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ARTICLE

PDGFRa mutations in humans with isolated cleft palate

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Isolated cleft palate (CP) is common in humans and has complex genetic etiologies. Many genes have been found to contribute to CP, but the full spectrum of genes remains unknown. PCR-sequencing of the entire coding regions and the 3′ untranslated region (UTR) of the platelet-derived growth factor receptor alpha (*PDGFRa*) and the microRNA (miR), *miR-140* identified seven novel single base-pair substitutions in the *PDGFRa* in 9/102 patients with CP (8.8%), compared with 5/500 ethnic-matched unaffected controls (1%) (the two-tailed *P*-value < 0.0001). Of these seven, four were missense mutations in the coding regions and three in the 3′UTR. Frequencies of four changes (three in coding, one in 3′UTR) were statistically different from those of controls (*P*-value < 0.05). The c.*34G > A was identified in 1/102 cases and 0/500 controls. This position is conserved in primates and located 10 bp away from a predicted binding site for the *miR-140*. Luciferase assay revealed that, in the presence of *miR-140*, the c.*34G > A significantly repressed luciferase activity compared with that of the wild type, suggesting functional significance of this variant. This is the first study providing evidence supporting a role of *PDGFRa* in human CP.
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INTRODUCTION

Isolated cleft palate (CP, MIM 119540) with a prevalence of 1.3–25.3 per 10 000 births is among the most common craniofacial anomalies in humans.[1] CP is a multifactorial disorder influenced by both genetic and environmental factors.[2] Several genes have been found to contribute to CP,[3] but the full spectrum of genes remains largely unknown. Approaches to identify causative genes for CP include analysis using linkage or linkage disequilibrium, candidate genes involving in craniofacial development during embryogenesis, animal models, and chromosomal aberrations.[4]

A very recent study revealed an association of Fas-associated factor-1 (*FAF1*) with cleft palate in humans. The role of *FAF1* in lower jaw development including palate was also strengthened by the presence of severe jaw defects in *faf1*-knockdown zebrafish.[5] A similar approach also identified another candidate gene for CP. Disruption of platelet-derived growth factor receptor alpha (*Pdgfra*) in zebrafish by Mirn140 was found to cause craniofacial abnormalities including cleft palate.[6] Consistent evidence supporting a significant role in palatal development came from previous studies in *Pdgfra* knockout mice.[7–10]

PDGFRa (MIM 173490) maps to chromosome 4q12, spans 65 kb, contains 23 exons, and encodes a protein with 743 amino-acid residues. *PDGFRa* has critical roles in mesenchymal cell migration and proliferation.[8] Involvement of *PDGFRa* in developmental anomalies such as neural tube defects (NTDs) was evidenced by a previous study demonstrating specific combinations of *PDGFRa* promoter haplotypes predisposed to NTDs in humans.[11] Somatic inactivating mutations in *PDGFRa* were also found to be associated with human gastrointestinal stromal tumors.[12] Although animal models suggest involvement of *PDGFRa* in the pathogenesis of cleft palate, there is no solid evidence supporting its involvement in human developmental defects. We hypothesized that germ-line mutations in *PDGFRa* had a role in CP in humans.

SUBJECTS AND METHODS

Study population

We recruited 102 unrelated individuals with non-syndromic CP under the auspices of the Thai Red Cross, from 33 medical centers throughout Thailand. This cohort also included patients who were previously screened for *TBX22* mutations as previously reported.[13] There were 44 male and 58 female. Ninety-six cases were sporadic and six cases had a positive family history. All patients were examined by a plastic surgeon (PS) and clinical geneticists (KS or VS). Patients with other major birth defects were excluded. Controls were Thai blood donors with no oral clefts, who denied history of oral clefts in family members.

The study was approved by the institutional review board of the Faculty of Medicine of Chulalongkorn University and written informed consent was obtained from each person included in the study.

Mutation analysis

DNA was extracted from leukocytes or Fargo Technology Alliance cards (Whatman, Inc., Clifton, NJ, USA). Genomic sequencing was performed for the entire coding regions, and a 708-bp region of the 3′ untranslated region (UTR) of *PDGFRa* and the entire single exon of *miR-140*. The 708-bp region covers the two miR-140 binding sites predicted by the Prediction Program (MicroInspecter, miRBase, Sanger Institute, Cambridge, UK), and microRNA.org (http://www.microrna.org).

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PCR amplifications were carried out using primers and annealing temperatures in Supplementary Table 1. PCR products were treated with ExoSAP-IT (USP Corporation, Cleveland, OH, USA), and sent for direct sequencing at Macrogen Inc. (Seoul, Korea). Analyses were performed by Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI, USA). PCR-RFLP was used to confirm the variants identified in the patients and unaffected controls.

Luciferase assay

We then attempted to determine the pathogenicity of the mutations found in the *PDGFRa* 3′ UTR by the luciferase reporter system. We first PCR amplified the *PDGFRa* 3′ UTR of the patient who was heterozygous for the c.*51G>A mutation using primers 5′-CGAGACCATTGAAGACATCG-3′ and 5′-GTTGTC AGGCTTCTAAATGACC-3′. The PCR product was gel extracted, purified, and then cloned with the pGEM-T Easy Vector System I (Promega, Madison, WI, USA). It was then transferred to the pMIR-REPORT expression vector (Ambion, Austin, TX, USA) using *Sac*I and *Hin*dIII restriction sites. The resulting two constructs, pMIR-REPORT–wt *PDGFRa*-3′UTR and pMIR-REPORT-c.*51G>A were verified by sequencing. Using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and the pMIR-REPORT–wt *PDGFRa*-3′UTR as template, we successfully generated two constructs containing the c.*34G>A and c.*479C>A. The primers used for site-directed mutagenesis according to the manufacturer′s protocol were in Supplementary Table 1. All constructs were verified by direct sequencing.

COS7 cells were plated on 24-well cell culture plates at 3.5×10^5 cells per well in DMEM (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum and sodium pyruvate without antibiotics. 24 h after plating, cell transfection was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). In each well, 24 ng of pMIR-REPORT–wt *PDGFRa*-3′UTR and 76 ng of pRL-TK-control vector (Promega) were co-transfected in the presence of 20, 5, 1 or 0.2 pmol of has-miR-140 pre-miR precursor molecules or pre-miR Neg-1, which was used as a control (Ambion). Six replicates were performed for each treatment. The dual-luciferase reporter assay (Promega) was used according to the manufacturer′s recommendation. Twenty-four hours after transfection, cells in each well were lysed using 150 μl of 1X passive lysis buffer. 20 μl of the cell lysates were then used for the assay. Measurements were taken using the TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). The raw luciferase data were presented in Supplementary Table 2.

All data of the luciferase activity were calculated as relative luciferase activity. Statistical analyses were performed using the Mann–Whitney *U*-test.

RESULTS

Mutation analysis

The entire coding and the 708-bp regions of the 3′ UTR of *PDGFRa* were analyzed by PCR-sequencing in 102 unrelated Thai patients with CP. Seven DNA changes in *PDGFRa* were found in nine sporadic patients. Of these seven mutations, three were not found in 1000 control chromosomes (Table 1). Four variants were non-synonymous mutations in the coding regions, which were c.1202C>A (p.A401D), c.1420C>T (p.T474M), c.1631T>C (p.V544A), and c.3155C>T (p.T1052M) (Figure 1a). The c.1202C>A (p.A401D) mutation was found in 3 of 102 patients with CP and 1 of 500 unaffected control individuals. These three sporadic cases were not related to one another. The c.1420C>T (p.T474M), which was identified in 1 of 102 cases with CP was also detected in 1 of 500 unaffected controls. Although three of the identified mutations changed the amino-acid polarity (Table 1), they were all predicted to be tolerated by the SIFT algorithm (http://sift.jcvi.org/). The multiple sequence alignments revealed the V at 544 and the T at 1052 amino-acid residues were conserved (Figure 2a). Polyphen indicates that the c.1631T>C (p.V544A) and c.3155C>T (p.T1052M) are possibly damaging with scores of 0.704 and 0.977, respectively (http://genetics.bwh.harvard.edu/pph2/index.shtml). Other DNA changes are summarized in Supplementary Table 3.

Three sequence variants were identified in the noncoding 3′ UTR: c.*34 G>A, c.*51 G>A, and c.*479C>A (Figure 1b). The c.*34G>A was not found in 500 controls and was conserved in primates (Figure 2b). All three variants were within or close to the predicted binding sites for the human miR-140 (Figures 3a and b).

Luciferase activity

Testing the effect of miR-140, it significantly reduced the luciferase activity of the pMIR-REPORT–wt *PDGFRa* -3′UTR when compared with that of the control miR (Figure 3d). In addition, we found that, with the presence of miR-140, the relative luciferase activity of the construct with the c.*34G>A mutation was significantly lower than that of the wild type ($P = 0.014$) (Figure 3e). The relative luciferase activities of the constructs with the other two variants were however not different from that of the wild type (Figures 3f and g).

Table 1 Identified *PDGFRa* mutations in our CP patients and their characteristics

Mutation	*c.1202C>A*	*c.1420C>T*	*c.1631T>C*	*c.3155C>T*	*c.*34G>A*	*c.*51G>A*	*c.*479C>A*
Nucleotide position	Exon 8	Exon 10	Exon 11	Exon 23	3′UTR	3′UTR	3′UTR
Protein	A401D	T474M	V544A	T1052M	NA	NA	NA
Polarity changes	Nonpolar>Polar	Polar>Nonpolar	Nonpolar>Nonpolar	Polar>Nonpolar	NA	NA	NA
Parental genotypes (father/mother)	NA	NA	NA	NA	GG/GA	GG/GA	CC/CC (*de novo*)
Evolutional conservation among primates	No	Yes	Yes	Yes	Yes	No	No
Case (chromosomes)	3/204	1/204	1/204	1/204	1/204	1/204	1/204
Control (chromosomes)	1/1000	1/1000	0/1000	0/1000	0/1000	1/1000	2/1000
P-value	0.002	0.21	0.02	0.02	0.02	0.21	0.45
PolyPhen (scoring)	Benign (0.000)	Benign (0.001)	Possibly damaging (0.704)	Possibly damaging (0.977)	NA	NA	NA
Position relative to the miR-140 binding sites[a]	NA	NA	NA	NA	−10	within	−9
miR-140's concentration decreasing luciferase activity (pmol)	NA	NA	NA	NA	0.2, 1, 5, 20	none	0.2

Abbreviations: CP, isolated cleft palate; NA, not available/not applicable; PDGFRa: platelet-derived growth factor receptor alpha.
[a]see figure 3.

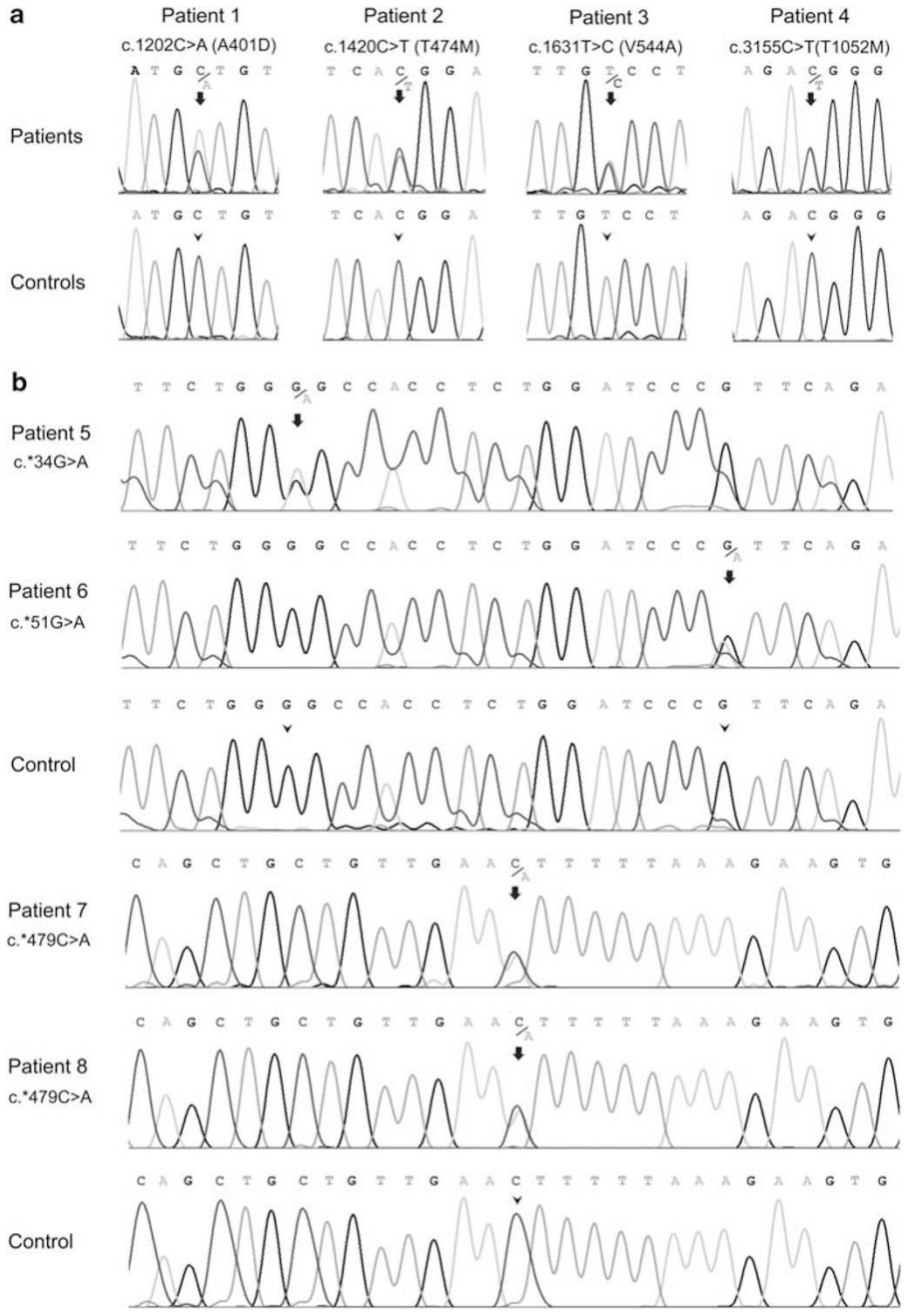

Figure 1 Mutation analysis. (**a**) The top row shows electropherograms of patients in coding regions, showing c.1202C>A (p.A401D), c.1420C>T (p.T474M), c.1631T>C (p.V544A), and c.3155C>T (p.T1052M) (arrows) in patients 1, 2, 3, and 4, respectively, and the lower row shows electropherograms of controls. (**b**) Electropherograms of patients in the 3′UTR, showing c.*34G>A, c.*51G>A, and c.*479C>A (arrows) in patients 5, 6, 7, and 8, respectively, and controls showing normal genotypes.

DISCUSSION

This study is the first to investigate the etiologic role of the *PDGFRa* gene in human CP. Seven novel single base-pair substitutions in the *PDGFRa* gene were identified in 9/102 patients with CP (8.8%), compared with 5/500 ethnic-matched unaffected controls (1%) (two-tailed *P*-value$<$0.0001). There were four and three missense mutations detected in the coding regions and in the 3′UTR, respectively. Comparing the frequency of all identified variants between individuals with CP and the unaffected controls, four of them (three in coding, one in 3′UTR) had significantly higher frequency in cases than in the unaffected controls (*P*-value$<$0.05). Functional analysis by the luciferase assay revealed that the c.*34G>A, the variant found in the 3′UTR, could significantly repress luciferase activity compared with that of the wild type in the presence of *miR-140*.

Similar to other Asian populations, the Thai population has a relatively high prevalence of isolated oral clefts. We previously found that *TBX22* mutations were a frequent cause of non-syndromic cleft palate in our population. However, they still contribute to a small

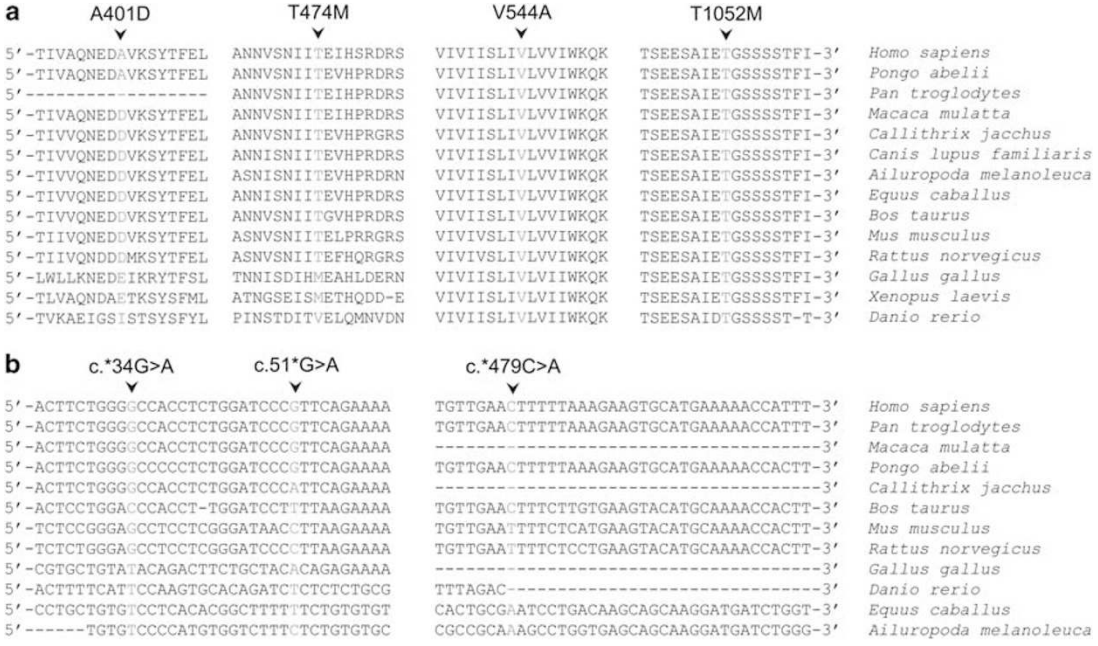

Figure 2 Conservation of PDGFRa. Sequence alignment of human PDGFRa and other orthologs in (**a**) the coding regions and (**b**) the 3′ UTR.

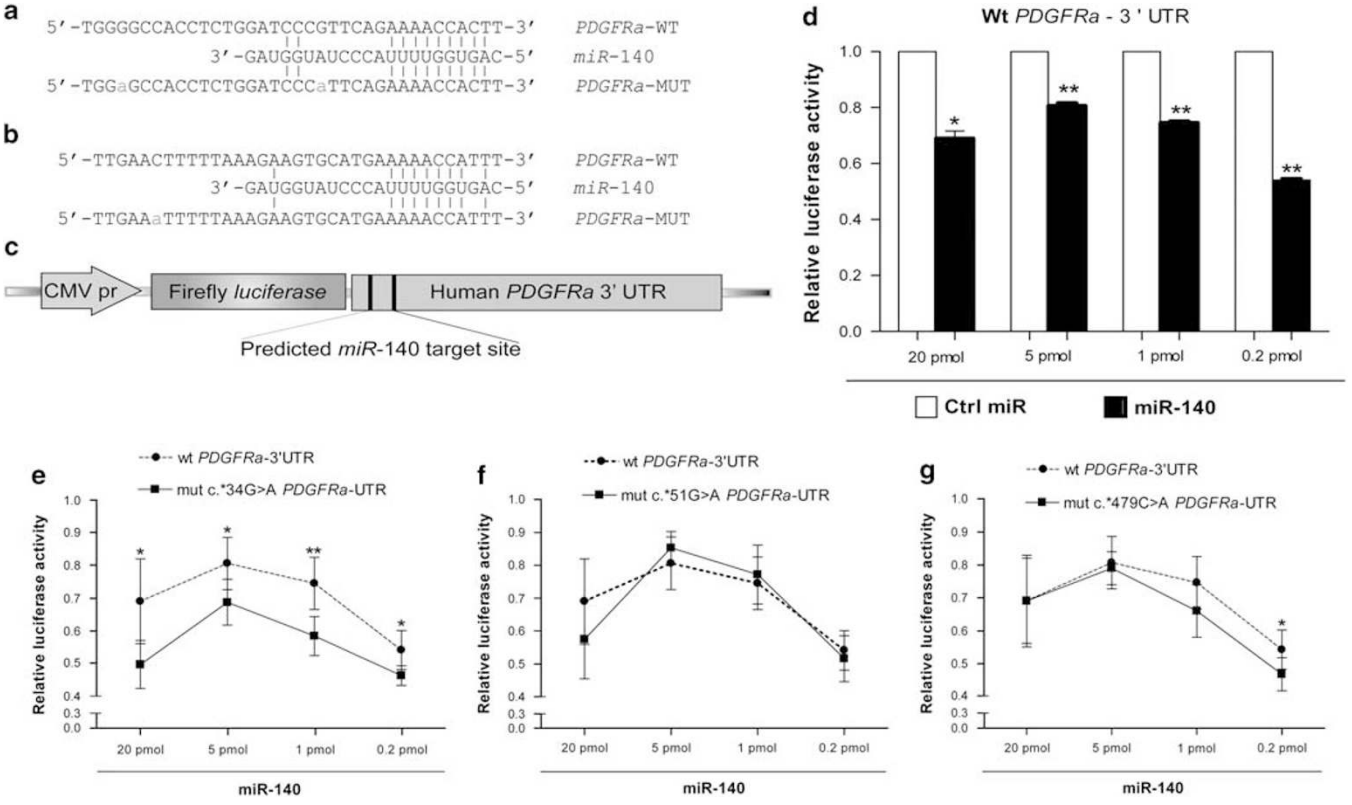

Figure 3 Characterization and functional analysis of the *PDGFRa* 3′UTR. (**a**) The c.*34G>A and c.*51G>A are located near and within the first predicted miRNA binding site for miR-140, respectively. (**b**) The c.*479C>A is located close to the second predicted miRNA binding site for miR-140. (**c**) The pMIR-REPORT-*PDGFRa*-3′UTR contains the CMV promoter, the *firefly luciferase* gene and the 708-bp fragment containing *PDGFRa* 3′UTR with two predicted binding sites for miR-140. (**d**) Relative luciferase activity of the pMIR-REPORT–wt *PDGFRa*-3′UTR that was co-expressed with different amounts of miR-140 (indicated in black) or negative control miRNA (indicated in white) in COS7 cells. Each experiment was repeated six times for each of the four different concentrations of miRNA. $^{**}P < 0.01$ and $^{*}P < 0.05$ (Mann–Whitney *U*-test). (**e**–**g**) Relative luciferase activity in the presence of miR-140 is shown for the wild-type (wt) *PDGFRa* 3′UTR (dashed line) and the mutant (mut) *PDGFRa* 3′UTR, containing the c.*34G>A, c.*51G>A, and c.*479C>A (solid lines), respectively.

fraction, approximately 7% of patients.[13,14] To better understand the genetic factors contributing to CP, more susceptible genes are needed to be identified.

PDGFRa was previously suggested to have a role in diaphragmatic hernia[15] and total anomalous pulmonary venous return (TAPVR). [16] A recently published article showed that disruption in Pdgf signaling in zebrafish caused craniofacial anomalies. *Pdgfra* mutations and Mirn 140-injected embryos shared a range of facial defects, including cleft palate.[6] A recent report suggests that the single-nucleotide polymorphisms (SNP) region in pre-miR-140 contributes to cleft palate susceptibility by influencing the processing of miR-140 in human PDGFRa.[17] In addition, a subsequent publication has revealed possible synergistic interaction between infants with CA/AA at rs7205289 located in the microRNA (miR)-*140* and maternal passive smoking in contributing to cleft palate risk.[18] We therefore investigated whether *PDGFRa* and *miR-140* had a role in human CP. Seven different mutations in *PDGFRa* were identified. None were found in patients with *TBX22* mutations. Of the four missense mutations identified in the coding region, the c.1202C>A (p.A401D) and the c.1420C>T (p.T474M) were also detected in unaffected controls. It is probable that both are SNPs, and therefore common variants present in a small percentage of the general population. The c.1631T>C (p.V544A) and c.3155C>T (p.T1052M) were located at well-conserved residues. The c.*34G>A was identified in one CP case. This position is conserved in primates and located 10 bp away from a predicted binding site for the *miR-140*. Luciferase assay revealed that, in the presence of miR-140, the c.*34G>A variant significantly repressed luciferase activity compared with that of the wild type. These findings suggest the functional significance of this variant.

Several studies have demonstrated that the pathogenic DNA variants in the 3′UTR do not need to reside within the miRNA binding sites.[19–21] Variants near binding sites were previously proved to be pathogenic. The c.*829C>T, a naturally occurring SNP, near the miR-24 binding site in the 3′UTR of human dihydrofolate reductase (*DHFR*) affects DHFR expression by interfering with miR-24 function, resulting in DHFR overexpression and methotrexate resistance.[19]

PDGFRa is ubiquitously expressed (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.74615); therefore, it is possible that mutations in its coding regions may affect several organs. However, miR-140 was expressed only at palate.[22] Therefore, mutations in the 3′UTR of *PDGFRa* increasing the binding affinity of the miR-140 would decrease *PDGFRa*′s mRNA. For the first time, we provided evidence supporting a role of *PDGFRa* in human palatal development.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on European Journal of Human Genetics website (http://www.nature.com/ejhg)