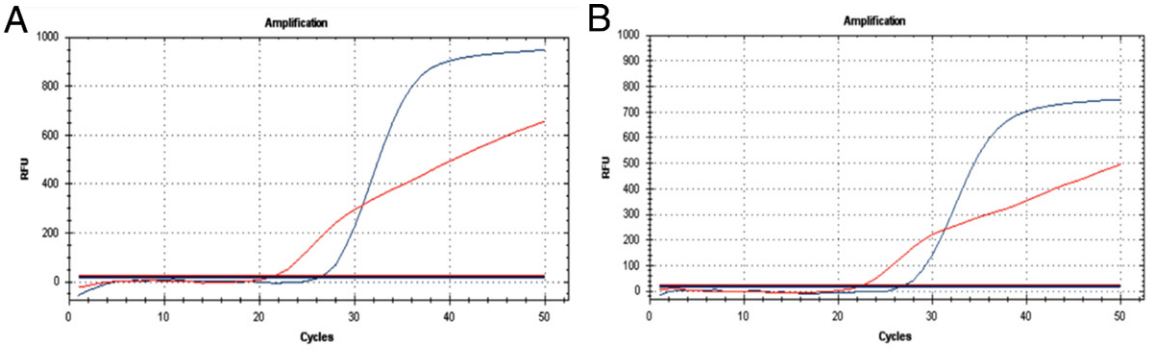
For the setup of assays able to detect the effects of therapeutic treatments on mutated *p63* levels, cDNAs from cultured oral mucosa epithelial cells were also analyzed. Standard curves showed profiles similar to those obtained with genomic DNA from blood samples (data not shown). Figure 3 shows the amplification plots from reactions in which cDNA carrying the *p63 R279H* mutation is present at 50.0% (as in the patient with heterozygous EEC syndrome; Figure 3A), 37.5% (Figure 3B), 12.5% (Figure 3C), and 0.0% (as in the wild-type healthy control subject; Figure 3D).

Testing p63/β-Actin Relative Gene Expression Analysis

To investigate *p63*/β-actin relative gene expression, we developed a new test able to run in a single analytical session. As shown in Figure 4, the curves from the β-actin amplification (in red) showed a lower cycle threshold when compared with those obtained after amplification with *p63* (in blue). However the *p63*/β-actin delta cycle threshold obtained was similar between mutated and wild-type samples, thus indicating that *p63* gene expression was unaffected by the presence/absence of the mutation. Overall these results clearly show that no difference in gene expression was observed between wild-type and mutated samples.

Discussion

Mutations in the *p63* gene result in familial and sporadic cases of EEC syndrome. Familial cases show an autosomal dominant inheritance with variable penetrance. However the majority of cases (≥60%) are caused by sporadic and *de novo* mutations arising during the first stages of embryonic development or, in rarer cases, in



C

	wt sample 1		wt sample 2		mutated sample 1		mutated sample 2	
	beta-actin	P63	beta-actin	P63	beta-actin	P63	beta-actin	P63
	22.1	27.6	23.3	27.9	21.9	28.3	21.1	26.9
	21.8	27.1	22	28.4	21.8	28	20.9	26.8
	22.9	28.2	21.3	28.7	22.8	28.9	22	27.5
AVERAGE	22.27	27.63	22.20	28.33	22.17	28.40	21.33	27.07
ST. DEV.	0.57	0.55	1.01	0.40	0.55	0.46	0.59	0.38
ST. DEV. %	2.55%	1.99%	4.57%	1.43%	2.48%	1.61%	2.75%	1.40%
P63/Actin Delta CT	1.24		1.28		1.28		1.27	

Figure 4. p63/β-Actin relative gene expression analysis. To investigate *p63*/β-actin relative gene expression, a new test assessing both genes in one single run was developed. Real-time PCR curves obtained from healthy (**A**) and mutated samples (**B**). **C:** For both the patient with EEC syndrome and the healthy subject, two samples were run in triplicate (12 different reactions overall). The curves from the β-actin amplification (red curves) showed lower cycle thresholds when compared with those obtained after *p63* amplification (blue curves). However, the cycle threshold delta values between *p63* and β-actin were not different when samples from the patient with mutated alleles and the healthy control were analyzed, thus indicating that *p63* gene expression is unaffected by the mutation. The unvaried ratios seen in mutated and wild-type samples do support the hypothesis that the mutation does not have any role in gene expression regulation.

the parental germline. The latter give rise to mutated sperm or eggs, a process also known as gonadal or germline mosaicism.

Mosaicism is defined as the presence of two genetically different cell lines arising after fertilization, and it informs the timing of postzygotic mutagenesis. Somatic mosaicism occurs in somatic cells, whereas germline mosaicism occurs in the gamete-forming cells.[10] In both conditions, the acquired mutation may be present in only a small proportion of cells, and a highly sensitive detection assay is therefore required.

Typically, germline mosaicism in fathers occurs in the adult testis, whereas in mothers it occurs during early development, since oocytes begin to mature from the fifth month of fetal life.[10] When a *de novo* mutation first appears in a pedigree, the risk of having a second affected child is normally thought to be low or at least equal to the chances of another *de novo* mutation occurring in the offspring. However even if a *de novo* mutation is suspected for the first child, there are cases in which a second affected child is born unexpectedly and this is likely to be due to gonadal or germline mosaicism. Recurrence of a *de novo* mutation is always considered as proof of the presence of germline mosaicism and, on the basis of theoretical and empirical data, it is estimated to be around 5%.[11,12] For this reason, germline mosaicism should be seriously considered for the assessment of recurrence risk during genetic counseling, and prenatal diagnosis should therefore be proposed to all couples, even when the mutation is apparently considered *de novo*.

The real genetic predisposition of the condition is difficult to ascertain if the analysis of *p63* mutation is performed by conventional genotyping. Although conventional genotyping is sensitive, it is in fact not quantitative and, more importantly, it is not able to detect mutations that are present in low abundance and with sequences that may differ from wild-type only for a single nucleotide. Alternatively, quantitative real-time PCR is a very sensitive method able to quantify DNA and mRNA rapidly and accurately. In addition, it has a number of advantages such as high throughput, fast turnaround time, and an accurate target quantitation over a wide range of DNA/RNA concentrations.

Such an approach would be very useful in detecting and quantifying *p63 de novo* mutation as early as possible—in spermatic cells, oocytes, or even in fetal cells. The latter are considered very interesting, as they can be extracted from maternal blood and could potentially be used for prenatal diagnosis as an alternative to chorionic villi sampling, amniocentesis, and cordocentesis. However recovery and analysis of fetal cells from maternal blood is complex and sensitivity is low because of the rarity of these cells in the maternal circulation. Real-time PCR has been shown to detect low levels of fetal DNA with sensitivity of approximately 95% to 100% and specificity of nearly 100%,[13] thus demonstrating it to be an accurate and rapid method for prenatal diagnosis.[14] The only caveat is that fetal cells from earlier pregnancies could still be present in the maternal circulation, thus affecting the outcome of the test.

Because of its sensitivity, we have developed a novel quantitative real-time PCR approach for the detection of the *R279H* mutation in the *p63* gene associated with EEC syndrome, with the perspective to use it for clinical purposes (prenatal diagnosis using fetal DNA from maternal blood, pre-implantation genetic diagnosis, and prenatal counseling) and as a diagnostic test to evaluate the effectiveness of therapies aimed at reducing the levels of the mutated allele. Standard curves obtained to evaluate the ability of the assay to detect differences even at $<10\%$ mutated/wild-type allele ratios showed that significant differences could be appreciated between 0% and 2.5% mutated/wild-type allele ratios ($P < 0.05$) and between 5% and 7.5% ($P < 0.01$), thus suggesting the great diagnostic potential of the assay we developed. Whether the assay was used for genotyping purposes or diagnosis of mutated *p63* levels, results appeared to be similar. The wild-type and mutated alleles appeared to be present at the same ratio, thus indicating unvaried allele-specific gene expression. In addition, the β-actin/*p63* relative gene expression showed unvaried ratios in mutated and wild-type samples, thus supporting the hypothesis that the presence of the mutation does not alter and/or regulate gene expression profiles.

In conclusion, the method we have developed and described in this article appears to be a specific, reproducible, and suitable molecular tool for both *p63* diagnostics and detection of the effects of therapeutic treatments aimed at decreasing mutated RNA concentration.

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