การวิเคราะห์การกลายพันธุ์และผลของการกลายพันธุ์ของยีน *SUMO1 PDGFRa* และ *miR-140* ในผู้ป่วยปากแหว่งเพดานโหว่ที่ไม่เกิดร่วมกับกลุ่มอาการ

นางสาวสาวิตรี รัตนโสภา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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MUTATION AND FUNCTIONAL ANALYSIS OF *SUMO1, PDGFRa* AND *miR-140* GENES IN NON-SYNDROMIC ORAL CLEFTS

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A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	MUTATION AND FUNCTIONAL ANALYSIS OF *SUMO1, PDGFRa* AND *miR-140* GENES IN NON-SYNDROMIC ORAL CLEFTS.
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สาวิตรี รัตนโสภา : การวิเคราะห์การกลายพันธุ์และผลของการกลายพันธุ์ของยีน *SUMO1, PDGFRa* และ *miR-140* ในผู้ป่วยปากแหว่งเพดานโหว่ที่ไม่เกิดร่วมกับกลุ่มอาการ (MUTATION AND FUNCTIONAL ANALYSIS OF *SUMO1, PDGFRa* AND *miR-140* GENES IN NON-SYNDROMIC ORAL CLEFTS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ศ.นพ.วรศักดิ์ โชติเลอศักดิ์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.พญ.ดร.กัญญา ศุภปีติพร,75 หน้า.

การเกิดปากแหว่งเพดานโหว่ชนิดที่ไม่เป็นกลุ่มอาการ มีความเกี่ยวข้องทั้งปัจจัยทางพันธุกรรมและปัจจัยทางสิ่งแวดล้อม การศึกษาก่อนหน้านี้ทั้งจากผู้ป่วยที่มี balanced chromosomal translocation และการศึกษาในสัตว์ทดลองพบว่ายีน *SUMO1 PDGFRa* และ miR 140 อาจเกี่ยวข้องกับโรคเพดานโหว่ แต่ยังไม่มีหลักฐานชัดเจน ในการทดลองนี้จึงทำการศึกษายีน *SUMO1, PDGFRa* และ *miR-140* ในคนไข้ไทยที่เป็นโรคปากแหว่งเพดานโหว่โดยใช้วิธีทำพีซีอาร์แล้วศึกษาลำดับเบส ซึ่งพบว่าในคนไข้ไทยที่เป็นปากแหว่งเพดานโหว่ไม่มีการกลายพันธุ์ในยีน *SUMO1* และ *miR-140* แต่ ในยีน *PDGFRa* พบการกลายพันธุ์ในตำแหน่งที่มีการจับกับ *miR-140* และรอบๆตำแหน่งที่จับอีกสองตำแหน่ง ซึ่งการกลายพันธุ์ตำแหน่งดังกล่าวไม่มีการรายงานมาก่อนหน้านี้ในกลุ่มประชากร ความถี่ของการกลายพันธุ์ทั้งสามระหว่างกลุ่มผู้ป่วยและกลุ่มควบคุมมีแนวโน้มที่จะแตกต่างกัน การศึกษาผลของการกลายพันธุ์ทั้งสามตำแหน่งต่อการควบคุมการการทำงานของยีน *PDGFRa* โดย *miR-140* โดยวิธี luciferase reporter system พบว่ามีเพียงตำแหน่งเดียวคือ c.*34G>A ที่มีผลในการลดการแสดงออกของ luciferase อย่างมีนัยสำคัญทางสถิติ ยีน *PDGFRa* อาจจะเป็นยีนอีกยีนหนึ่งที่มีความสำคัญต่อการเจริญพัฒนาของปากและเพดานปาก และการกลายพันธุ์ในยีน *PDGFRa* เป็นสาเหตุอีกสาเหตุหนึ่งของการเกิดโรคปากแหว่งเพดานโหว่ชนิดที่ไม่เป็นกลุ่มอาการในมนุษย์

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The *SUMO1*, *PDGFRa* and *miR-140* have been proposed as genes in which mutations may contribute to non-syndromic cleft lip and/or cleft palate. Strong evidence supporting the significant roles of these genes comes from a chromosomal translocation causing gene disruption resulting in haploinsufficiency, a cleft phenotype in a knockout mouse experiment and an expression study during palatal development. Mutation screening of the *SUMO1*, *PDGFRa* and *miR-140* genes was carried out in 227 Thai patients, 123 with cleft lip and/or palate and 104 with cleft palate only. Even though there were no potential pathogenic mutations identified in the *SUMO1* and *miR-140* genes, there were 3 potential pathogenic mutations identified in the 3' untranslated region (UTR) of the *PDGFRa*; namely c.*34G>A, c.*51G>A and c.*480C>A. They are all within or close to the predicted binding sites for the human microRNA, *miR-140*. Their frequencies between cases and controls are significantly or have a tendency to be different. Functional studies of these three variants by luciferase assay revealed that, in the presence of miR-140, the c.*34G>A variant significantly repressed luciferase compared to that of the wild type. This strongly suggests the functional significance of this variant. For the first time, we showed strong evidences that *PDGFRa* mutations cause human oral clefts.

Field of Study: Medical Science

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LIST OF ABBREVIATIONS

CLP	=	Cleft lip with or without cleft palate
CL/P	=	Cleft lip and/or palate
CP	=	Cleft palate
OR	=	Odd ratio
95%CI	=	95% Confidence Interval
HWE	=	Hardy-Weinberg equilibrium

CHAPTER I
INTRODUCTION

Background and Rationale

The most common craniofacial malformation in the newborn is the orofacial cleft, consisting of cleft lip with or without cleft palate and isolated cleft palate. Patients who have cleft lip or palate face significant lifelong communicative and aesthetic challenges, and difficulties with deglutition.

Epidemiology

The overall incidence of orofacial clefting is typically as 1 in 700 live births[1]. Cleft lip with or without cleft palate (CL/P), is an epidemiologically and etiologically distinct entity from isolated cleft palate (CP)[2]. Cleft lip is associated with cleft palate in 68% to 86% of cases[3]. The incidence of CL/P varies significantly by racial group and socioeconomic status, with an incidence of 1 in 1,000 births in whites, 1 in 500 births in Asians and Native Americans, and approximately 1 in 2,400 to 2,500 births in people of African descent[4]. The incidence of CP does not have the same ethnic heterogeneity and is typically quoted as 1 in 1,500 to 2,000 live births[1, 3].

Etiological Factors of Oral Clefts

Following these general rules, clefts are most often discussed as either those that involve the lip with or without the palate (CL/P) or those that involve the palate only (CPO). In addition, clefts can be divided into nonsyndromic and syndromic forms. In nonsyndromic clefts, affected individuals have no other physical or developmental anomalies. Most studies suggest that about 70% of cases of CL/P and 50% of CPO are nonsyndromic[2]. The syndromic cases can be subdivided into chromosomal syndromes, more than 350 Mendelian disorders (Online Mendelian Inheritance in Man, 2002), disorders caused by teratogens (e.g. phenytoin or alcohol) and uncategorized syndromes. The complex etiology of clefting to identify genes and gene–environment interactions and to learn more about human embryology and its disturbances[6]. Advances in both quantitative and molecular analysis make linkage and association

approaches to CL/P etiology practical[7]. Studies in animal models can provide genes and loci for studies in humans and animal models can be used to look at gene–gene and gene–environment interaction. Dense genetic maps [8] provide resources for family-based studies. Studies of twins have been particularly informative regarding the genetics of clefting. Concordance in monozygotic (MZ) twins ranges between 40% and 60%, and is 5% in dizygotic twins. The lack of 100% concordance in monozygotic twins suggests that genetic events alone are not responsible for the clefting phenotype.

Hypothesis

CL/P represents a significant public health problem due to the significant life-long morbidity and complex etiology of these disorders. The extensive psychological outcomes emphasize the importance of understanding the underlying causes of CL/P to optimize treatment planning, to predict the long-term course of any affected individual's development, to improve recurrence risk estimation, and to provide pre-reproductive counseling. Furthermore, a better understanding of the embryology and genetics of orofacial clefting is crucial for the development of a biologically relevant orofacial cleft classification system.

In this work, we studied patients with nonsyndromic CL/P by the concept that mutations in a single gene can contribute to a spectrum of phenotypes. The potential candidate genes for CL/P selected from animal model palatogenic genes included the *PDGFRa*, *miR-140* [9, 10, 11]and *SUMO1* genes [12].

Research Questions

1. Do patients with non-syndromic oral clefts have intragenic mutations in the *SUMO1, PDGFRa* and *miR-140*?
2. Are novel DNA variants found in patients with non-syndromic oral clefts pathogenic?

Objectives

1. To identify mutations in the *SUMO1, PDGFRa* and *miR-140* in individualswith clinically diagnosed non-syndromic oral clefts.

2. To determine the pathogenic roles of novel DNA variants of the *SUMO1, PDGFRa* and *miR-140* found in patients with oral clefts.

Hypothesis

Mutations in the *SUMO1, PDGFRa* and *miR-140* genes can cause oral clefts in humans.

Assumption

Target population; Thai patients with clinically diagnosed oral clefts

Research Design

Descriptive and *in vitro* studies.

Limitation

Some samples obtained from the patients could not be completely analyzed due to poor quality or insufficient quantity of DNA.

Expected Benefit

Identification of additional candidate genes for non-syndromic oral clefts in humans and further characterization of their pathogenic mutations will provide more understanding of the underlying disease mechanism leading to more effective treatment and prevention strategies.

Ethical Consideration

This study has been approved by the local Ethics Committee. Written informed consent was obtained from all patients or their parents who participated in the study.

Research Methodology

1. Sample collection

The subjects of this study were 123 sporadic cases of cleft lip with/or without cleft palate (CL/P) and 104 patients with nonsyndromic cleft palate only. The subjects were recruited between 1999 and 2008 from 14 medical centers throughout Thailand. For sample collection in remote areas, the FTA filter paper was used. The control samples for mutation screening were Thai blood donors with no oral clefts, who denied history of oral clefts in other family members.

2. Study process

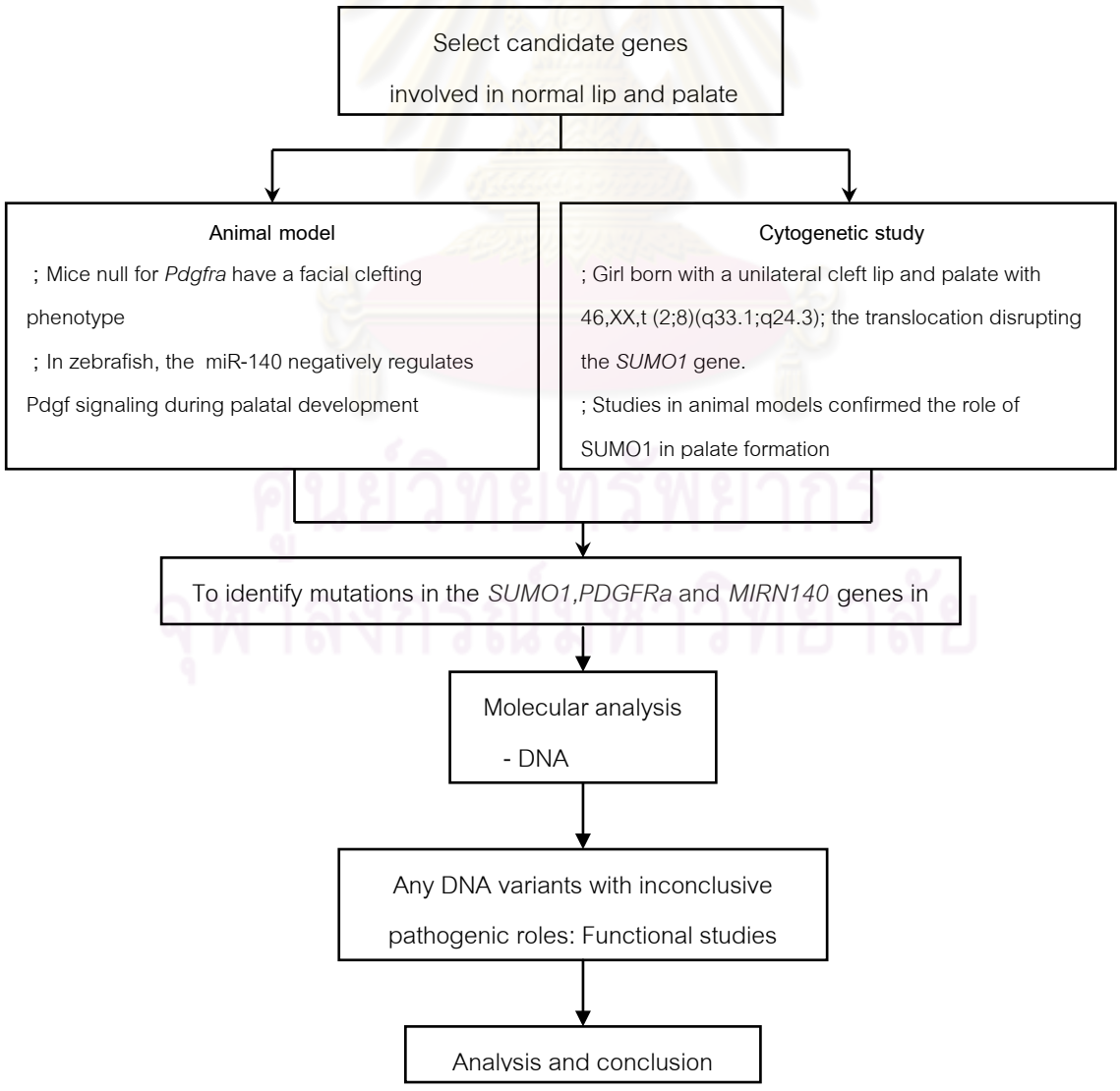
2.1 Blood collection

2.2 Mutation analysis

2.3 Functional analysis by luciferase reporter system

3. Data collection and analysis

Conceptual Framework



CHAPTER II

BACKGROUND AND LITERATURE REVIEWS

Oral clefts as a model system

Development of the primary palate leads to formation of the upper lip and the maxillary dental alveolus[13]. In humans, primary palate development initiates at approximately day 28 (ED9.5 in mice) through an invagination of the nasal placodes. This invagination deepens to form the medial and lateral nasal processes, which continue to proliferate and expand. Also at this time, the bilateral maxillary processes, which are derived from neural crest cells that have migrated to the first branchial arch mesenchyme, grow forward to meet the lateral surface of the medial nasal processes. Shortly thereafter, the lateral nasal processes fuse with the medial nasal processes so that by the sixth to seventh week (ED11 in mice), the primary palate is complete. The epithelial layer at the points of fusion is replaced by mesenchymal tissue. The secondary palate, which has been investigated more exhaustively than the primary palate, develops later and forms the roof of the mouth and the nasal floor[14].The secondary palate is first evident at approximately six weeks as two vertical maxillary processes along the lateral edges of the tongue. At about nine weeks in humans (ED14.5 in mice) the shelves approximate each other and fuse by week 12 (ED15 in mice). Since the primary palate develops prior to the secondary palate, improper fusion of the primary palate, leading to a cleft lip, often causes the shelves of the secondary palate to be malpositioned such that palatal shelf contact and fusion may not occur. Hence, cleft lip can occur with or without cleft palate (CL/P), while clefts of the secondary palate can occur as an isolated defect (CP).

Birth Defects Genetics

From a genetic perspective, the different inheritance patterns in CL/P and CP could be due to mutations in different genes (genetic heterogeneity) or different mutations in the same gene (allelic heterogeneity). However, given that similar

developmental processes occur during the both primary and secondary palatogenesis, it is not unexpected that some genes may play a role in both types of clefting.
Finally, CP may also be caused by mechanisms that interfere with palatal shelf elevation, such as micrognathia. These factors add to the genetic heterogeneity for CP and are unlikely to be causal for CL/P. Most human researches in orofacial clefting etiology have focused on CL/P. Isolated CL/P has no clearly recognizable mode of inheritance. Instead, complex inheritance patterns exist, as evidenced by a positive family history for clefting in 33% of the patients, and reduced penetrance[15] . The relative risk for siblings, defined as the prevalence of clefting in siblings relative to the population prevalence, is 40 for CL/P in Caucasian populations. In addition, there is an increased risk for children of individuals with CL/P and a greater concordance for CL/P in monozygotic than dizygotic twins. All of these observations indicate that genetic factors play a major etiologic role for CL/P[16]. Segregation analyses have been unable to conclusively define the mode of inheritance for clefting. These studies have suggested inheritance patterns for CL/P as being autosomal dominant, autosomal recessive, or the interaction of a major gene with other genetic and environmental factors[17]. Efforts to identify genes for isolated CL/P and CP have generally employed candidate gene strategies looking for linkage or linkage disequilibrium (LD). Candidate genes can be selected from those known to cause syndromic forms of clefting. Furthermore, mice are providing excellent data through expression studies or knockout/transgenic models with cleft phenotypes[18].

Genetic Approaches to Identify Disease Genes

Linkage

Linkage mapping looks for the co-segregation of alleles for genetic markers with a disease phenotype in a family. Chromosome recombination occurs during meiosis, resulting in crossing over of genetic material from one parental chromosome to the other parental chromosome. Recombination is correlated with distance, such that markers that are far apart will appear to be transmitted randomly, while markers close together will be co-transmitted or linked. Linkage is very powerful for single-gene disorders, assuming that the disease is homogeneous. Most cloned disease genes have

been identified using linkage approaches in a few large families with single-gene disorders. In heterogeneous diseases, meaning more than one gene may be sufficient to cause the disease, linkage may not be observed when several smaller families are used. This occurs when one family cancels another with a causal gene at another locus. The application of traditional parametric linkage analyses (the logarithmic odds [LOD] score method) to complex diseases such as CL/P or CP has been complicated by the underlying genetic heterogeneity and the inability to properly specify the genetic model. Despite these limitations, parametric linkage approaches have proven to be effective for complex traits and new powerful nonparametric analyses have been developed as complementary approaches. There has been considerable success in identifying etiologic genes for syndromic forms of birth defects including orofacial clefting. Specifically relevant to CL/P are syndromes involving clefts of the primary palate in which genes have been cloned.

Linkage Disequilibrium (LD)

Another genetic mapping approach is the measurement of LD between a marker and disease, using either a population of affected and control individuals or a collection of nuclear families. LD mapping is the identification of nonrandom correlations (associations) between alleles at two loci in a population[19]. The assumption is that affected individuals would have a shared ancestor on whose chromosomes the original mutation arose. This would result in shared alleles for markers near the mutation among affected individuals, while recombination, which has occurred throughout the entire history of the population, would result in random segregation of the regions outside of the disease locus. Thus, there should be decreasing LD over larger genetic distances, which is the limiting factor for LD mapping. LD has been shown to have greater power than linkage strategies when applied to complex diseases[20]. LD is ideally applied when a homogenous mutation exists, meaning that all affected individuals share the same mutation. This is a founder effect observed more commonly in isolated populations and may result in a high degree of homogeneity, such that if a disease mutation

arose earlier, most currently affected individuals would share the same genetic material over relatively large genetic distances near the disease mutation.

Single Nucleotide Polymorphisms(SNP)

Genetic studies require the ability to characterize DNA variation in patient samples. Genotyping of human DNA samples has improved dramatically in the 20 years since restriction length fragment polymorphisms (RLFPs) replaced protein variant testing. Short tandem repeat polymorphisms (STRPs) consisting of di-, tri-, and tetranucleotide repeats are currently the standard for genome-wide searches. The development of single nucleotide polymorphisms (SNPs) is now providing the opportunity to develop assays that can be functionally relevant and take advantage of the far larger set of polymorphic variants resulting from single nucleotide changes. With the advent of real time PCR and fluorescent tagging, high-throughput gene-specific genotyping is a reality. The identification of SNPs is ongoing, both through lab specific projects and genome-wide efforts, including the recently initiated HapMap project [21]. More than 5.0 million validated SNPs can be found in public databases (http://www.ncbi.nlm.nih. gov/SNP).

HapMap project

The use of LD is also constrained by concerns that as many as 1 million markers might be required to detect LD in a genome-wide approach, and the potential need for a high marker density may even create problems for studies of a targeted locus. A partial alleviation of the most severe predictions comes from recent data showing that SNPs cluster in haplotype blocks so that the observed number of haplotype arrangements arising from alleles at multiple loci is often far lower than the number theoretically possible. There remain substantial concerns about how useful haplotype blocks will be for constructing tagging SNPs given the known heterogeneity of blocks, which vary by ancestry, SNP density, polymorphism content, and confounding by large block-free regions. The HapMap project has already made available an impressive collection of genotyped SNPs and analytic software to facilitate haplotype construction for fine structure gene mapping (http://www.hapmap.org/)[22]

Breakpoint mapping.

Adventitious chromosomal anomalies also provide important clues for genes involved in birth defects including clefting. Chromosomal deletions and duplications can result in birth defects, and regions highly associated with an array of structural anomalies have been reported, including cleft lip or cleft palate[23]. Mapping of breakpoints in individuals with these defects can be used to find genes transected by or adjacent to the breaks that are then candidates for causing that birth defect. These genes will be especially attractive candidates when the phenotype includes a birth defect of interest and/or when a given birth defect is found in multiple members of a family segregating with the translocation. The *SATB2* gene at 2q32 is transected[24] and encodes a 733 amino acid DNA binding protein of remarkable conservation between human and mouse (only 3 of 733 amino acids differ) and the gene spanning 208 kb is confined to one large haplotype block. Furthermore, *SATB2* is highly expressed in the lip, as well as in the palate, making it an excellent candidate for contributing to isolated CL/P. Both of these genes/loci can now be investigated in more detail for the role they might play in clefting.

Candidate Genes

Three candidate genes were selected for studying their roles in oral clefts. These included *SUMO1, PDGFRa* and *miR-140.*

I. PLATELET-DERIVED GROWTH FACTOR RECEPTOR, ALPHA (PDGFRA)

Function

Platelet-derived growth factor (PDGF) and its receptor (PDGFR) are important regulators for tissue–tissue interactions to control cell migration, proliferation, survival, and deposition of extracellular matrix during mammalian embryonic development[25; 26; 27; 28; 29; 30]. Functionally, PDGF signaling is critical for regulating progenitor cell proliferation and cell migration during early embryonic development where it controls extracellular matrix formation, tissue remodeling, and patterning determination during later stages of embryogenesis. During craniofacial development, *PDGFRa* expression is specifically

associated with the cranial neural crest derived ectomesenchyme and may regulate the development of cranial neural crest (CNC) derivatives. The homozygous *Patch* mutant mice showed pleiotropic defects associated with cranial and trunk neural crest cells and produced significant first branchial arch deformities, including facial clefting, hemifacial microsomia, micrognathia, adontia, open neural tube, heart deformities, large interstitial "blebs," and were embryonic lethal by E12.5[29; 31; 32]. Loss of- function of *Pdgfra* signaling results in early embryonic lethality, incomplete cephalic closure similar to that seen in a subset of *Patch* mutants, and increased cell death in the CNC migration pathway.

Correlation between *PDGFRa* and CL/P

Conditional inactivation of the *Pdgfra* gene in the neural crest results in multiple craniofacial malformations, including cranial, skeletal and cartilage defects and mid-facial cleft suggesting that there is a cell autonomous requirement for PDGFRa signaling in the CNC cells[29; 33]. The *Pdgfra* gene is specifically expressed in the CNC-derived palatal mesenchyme and loss of *Pdgfra* results in complete cleft palate with 100% phenotype penetrance[34]. The successful fusion of the palatal shelves of the *Pdgfra* null mutant in the palatal organ culture model suggests that the midline epithelial cells are competent to mediate palatal fusion, despite the loss of PDGFRa signaling in the CNC-derived palatal mesenchyme, and there is a cell-autonomous requirement for PDGFRa signaling in the mesenchyme of the developing palatal shelf. In human clefting, failure of palatal fusion after proper palatal adhesion (such as the one associated with the transforming growth factor-α3 (*TGF-α 3*) null mutation[35] only represents a small percentage of the cleft palate cases, whereas failure of palatal shelf extension (such as the one in *Pdgfra* null or conditional mutant mice) is associated with the majority of cleft palate cases. Hence, the *Pdgfra* mutant mice will serve as an important animal model for the investigation of the molecular etiology of human cleft palate. It is important to note that there is a prominent midfacial cleft present in both the *Pdgfra* null and the neural crest specific conditional *Pdgfra* mutant mice[29; 33]. Midfacial cleft may alter the width of the developing embryonic head, resulting in failure of the head to lift up and may interfere with the descent of tongue to prevent normal palatal fusion. Future studies

using a palatal mesenchyme specific knockout animal models will be very informative to investigate the functional significance of PDGFRa signaling in regulating palatal fusion. Endogenous PDGFa and its cognate receptor PDGFRa regulate dental cusp development and extension of the palatal shelf during craniofacial development. This regulatory function may be carried out through the regulation of deposition and remodeling of extracellular matrix in the CNC-derived ectomesenchyme. Loss of function of PDGFRa signaling provides a model for investigating the molecular mechanism of dental cusp defect and cleft palate. Future studies using this animal model will provide useful information on the mechanism of PDGFRa signaling in both normal and abnormal human development. Genetic screening of the *Pdgfra* mutation among individuals with dental cusp defect and cleft palate may provide crucial information to understand the etiology of congenital malformation.

II. MICRO RNA140 (MIRN140)

Function

MicroRNAs (miRNAs) have emerged as a new class of gene expression regulators. MiRNAs are 20–24 nucleotide non-coding RNA molecules that post-transcriptionally regulate gene expression. They are generated from precursor RNA molecules with hairpin structure – the enzyme Dicer cleaves the partially double-stranded stem and releases the mature miRNA[5]. One of the mature miRNA strands forms an effector ribonucleoprotein complex termed RISC (RNA induced silencing complex) which guides the miRNAs to specific mRNAs[36]. RISC either cleaves or blocks translation of the target mRNA, depending on the degree of sequence complementarity. The majority of miRNA targets in animals are not cleaved but translationally suppressed due to the mismatches between miRNAs and target sites. The number of experimentally validated miRNA targets is growing but is still low[37] due to the lack of a generally applicable miRNA target identification protocol. Several computer programmes are available to predict targets for miRNAs[38] (http://microrna.sanger.ac.uk/targets/v2/).

Correlation between *PDGFRa, miR-140* and CL/P

Although crest cells must receive Pdgf signals for correct palatogenesis in mouse[34], the palatogenic cell behaviors regulated by Pdgf signaling and the modulation of Pdgf signaling during palatogenesis are unknown. MiRNAs provide an important mechanism for modulating signaling pathways[9, 10, 11, 39]. Skeletogenic, including palatal, precursors express mirn140 (miR-140) in teleosts[40]and amniotes[41, 42, 43] suggesting that Mirn140 may modulate signaling during palatogenesis across vertebrate species. Despite the function of miRNAs in development and the importance of neural crest in evolution and disease, no miRNA has, to our knowledge, yet been shown to regulate neural crest development or cellular behaviors. One important neural crest cell behavior is their migration along highly stereotyped pathways to give rise to a diverse array of differentiated cell types. Across vertebrate species, crest cells at cranial levels migrate in one of three crest streams. Cells in the most anterior, or first, stream migrate rostrally and caudally around the eye into the first pharyngeal arch and contribute to the jaw and palatal skeleton[44]. Although research in zebrafish and amniotes has uncovered cues that regulate migration of cranial neural crest cells in all crest streams[45, 46, 47], we lack knowledge of the cues that specifically guide neural crest–derived palatal precursors to the first pharyngeal arch. Eberhart *et al*[48] showed that Mirn140 attenuated Pdgf-mediated attraction during migration of neural crest–derived palatal precursors. Embryos injected with Mirn140 duplex and pdgfra mutants shared craniofacial phenotypes, including cleft palate and loss of oral ectoderm gene expression, suggesting an interaction between Mirn140 and pdgfra. Binding sites for Mirn140 were conserved in the 3' UTR of pdgfra across vertebrate species, and Mirn140 interacted with the 3' UTR of the pdgfra transcript to negatively regulate Pdgfra protein production. Palatal precursors expressed both mirn140 and pdgfra as they followed a migratory pathway delimited by expression of the ligand Pdgfaa. Attenuation of Pdgf signaling by Mirn140 was critical for rostrally migrating neural crests to migrate beyond the optic stalk, a Pdgfaa source, onward to the oral ectoderm, another Pdgfra source. These findings have demonstrated how delicately orchestrated modulation of Pdgf signaling regulates palatal morphogenesis.

III. SMALL UBIQUITIN-LIKE MODIFIER1 (SUMO1)

Function

The SUMO1 gene encodes a protein that is a member of the SUMO (small ubiquitin-like modifier) protein family. It functions in a manner similar to ubiquitin in that it is bound to target proteins as part of a post-translational modification system. However, unlike ubiquitin which targets proteins for degradation, this protein is involved in a variety of cellular processes, such as nuclear transport, transcriptional regulation, apoptosis, and protein stability. It is not active until the last four amino acids of the carboxy-terminus have been cleaved off. Several pseudogenes have been reported for this gene. Alternate transcriptional splice variants encoding different isoforms have been characterized. SUMO proteins, such as SUMO1, and ubiquitin modify numerous cellular proteins and affect their metabolism and function. However, unlike ubiquitination, which targets proteins for degradation, sumoylation participates in a number of cellular processes, such as nuclear transport, transcriptional
regulation, apoptosis, and protein stability[49]. Ubiquitin and SUMO compete for modification of proliferating cell nuclear antigen (PCNA), an essential processivity factor for DNA replication and repair[50].

Correlation between *SUMO1* and CL/P

Alkuraya et al. [12] Identified a girl born with a unilateral cleft lip and palate (primary and secondary) who was otherwise phenotypically normal. Her karyotype was 46,XX,t (2;8)(q33.1;q24.3). They showed that this balanced translocation disrupted the *SUMO1* gene. The authors found SUMO1 to be expressed on mouse embryonic day 13.5 in the upper lip, primary palate, and medial edge epithelia of the secondary palate. Additional studies in animal models confirmed the role of SUMO1 in palate formation. More recently in [51], it has demonstrated that SUMO1 is able to regulate MSX1. Genetic studies in both humans and mice have indicated that the Msx1 transcription factor is associated with specific disorders, including cleft palate andfound that TBX22 is a target for the small ubiquitinlike modifier SUMO-1[52]. This modification is required for TBX22 repressor

activity. Although the site of SUMO attachment at the lysine at position 63 is upstream of the T-box domain, loss of SUMO-1 modification is consistently found in all pathogenic CPX missense mutations. This implies a general mechanism linking the loss of SUMO conjugation to the loss of TBX22 function. Proteins encoded by three other genes *SATB2*, and *SMAD4*, are sumoylated and are either involved in or linked to pathways involved in palate morphogenesis[53, 54, 55, 56]. This suggests that SUMO1 might control the activity of a repertoire of downstream effectors involved in palatogenesis, accounting for the sensitivity of palatal development to SUMO1 gene dosage.

CHAPTER III

Materials and Methods

Research Instruments

1. Pipette tip : 10 µl, 100 µl, 200 µl, 1,000 µl (Elkay, USA)
2. Microcentrifuge tube : 0.2 ml, 0.5 ml, 1.5 ml (Bio-RAD, Elkay, USA)
3. Polypropylene conical tube : 15 ml (Elkay, USA)
4. Beaker : 50 ml, 100 ml, 200ml, 500 ml, 1,000 ml (Pyrex)
5. Flask : 250 ml, 500 ml, 1,000 ml (Pyrex)
6. Reagent bottle : 100 ml, 250 ml, 500 ml, 1,000 ml (Duran, USA)
7. Cylinder : 25 ml, 50 ml, 100 ml, 250 ml, 500 ml, 1,000 ml (Witeg, Germany)
8. Glass pipette : 5 ml, 10 ml (Witeg, Germany)
9. Pipette rack (Autopack, USA)
10. Thermometer (Precision, Germany)
11. Parafilm (American National Can, USA)
12. Plastic wrap
13. Stirring-magnetic bar
14. Combs
15. Automatic adjustable micropipette : P2 (0.1-2 µl), P10 (0.5-10 µl),
16. P20 (5-20 µl), P100 (20-100 µl), P1000 (0.1-1 ml) (Gilson, France)
17. Pipette boy (Tecnomara, Switzerland)
18. Vortex (Scientific Industry, USA)
19. pH meter (Eutech Cybernatics)

20. Stirring hot plate (Bamstead/Thermolyne, USA)
21. Balance (Precisa, Switzerland)
22. Centrifuge (J.P.Selecta, Span)
23. Microcentrifuge (Eppendorf, Germany)
24. Mastercycler personal (Eppendorf, Germany)
25. Thermal cycler (Touch Down, Hybraid USA)
26. Power supply model 250 (Gibco BRL, Scothland)
27. Power poc 3000 (Bio-RAD)
28. Horizon 11-14 (Gibco BRL, Scothland)
29. Sequi-gen sequencing cell (Bio-RAD)
30. Heat block (Bockel)
31. Incubator (Memmert)
32. Thermostat shaking-water bath (Heto, Denmark)
33. Spectronic spectrophotometers (Genesys5, Milon Roy USA)
34. UV Transilluminator (Fotodyne USA)
35. UV-absorbing face shield (Spectronic, USA)
36. Gel doc 1000 (Bio-RAD)
37. Refrigerator 4 ^{0}C (Misubishi, Japan)
38. Deep freeze -20 ^{0}C, -80 ^{0}C (Revco)
39. Water purification equipment (Water pro Ps, Labconco USA)
40. Water bath (J.P.Selecta, Span)
41. Storm 840 and ImageQuaNT solfware (Molecular dynamics)
42. 12-well culture plates (Corning, New York)
43. T-25 and T-75 Flasks (Corning, New York)

44. Costar® Stirpipette®: 0.2 ml, 10 ml, 25 ml (Corning, New York)
45. Haematocytometer counting chamber
46. Petridish (Sterilin limited, UK)
47. Cryotube vial 2.0 ml (Corning, New York)
48. Cryo 1°C Freezing container (Nalgene® Labware)
49. GloMax. 20/20 Luminometer (Promega)

Reagents

1. General reagents

1. Absolute ethanol (Merck)
2. Agarose, molecular glade (Promega)
3. Ammonium acetate (Merck)
4. Boric acid (Merck)
5. Bromphenol blue (Pharmacia)
6. Disodium ethylenediamine tetracetic acid : EDTA (Merck)
7. Ethidium bromide (Gibco BRL)
8. Ficoll 400 (Pharmacia)
9. Hydrochloric acid (Merck)
10. Mineral oil (Sigma)
11. Phenol (Sigma)
12. Chloroform (Merck)
13. Isoamyl alcohol (Merck)
14. Sodium chloride (Merck)
15. Sodium dodecyl sulfate (Sigma)
16. Sodium hydroxide (Merck)

17. Sucrose (BDH)
18. Tris base (USB)
19. Triton X-100 (Pharmacia)
20. 100 base pair DNA ladder (Biolabs)
21. 40%acrylamide/bis solution 19:1 (Bio-RAD)
22. GelStar (Camberx)
23. TE buffer (10mM Tris-HCl, 0.1 mM EDTA, pH 8.0)
24. FTA purification

2. PCR reagents

1. 10X PCR buffer (500 mM KCl, 200 mM Tris-HCl pH 8.4) (Promega)
2. 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl pH 8.8, 0.8% Nonidet P40) (Fermentas)
3. Magnesium chloride (Promega)
4. Magnesium chloride (Fermentas)
5. Deoxynucleotide triphosphates (dNTPs) (Promega)
6. Deoxynucleotide triphosphates (dNTPs) (Fermentas)
7. Oligonucleotide primers (BSU)
8. Oligonucleotide primers (Biogenomed)
9. *Taq* DNA polymerase (Promega)
10. *Taq* DNA polymerase (Fermentas)
11. 100% DMSO
12. Genomic DNA sample

3. Restriction enzymes

1. *BamH*I (Biolabs)

2. *EcoR*I (Biolabs)
3. *Bgl*II (Biolabs)
4. *Taq*$^{\alpha}$I(Biolabs)
5. *Btg*I(Biolabs)
6. *Apo*I(Biolabs)
7. *Sau96*I(Biolabs)
8. *Sac*I(Biolabs)
9. *Hind*III(Biolabs)
10. *HpyCH4*IV(Biolabs)
11. *BsaJ*I(Biolabs)
12. *Nla*III(Biolabs)
13. *EcoR*V(Biolabs)
14. *Bst*XI(Biolabs)

4. Bacterial culture media

1. Yeast extract powder (Bio Basic Inc.)
2. Agar bacterial powder (Conda, Spain)
3. Tryptone powder (Bio Basic Inc., Canada)
4. Sodium chloride (BDH AnalaR®, Merk group)

5. Cell culture

1. Dulbecco's Modified Eagle's Medium (DMEM) (Hyclone)
2. Fetal Bovine Serum (FBS) (GIBCO, Invitrogen)
3. PenStrep (GIBCO, Invitrogen)
4. Trypsin-EDTA (GIBCO, Invitrogen)
5. Phosphate-buffered saline (PBS)

6. Charcoal-stripped FBS (Hyclone)

7. 3, 3', 5-triiodothyronine (Sigma)

8. Tryphan blue

6 Transfection reagents

1. Lipofectamine™ 2000 (Invitrogen)

2. Opti-MEMI Reduced Serum Medium (GIBCO, Invitrogen)

7. Dual-luciferase reporter assay (Promega)

Experimental Procedure

2. Subjects and sample collection

Oral cleft sample: 123 probands with cleft lip with/or without cleft palate (CL/P) and 104 cases with cleft palate only were studied under the auspices of the Thai Red Cross, a national charity organization devoted to providing clinical care for the poor. Subjects were recruited between 1999 and 2008 from 13 medical centers throughout Thailand. Kalasin, Nakhonrachsima, Mahasarakham, Meahongson, Nan, Sakaew, Trang, Uthaithanee, Chaiyapoom, NongKhai, Yasothon, Leoy, Kumpakpetch.

And in this report, we use FTA filter paper as a method for blood sample collection and subsequent mutation analysis in remote areas where obtaining sample is more difficult.

Control sample: Controls for mutation screening and SNP were Thai blood donors with no oral cleft, who denied history of oral clefts in the other family members.

2. Genetic analysis

2.1 DNA extraction

After informed consent, genomic DNA was isolated from peripheral blood leukocytes. This procedure was performed as follows:

Preparation of DNA sample from peripheral blood leukocytes

1. 3 ml. of whole blood were centrifuged for 10 minutes at 3,000 rpm.
2. Remove supernatant and transfer buffy coat to a new polypropylene tube. Then add 10 volumes of cold lysis buffer 1 (or 10 ml.), mix thoroughly and incubate at –20°C for 5 minutes.
3. Centrifuge for 8 minutes at 1,000 g, and remove supernatant.
4. Add 3 ml. of cold lysis buffer 1, mix thoroughly and centrifuge for 8 minutes at 1,000 g.
5. Discard supernatant and add 900 µl of lysis buffer 2, 10 µl of proteinase K solution (20 mg of proteinase K in 1.0 ml. of 1% SDS-2 mM EDTA), and 50µl of 10% SDS. Mix vigorously for 15 seconds.
6. Incubate the tube(s) in a 37°C shaking waterbath overnight for complete digestion.
7. Add 1 ml. of phenol-chloroform-isoamyl alcohol and shake vigorously for 15 seconds and centrifuge at 6,000 rpm for 5 minutes.
9. Transfer the supernatant from each tube (containing DNA) to a new microcentrifuge tube.
10. Add 0.5 volume of 7.5 M CH_3COONH_4 and 1 volume of 100% ethanol and mix by inversion. The DNA should immediately form a stringy precipitate. Recover the DNA by centrifugation at 14,000 rpm for 15 minutes. Then remove supernatant.
11. Rinse the pellet with 70% ethanol. Decant the ethanol and air-dry the pellet. (It is important to rinse well to remove any residual salt and phenol.)
12. Resuspend the DNA in 20-300 µl of the double distilled water at 37°C until dissolved.
13. Calculation of DNA concentration, the reading at 260 nm is used for

calculating the concentration of nucleic acid of the samples. An OD of 1 corresponds to approximately 50 µl/ml for double-strand DNA. Therefore DNA concentration is calculated from the following

DNA concentration = ODx50xdilution ratio (µg/ml)

FTA Cards for DNA extraction from blood samples

Application of blood samples

1. Label the FTA card with appropriate sample identification.

2. Drop the blood in concentric circular motion (avoid puddling of the liquid sample; do not rub or smear the blood onto the card).

3. Allow the samples to dry at room temperature.

4. Dried blood spots will appear darker than freshly spotted ones.

Preparation of DNA sample from FTA card

1. Take a sample disc from the desired sample spot using a coring device(puncher).

2. Place sample disc in a PCR amplification tube.

3. Add 200 µl of FTA purification Reagent to the PCR tube.

4. Incubate for 5 minutes at room temperature (moderate manual mixing may be done if desired).

5. Remove and discard all spent FTA purification reagent using a pipette (Do not remove the sample disc).

6. Repeat steps 3-4 twice for a total of 3 washes with FTA Purification Reagent.

7. Add 200µl of TE buffer (10mM Tris-HCl, 0.1 mM EDTA, pH 8.0) to PCR tube.

8. Incubate for 5 minutes at room temperature.

9. Remove and discard all spent TE buffer with a pipette.

10. Repeat steps 7-9 for a total of 2 washes with TE buffer.

11. Allow disc to dry at room temperature for 1 hour or heat assist the disc at 56 ^{o}C for 10 minutes.

* The FTA disc is now ready for DNA amplification reaction

2.2 Polymerase Chain Reaction (PCR) analysis

I. PCR for the *SUMO1* gene

The primers were designed within introns to allow genomic amplification and sequencing of exons 1-5 including exon-intron boundaries as shown in Table1.

Table1 Primers sequences for *SUMO1* mutation analysis

Primer name	Nucleotide sequence (5'>3')	Length (base pair)	Product size (base pair)	Annealing Temperature (°C)
Sumo1-F1	GTAGCGGAAGTTACTGCAGC	20	131	60
SumoR1-2	AGGCAGACCTGCAGGGAAGG	20		
Sumo1-F2	CTGTTTGTATTCTCAGGTGC	20	334	55
Sumo1-R2	CTTCTACCTCTAACAGATGC	20		
Sumo1-F3	TTCAGTGACACTTCACTTGG	20	709	55
Sumo1-R3	GAGTGTTTTCCTTGAGTTGC	20		
Sumo1-F4	GTGTTCTAAGGCTTTCATGC	20	258	55
Sumo1-R4	GGGCAGTTTTAACACCAGTG	20		
Sumo1-F5	GAGGAGTGTAAGTATGGGTC	20	288	55
Sumo1-R5	GGGTGCCAGTTTTCAATTCC	20		

1. PCR reaction for amplification of DNA is obtained by combining the following components in a 20 µl reaction (Table2)
2. Centrifuge the reaction mixture briefly
3. PCR is performed in a Perkin-Elmer/ DNA thermal Cycle 480. The details of PCR cycles were shown in Table3.

Table2 Mixture of *SUMO1* PCR reactions.

Components	Final concentration	Per reaction (µl)
10X PCR reaction buffer	1X	2
50 mM $Mgcl_2$	1.5 mM	1.5
10 mM dNTPs	5.0 (250 ng)	0.4
10 µM Forward primer	1.25	0.4
10 µM Reverse primer	1.25	0.4
Taq DNA polymerase (5U/µl)	1	0.1
Distilled water	36.5	13.2
DNA template (50 ng/µl)	1	2
Total volume (µl)	-	20

Table 3 PCR condition of *SUMO1* amplification

Step	Temperature	Incubation time
Initial denaturation	94°C	3 min
PCR cycle of	35 cycles	-
Denature	94°C	30 sec
Annealing	Table	-
Extension	72°C	45 sec
Final extension	72°C	10 min

II. PCR for *miR-140* gene

The primers were designed within introns to allow genomic amplification and sequencing of amplicon as shown in Table 4

1. PCR reaction for amplification of DNA is obtained by combining the following in a 20 µl reaction (Table 5)
2. Centrifuge the reaction mixture briefly
3. PCR is performed in a Perkin-Elmer/ DNA thermal Cycle 480. The details of PCR cycles were shown in Table 6.

Table 4 Primer sequences for *miR-140* mutation analysis

Primer name	Nucleotide sequence (5'>3')	Length (base pair)	Product size (base pair)	Annealing Temperature (°C)
miR-140-F	GTGGTGGCGTTGCCTTCTGC	20	357	64
miR-140-R	GGTGCG AGCCTCAGGCATGA	20		

Table 5 Mixture of *miR-140* PCR reactions.

Step	Temperature	Incubation time
Initial denaturation	94°C	3 min
PCR cycle of	35 cycles	-
Denature	94°C	30 sec
Annealing	64°C	30 sec
Extension	72°C	45 sec
Final extension	72°C	10 min

Table 6 PCR condition of *miR-140* amplification

Components	Final concentration	Per reaction (µl)
10X PCR reaction buffer	1X	2
50 mM $Mgcl_2$	1.5mM	1.5
10 mM dNTPs	5.0 (250 ng)	0.4
10µM Forward primer	1.25	0.4
10µM Reverse primer	1.25	0.4
Taq DNA polymerase(5U/µl)	1	0.1
Distilled water	36.5	13.2
DNA template(50 ng/µl)	1	2
Total volume (µl)	-	20

III. PCR for the *PDGFRa* gene

The primers were designed within introns to allow genomic amplification and sequencing of exons 2-23 including exon-intron boundaries and amplified 708-bp fragment containing the *PDGFRa* 3' UTR as shown in Table 7.

1. PCR reaction for amplification of DNA is obtained by combining the following components in a 20 µl reaction (Table 8)
2. Centrifuge the reaction mixture briefly
3. PCR is performed in a Perkin-Elmer/ DNA thermal Cycle 480. The details of PCR cycles were shown in Table9.

Table 7 Primer sequences for *PDGFRa* mutation analysis

Primer name	Nucleotide sequence (5'>3')	Length (bp)	Product size (bp)	Annealing Temperature (°C)
PDGFRa3'UTR-F	CGAGACCATTGAAGACATCG	20	708	64
PDGFRa3'UTR -R	GTTGTCAGGCTTCTAAATGACC	22		
PDGFRa-E2F	CTGCATGCAATCACAGAAGG	20	471	62
PDGFRa-E2R	ACAGCAAAGGGAACTGAAGG	20		
PDGFRa-E3F	GAGCTTTCATGGGCATCCAG	20	770	60
PDGFRa-E3R	CACACTGTGAGTATGTGTGC	20		
PDGFRa-E4F	CATGAGCCACAACTCCTGTC	20	659	61
PDGFRa-E4R	TTAGCTGGGGTCCTAAATCC	20		
PDGFRa-E5F	TACACCATCTCACAATCAAG	20	428	60
PDGFRa-E5R	CAGAGGTGGGAAGCTAAGGT	20		
PDGFRa-E6-7F	ATGTGTAGCCTCCCACCTTG	20	709	64
PDGFRa-E6-7R	CTGACTTTGGGTCTGGGTTG	20		
PDGFRa-E8F	TCCAGACCTTCAGTGTGTGC	20	514	62
PDGFRa-E8R	CCTAGGGATTCCTCGCCTAC	20		
PDGFRa-E9F	CTGCTAACCATGTGGGTCTG	20	471	60
PDGFRa-E9R	GAGCCCTGGACCTTCTTCTC	20		
PDGFRa-E10F	GCCATCTTAGAGTGTTCCCG	20	524	60
PDGFRa-E10R	GTCCTAGGTAGAGGTTGCAG	20		

Table 7 Primers sequences for *PDGFRa* mutation analysis (cont.)

Primer name	Nucleotide sequence (5'>3')	Length (bp)	Product size (bp)	Annealing Temperature (°C)
PDGFRa-E10F	GCCATCTTAGAGTGTTCCCG	20	524	60
PDGFRa-E10R	GTCCTAGGTAGAGGTTGCAG	20		
PDGFRa-E11-12F	TGAGAGATTCCTGGCTCAGACACA	24	617	60
PDGFRa-E11-12R	GCTCAGATCTCTATTCTGCCAAGG	24		
PDGFRa-E13-14F	CAGGAAAGACACTCGCCCAG	20	860	62
PDGFRa-E13-14R	GTCAGCTCCATTCAAGGGAC	20		
PDGFRa-E15F	GGCTTAAATCCTCCACTCTC	20	559	62
PDGFRa-E15R	AGTCTTGCTACCTTGGGCAC	20		
PDGFRa-E16F	TACCCAGTTAGCTCCCATGC	20	389	60
PDGFRa-E16R	GCCAGGTTCACTTACCATCC	20		
PDGFRa-E17-18F	TGTGTTCTTTGGGCATGCCT	20	804	55
PDGFRa-E17-18R	CAGTGTACTGACCCCTTGAA	20		
PDGFRa-E19F	CTGGTCCATTCAGGGCTTAC	20	533	62
PDGFRa-E19R	TGGCCTCACACCAGGTTATC	20		
PDGFRa-E20-21F	CTTGGACTTGACCAACCTGG	20	686	62
PDGFRa-E20-21R	ACTAGGGTCTCGTGGAGCAC	20		
PDGFRa-E22F	TTGGAGAAGGGGCTACTGGT	20	751	62
PDGFRa-E22R	ACGTGGACCTCCTGAGGATT	20		
PDGFRa-E23F	AATGATGGGTGGACCTTGGT	20	546	55
PDGFRa-E23R	TACTGAGGCATTGCAAGAGG	20		

Table 8 Mixture of *PDGFRa* PCR reactions.

Components	Final concentration	Volume per reaction (µl)
10X PCR reaction buffer	1X	2
50 mM $Mgcl_2$	1.5mM	1.5
10 mM dNTPs	5.0 (250 ng)	0.4
10µM Forward primer	1.25	0.4
10µM Reverse primer	1.25	0.4
Taq DNA polymerase(5U/µl)	1	0.1
Distilled water	36.5	13.2
DNA template(50 ng/µl)	1	2
Total volume (µl)	-	20

Table 9 PCR condition of *PDGFRa* amplification

Step	Temperature	Incubation time
Initial denaturation	94°C	3 min
PCR cycle of	35 cycles	-
Denature	94°C	30 sec
Annealing	Table 7	Table 7
Extension	72°C	45 sec
Final extension	72°C	10 min

2.3 Direct Sequencing

PCR products were treated with ExoSAP-IT (USP, Cleveland, OH, USA) according to the manufacturer's recommendation, and sent for direct sequencing to Macrogen, Seoul, Korea. The sequences were analyzed using Sequencher (version 4.2 Gene Codes Corporation, Ann Arbor).

2.4 Restriction Fragment Length Polymorphism (RFLP) assay

The polymorphism study of entire three genes was determined by RFLP analysis. Each gene has specific restriction enzyme for identification of its polymorphism.

I. RFLP for the *SUMO1* gene

Make up mixture for restriction endonuclease digestion in intron 3 88-150A>G

Table10 Restriction nuclease site for *SUMO1*

Restriction enzyme	Restriction site	Product size (base pair)	Digested products (base pair)
*Mbo*II	5' GAAGANNNNNNNN▼ 3' 3' CTTCTNNNNNNN▲ N 3'	709	330, 287, 49,43

Table11 Mixture for restriction endonuclease digestion for *SUMO1*

Components	Volume per reaction* (µl)
10X NEBuffer2	2
Distilled water	12
5U/µl *Mbo*II	1
PCR product	2
Incubation time	37^0 overnight

II. RFLP for the *miR-140* gene

Make up mixture for restriction endonuclease digestion +66G>A from coding sequence (Table 12 and 13)

Table12 Restriction nuclease site for *miR-140*

Restriction enzyme	Restriction site	Product size (base pair)	Digested products (base pair)
Taq$^{\alpha}$I	5' NNNNT▼CGANNNN 3' 3' NNNNAGC▲TNNNN 3'	357	221,136

Table13 Mixture for restriction endonuclease digestion for *miR-140*

Components	Volume per reaction* (µl)
10X NEBuffer3	2
100X BSA	0.15
Distilled water	3.65
20U/µl *Taq*$^{\alpha}$I	0.2
PCR product	9
Incubation time	65^{0} overnight

III. RFLP for the *PDGFRa* gene

Make up mixture for restriction endonuclease digestion for *PDGFRa* (Table14, 15 and 16)

Table14 Restriction nuclease site for *PDGFRa*

Restriction enzyme	Restriction site	Product size (base pair)	Digested products (base pair)
*Btg*I	'5' NNNNC▼CRYGGNNNN 3' 3' NNNNGGYRC▲CNNNN 3'	708	416, 292
*Apo*I	5' NNNNR▼AATTYNNNN 3' 3' NNNNYTTAA▲RNNNN 3'	708	556, 152
*Sau96*I	5' NNNNG▼GNCCNNNN 3' 3' NNNNCCNG▲GNNNN 3'	708	96,112,208,500
*HpyCH4*IV	5' NNNNA▼CGTNNNN 3' 3' NNNNTGC▲ANNNN 3'	619	39, 580
*EcoR*V	5' NNNNGAT▼ATCNNNN 3' 3' NNNNCTA▲TAGNNNN 3'	164	130, 34
*BsaJ*I	5' NNNNC▼CNNGGNNNN 3' 3' NNNNGGNNC▲CNNNN 3'	617	412, 137, 59, 19
*Nla*III	5' NNNNCATG▼NNNN 3' 3' NNNN▲GTACNNNN 3'	524	376, 108,40
*Bst*XI	5' NCCANNNNNN▼NTGGNNN 3' 3' NNNGGTN▲NNNNNACCN 3'	546	216, 330

Table15 Mixture for restriction endonuclease digestion for *PDGFRa* 3'UTR

Components	Volume per reaction* (µl)			
	c.*455A>C	c.*34G>A	c.*51G>A	c.*479C>A
10X NEBuffer1	-	-	1.5 (1X)	-
10X NEBuffer3	1.5 (1X)	-	-	-
10X NEBuffer4	-	1.5 (1X)	-	1.5 (1X)
100X BSA	0.15	-	-	0.15
Distilled water	3.75	6.2	5.2	6.2
10U/µl *Btg*I	0.5	-	-	-
10U/µl *Apo*I	-	-	-	0.25
5U/µl *Sau96*I	-	0.3	-	-
10U/µl *HpyCH4*IV		-	0.3	-
PCR product	9	7	8	8
Total volume (µl)	15	15	15	15
Incubation time	37^0 overnight	37^0 overnight	37^0 overnight	50^0 4hr

Table16 Mixture for restriction endonuclease digestion for *PDGFRa*

Components	Volume per reaction* (µl)			
	A401D (Exon8)	T474M (Exon10)	V544P(Exon11)	T1052M (Exon23)
10X NEBuffer2			2 (1X)	-
10X NEBuffer3	2(1X)	-	-	2 (1X)
10X NEBuffer4	-	2 (1X)	-	-
100X BSA	0.2	0.2	-	-
Distilled water	12.3	7.8	7	7.25
20U/µl *EcoR*V	0.5	-	-	-
2.5U/µl *BsaJ*I	-	-	1	0.25
10U/µl *Nla*III	-	1	-	-
5U/µl *Bst*XI		-	0.3	0.5
PCR product	5	10	10	10
Total volume (µl)	20	20	20	20
Incubation time	37^0 overnight	37^0 overnight	60^0 4 hr	37^0 overnight

Artificial Restriction Fragment Length Polymorphism (A-RFLP) Analysis

Restriction analysis of polymerase chain reaction (PCR) products is one of the earliest techniques used for analyzing amplification products. This approach is applicable for distinguishing alleles in which the polymorphic residue results in the creation or removal of a restriction enzyme site. Unfortunately, many polymorphisms are not associated with restriction enzyme site change and thus are not amenable to this analysis. However, by using site-directed mutagenesis using primers with mismatches near the 3' ends, it is possible to create an artificial restriction fragment length polymorphism (A-RFLP) for almost all naturally occurring DNA polymorphisms (Figure1)

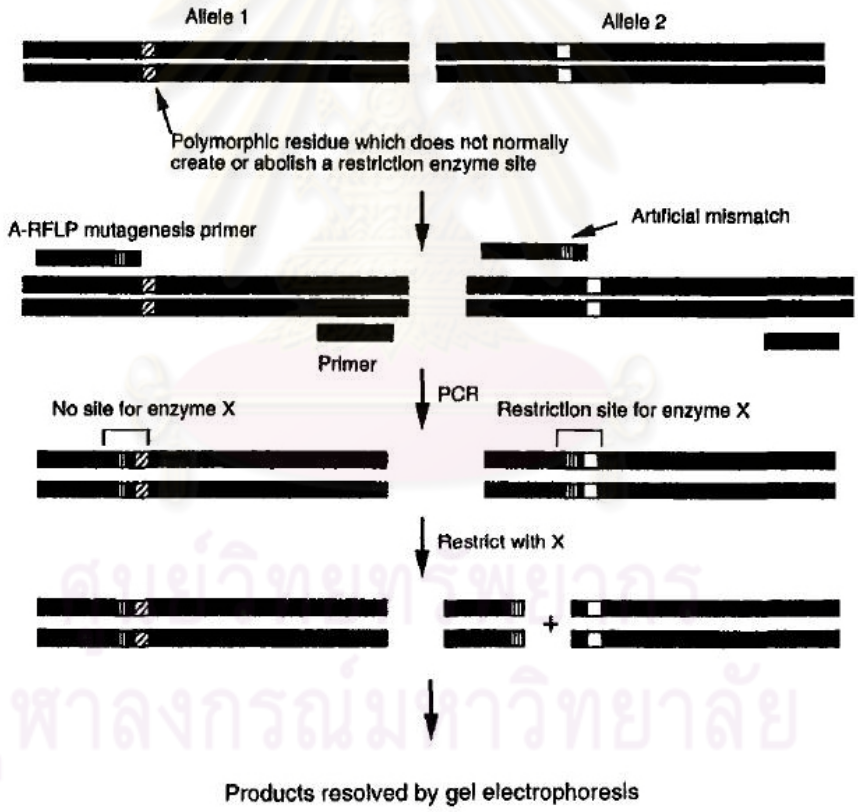


Figure1 Principles of artificial RFLP (REF)

Design of an A-RFLP primer can be accomplished easily and rapidly using a semiautomatic approach using a computer program that will search for restriction enzyme sites for a given sequence, e.g., DNA Strider. The process is illustrated in Figure 2. For the following discussion, let us assume that the polymorphic residue is P (Figure 2) and that we are searching for restriction enzymes with recognition sites of up to six bases. Five bases on either side of P are entered into the computer (from-5 to +5) and the program is used to search for a restriction enzyme site encompassing P. If a restriction enzyme site is found that is only present in one allele but not m the other one, then an A-RFLP site is found. If no restriction site polymorphism is found, then the nucleotides from -2 to -5 and from +2 to +5 are systematically changed one at a time, and a computer-assisted search for restriction enzyme sites is carried out after each alteration. For each position, the nucleotides A, T, C, and G is substituted in turn (one of them will be found in the naturally occurring sequence)

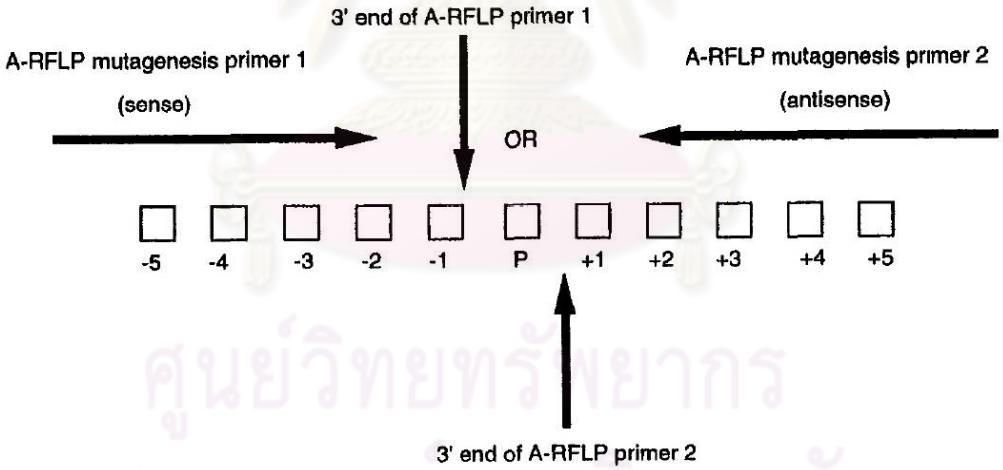


Figure 2 Semiautomatic approach for designing A-RFLP primers. See text for details. Following the design stage, only one of the A-RFLP primers would be chosen, either in direction 1 (sense) or direction 2 (antisense). PCR amplification would then be carried out using the A-RFLP primer and a downstream primer. (REF)

2.3 Agarose gel electrophoresis and DNA sequencing

The PCR products were verified for correct size on ethidium bromide-stained 1.5% agarose gel. The PCR products were then treated with ExoSAP-IT (USP Corporation,

Cleveland, OH) according to the manufacturer's recommendations, and sent for direct sequencing at the Macrogen Inc., Seoul, Korea. The sequence was analyzed using Sequencher (version 4.2; Gene Codes Corporation, Ann Arbor, MI).

3. Functional analysis

This procedure was performed to investigate an effect of the mutation detected in patients with mutation in the 3'UTR of the *PDGFRa* gene. The luciferase reporter gene assay was used to investigate that . The construction of plasmids, *in vitro* site-directed mutagenesis, and transient transfection procedures are described below.

3.1 Construction of plasmids

3.1.1 Construction of mammalian expression vector

Using the pGEMR-T Easy Vector (Figure 3) as a template, genomic DNA from the patient with heterozygous 51G>A mutation was amplified using forward mutagenesis primer CGAGCTCATTGAAGACATCG for *Sac*I (5' GAGCT▼C 3') and reverse primer GTTGTCAAGCTTCTAAATGACC for *HinD*III (5' A/AGCTT 3') to generate a 708 base pair fragment containing the *PDGFRa* 3' UTR.

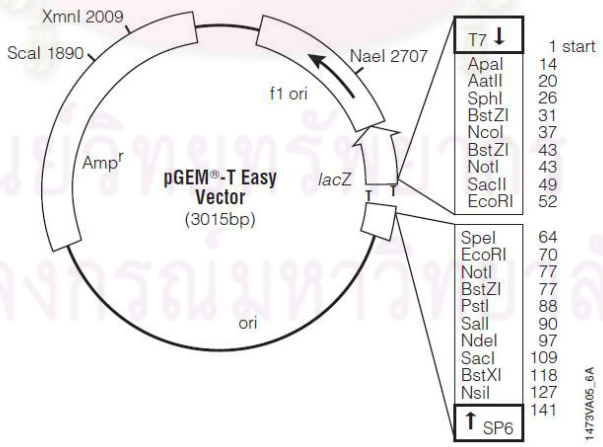


Figure 3 pGEMR-T Easy Vector

Ligation Using the pGEM®-T Easy Vectors

1. Briefly centrifuge the pGEMR-T Easy Vector tubes to collect the contents at the bottom of the tubes.

2. Set up ligation reactions as described below (Table 17).

Table 17 Reaction component of ligation

Reaction Component	Standard Reaction (µl)
10X Ligation Buffer	2
pGEMR-T Easy Vector (50 ng)	5
PCR product (CLP140)	12
T4 DNA Ligase (3 Weiss units/µl)	1
nuclease-free water	10

3. Mix the reactions by pipetting. Incubate the reactions for 1 hour at room temperature.

Optimizing Insert : Vector Molar Ratios

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert : vector molar ratio} = \text{ng of insert}$$

4. An aliquot of the PCR reaction should be analyzed on an agarose gel before use in the ligation reaction to verify that the reaction produced the desired product. The PCR product to be ligated can be gel-purified or purified. Clean-up of reactions prior to ligation is recommended to remove primer dimers or other undesired reaction products, and to improve ligation efficiency. Exposure of PCR products to shortwave ultraviolet light should be minimized in order to avoid the formation of pyrimidine dimers.

Transformation

1. Prepare LB/ampicillin plates.

2. Add 2 µl of each ligation reaction to a sterile 1.5 ml microcentrifuge tube on ice.

3. Remove tube(s) containing Competent Cells from storage and place in an ice bath until just thawed (about 5 minutes). Mix the cells by gently flicking the tube. Avoid excessive pipetting, as the competent cells are extremely fragile.

4. Carefully transfer 50 µl of cells into each tube prepared in Step 2.

5. Gently flick the tubes to mix and place them on ice for 20 minutes.

6. Heat-shock the cells for 45–50 seconds in a water bath at exactly 42°C.

7. Immediately return the tubes to ice for 2 minutes.

8. Add 980µl room-temperature SOC medium to the tubes containing cells transformed with ligation reactions.

9. Incubate for 1.5 hours at 37°C with shaking (~150rpm).

10. Plate 200 µl of each transformation culture onto duplicate LB/ampicillin plates. If a higher number of colonies are desired, the cells may be pelleted by centrifugation at 1,000 × *g* for 10 minutes, resuspended in 200 µl of SOC medium, and 200 µl plated on each of two plates.

11. Incubate the plates overnight (16–24 hours) at 37°C.

Screening transformants for inserts

Successful cloning of an insert into the pGEMR-T Easy Vector interrupts the coding sequence of β-galactosidase; recombinant clones can be identified by color screening on indicator plates. However, the characteristics of the PCR products cloned into the vectors can significantly affect the ratio of blue:white colonies obtained. Usually clones containing PCR products produce white colonies. TA –WT *PDGFRa* -3'UTR and TA -MUT *PDGFRa* -3'UTR were verified by sequencing.

Mutant strand of *PDGFRa* 3'UTR synthesis

Two uncharacterized *PDGFRa* -3'UTR mutants constructs were generated by *in vitro* site-directed mutagenesis (Stratagene's QuickChange site directed mutagenesis kit) from wild type allele (TA –WT *PDGFRa* -3'UTR).

Mutant strand synthesis reaction (thermal cycling)

1. Synthesize two complimentary oligonucleotide primers containing the desired point mutation. The mutagenesis primer sets were designed by using *Stratagene's web-based QuikChange Primer Design Program* available online at http://www.stratagene.com/qcprimerdesign. All mutagenesis primer sequences were shown in table 18.

Table 18 Mutagenesis primer sequences for site directed mutagenesis by using PCR.

Mutant Types	Mutagenesis primer sequences for PCR (5' to 3')
1. 34G>A	PDGFRa G34A F:5'-GTTCCTTCCACTTCTGGAGCCACCTCTGGATCCCG-3'
	PDGFRa G34A R:5'-CGGGATCCAGAGGTGGCTCCAGAAGTGGAAGGAAC-3'
2. 479C>A	PDGFRa G479A F:5'-CCTGATGTCAGCTGCTGTTGAAATTTTTAAAGAAGTGCATGAAA-3'
	PDGFRa G479A R:5'-TTTCATGCACTTCTTTAAAAATTTCAACAGCAGCTGACATCAGG-3'

2. **Prepare the sample reaction(s) as indicated in table 19.**

Table 19 Mixture of PCR reactions for site directed mutagenesis

Components	Volume per reaction (µl)
1. 10X PCR reaction buffer	5
2. 50 ng/µl TA-*PDGFRa*-CLP140	4
3. 125 ng Forward primer	2
4. 125 ng Reverse primer	2
5. dNTP mix	1
6. Distilled water	36
7. 5U/µl Pfu.Turbo polymerase	1
Total volume (µl)	51

3. Cycle each reaction using the cycling outlined in table 20.

Table 20 PCR cycle for site directed mutagenesis.

Step	Temperature and incubation time
1. Initial denaturation	95°C/ 30 sec
2. PCR cycle	12 cycles
Denature	95°C/ 30 sec
Annealing	55°C/ 1 minute
Extension	68°C/ 6 minutes
3. Final extension	72°C/ 7 minutes

*Final concentration per reaction in each PCR reaction

_Dpn_I digestion of the amplification products

This step was performed for digestion of the nonmutated parental DNA template with *Dpn*I.

1. Add 1 µl of the *Dpn*I restriction enzyme (10 U/µl) directly to each amplification reaction.

2. Gently and thoroughly mix each reaction mixture by pipetting the solution up and down several times. Spin down the reaction mixtures in a microcentrifuge for 1 minute and immediately incubate each reaction at 37°C for 1 hour to digest the parental DNA.

Transformation & Screening Transformants for Inserts

Human *PDGFRa* 3'UTR Luciferase Constructs

Used *Sac*I (5' GAGCT▼C 3') and *HinD*III (5' A/AGCTT 3') enzymes to liberate the 3'UTR region of the *PDGFRa* gene from TA constructs (Table 21) and ligated the fragments into pMIR-REPORT™(ambion, Figure 4) between *Sac*I and *HinD*III sites (Table 22), making *PDGFRa* 3'UTR Luciferase Constructs. pMIR –WT *PDGFRa* -3'UTR and pMIR -MUT *PDGFRa* -3'UTR were verified by sequencing.

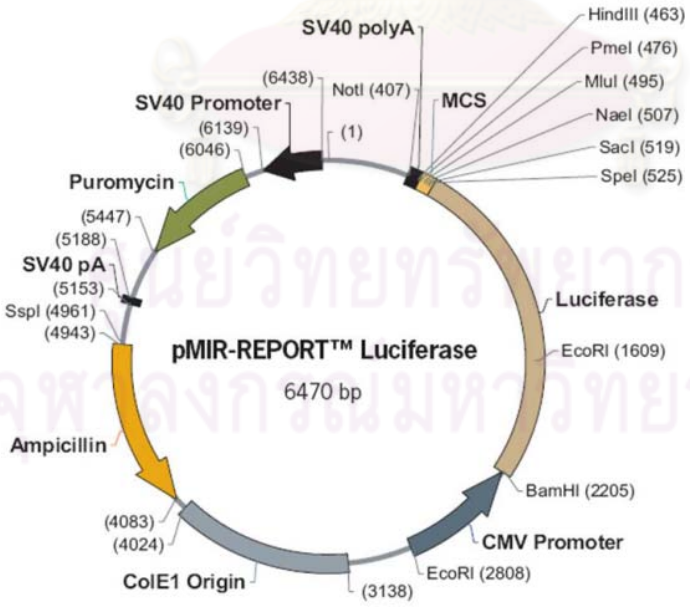


Figure 4 pMIR-REPORT™ vector (ambion)

The pMIR-REPORT™ miRNA Expression Reporter Vector System provides accurate, quantitative, in-cell measurement of miRNA expression. This validated reporter system contain mammalian expression vectors (Figure 3). The pMIR-REPORT™ Luciferase miRNA Expression Reporter Vector contains firefly luciferase under the control of a mammalian promoter/terminator system, with a miRNA target cloning region downstream of the luciferase translation sequence. This vector is optimized for cloning of miRNA targets and evaluation of miRNA regulation. pMIR-REPORT Luciferase utilizes the powerful CMV promoter to drive high-level expression of firefly luciferase in mammalian cells. A miRNA target multiple cloning site follows the luciferase gene and is itself followed by a SV40 polyA region. The vector utilizes puromycin for selection in cell culture and a ColE1 Ori/Ampicillin-resistance gene for maintenance in *E. coli*. pMIR-REPORT Luciferase is designed for the cloning and testing of putative miRNA binding sites. pMIR-REPORT Luciferase can be transfected into mammalian cells to evaluate endogenous miRNA expression, or used to evaluate the up- or down-regulation resulting from the transfection of Pre-miR™ miRNA Molecules or Anti-miR™ miRNA Inhibitor Molecules respectively. pMIR-REPORT Luciferase can also be used as a sequence screening tool to identify miRNA targets or screen libraries of Pre-miR miRNA Molecules to identify genes that regulate expression.

Table 21 Mixture for restriction endonuclease double digestion for *Sac*I and *Hind*III

Components	Volume per reaction* (µl)
10X NEBuffer2	2
100X BSA	0.2
Distilled water	6.13
20U/µl *Sac*I	1
20U/µl *Hind*III	0.5
3'UTR-TA	10
Incubation time	37^0 overnight

Table 22 Reaction Component of ligation for pMIR-*PDGFRa* -3'UTR

Reaction Component	Standard Reaction (µl)
10X Ligation Buffer	2
pGEMR-T Easy Vector (50ng)	5
PCR product (CLP140)	12
T4 DNA Ligase (3 Weiss units/µl)	1
nuclease-free water	10

3.1.2 Preparation of internal control and empty vector

The pRL-TK is a commercially available vector containing the native *Renilla* luciferase gene under the transcriptional control of the herpes simplex virus thymidine kinase (TK) promoter region. It was used as an internal control for determination of transfection efficiency. Normalization of the levels of transcription of the experimental reporter gene (TREpalx3-Luc) to the internal control reporter gene minimizes the variability of the obtained results caused by differences in the transfection efficiency between different samples of transfected cells (Figure 5A).

For a control, the empty vector, the pcDNA™ 3.1/myc-His B (Figure 4B) was used. The pcDNA™ 3.1/myc-His B is a commercially available vector (Invitrogen).

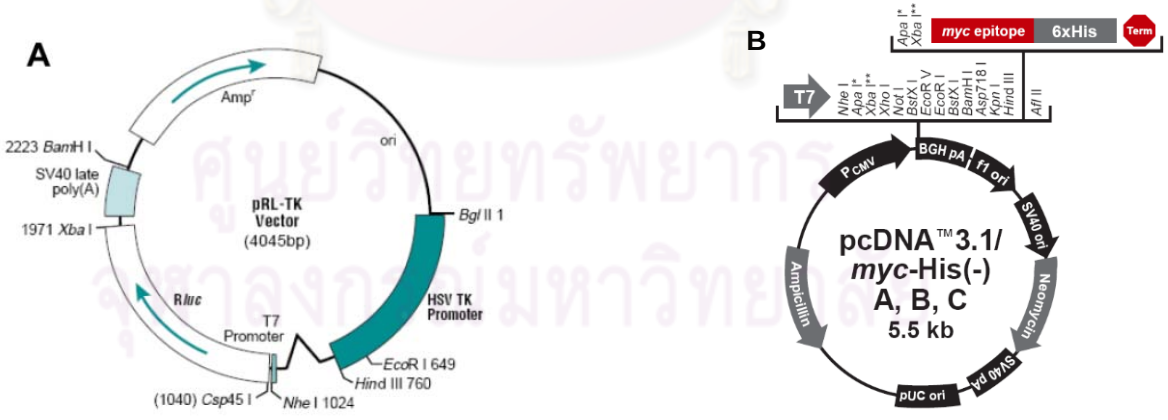


Figure 5 Circle maps of the pRL-TK and the pcDNA™3.1/myc-His B.

Panel A shows the pRL-TK construct.

Panel B shows the pcDNA™ 3.1/myc-His B.

3.2 Amplification of expression vectors for transfection experiment

3.2.1 Preparation of competent cells

1. Grow bacteria (DH5α and XL-1 blue) from glycerol stock by streaking on the LB plate (without antibiotic) and incubate for 18 hours at 37°C.

2. Pick a single colony and grow them in 30 ml of LB broth without antibiotic (starter media) in a 100-ml flask. Incubate the culture for 18 hours at 37°C with shaking at 225 rpm.

3. Dilute the starter media at ratio 1:10 with fresh LB broth without antibiotic (original media). Incubate the culture for 90 minutes at 37°C with shaking at 225 rpm.

4. Transfer 10 ml of the culture to a pre-chilled sterile 15-ml centrifuge tube. Pellet the bacteria with a 4000 rpm spin for 10 minutes at 4°C. Discard supernatant and place the cell pellet on ice.

5. Resuspend cells in 10 ml of cold 0.1 M $CaCl_2$ solution. Pellet the bacteria with a 4000 rpm spin for 10 minutes at 4°C.

6. Discard supernatant and resuspend cells in 2 ml of cold 0.1 M $CaCl_2$ solution per original media and add 10% glycerol. Mix by slowly pipetting up and down and store cells at –80°C.

7. Test for cell competency by transformation with the control plasmid vector using heat shock (see 3.2.2).

3.2.2 Transformation

To make bacterial cells take up the plasmid/foreign DNA by using heat shock.

1. Take out competent (DH5α or XL-1 blue) cells from –80 ^{0}C and thaw on ice for 5 minutes.

2. Add 5 µl of plasmid DNA into 50 µl of competent cells in a 1.5-ml microcentrifuge tube and gently stir with tip. Incubate for 30 min at –4 ^{0}C.

3. Put tubes with DNA into heat block at 42 ^{0}C for 45 seconds.

4. Put tubes back on ice for 2 minutes to reduce damage to the cells.

5. Add 1 ml of culture medium at 37 ^{0}C containing SOC 96 µl, Mg^{2+} 2 µl, and 10 M glucose 2 µl (without antibiotic added). Incubate tubes for 90 minutes at 37 ^{0}C with shaking at 225 rpm.

6. Spread 20-50 µl of culture by the spreader on warmed LB plates (with 100mg/µl of ampicillin). Grow them overnight at 37 ^{0}C for 18 hours.

7. Pick a fresh single colony and place in 5 ml of LB broth (with 100mg/µl of ampicillin), and then incubate at 37 ^{0}C with shaking at 225 rpm for 16 hours.

8. Extract and purify the plasmid DNA with mini prep.

3.2.3 Plasmid DNA extraction

1. Harvest bacteria from culture tubes into 1.5-ml microcentrifuge tubes. Centrifuge at 13,400 rpm for 3 minutes. Bacterial cells may be harvested in 15 ml tubes. Centrifuge at 5,400 x g for 10 minutes at 4 ^{0}C.

2. Discard supernatant and add 250 µl of chilled complete Qiagen suspension solution (P1), vortex or pipette up and down until no cell clumps remain. Transfer suspension cells to a 1.5 ml-microcentrifuge tube.

3. Add 250 µl of Qiagen lysis solution (P2) and mix thoroughly by inverting 10 times and let stand for 2 minutes at room temperature or until the lysate solution is clear.

4. Add 350 µl of Qiagen neutralize solution (N3) and mix thoroughly by inverting 10 times. Centrifuge for 10 minutes at 13,400 rpm in a table-top microcentrifuge. A compact white pellet will form.

5. Transfer the supernatant from step 4 to spin column tubes and centrifuge for 1 minute. Discard the flow-through.

6. Add 500 µl of Qiagen wash buffer (PB) and centrifuge for 1 minute. Discard the flow-through.

7. Remove all residual buffer PB by adding 750 µl of Qiagen wash buffer (PE) and centrifuge for 1 minute. Discard the flow-through and centrifuge again for 1 minute.

8. Transfer spin columns to a new 1.5-ml microcentrifuge tube, add 30- 50 µl of filtered dH_2O or elution buffer (EB), and let stand for 5 minutes at room temperature. Centrifuge for 5 minutes at 13,400 rpm in a table-top microcentrifuge. Remove the column and store DNA at -20 ^{0}C.

3.2.4 DNA digestion

1. Obtain a buffer that works for all of the enzymes being used in the digestion. Vortex well and keep on ice.

2. Set-up a master mix consisting the buffer (1, 2, 3, or 4) that works for all enzymes being used, filtered dH_2O, and the enzymes. The digestion reaction was summarized in table 4.

3. Incubate the reaction (see in table 4) in the 37°C water bath for at least one hour.

4. Check the cut sizes by electrophoresis of 20 µl of the digestion products on a 0.8% agarose gel.

Table 23 Mixture of DNA digestion reactions for plasmid detection.

Components	Volume per reaction* (µl)	
	Renilla-TK	pcDNA™ 3.1/myc-His B
1.10X NEBuffer3	2 (1X)	2 (1X)
2.100X BSA	0.2 (1X)	0.2 (1X)
3.Plasmids	5	5
4.Distilled water	10.8	11.8
5.20U/µl *BamH*I	1 (20U)	1 (20U)
6.20U/µl *EcoR*I	-	-
7.10U/µl *Bgl*II	1 (10U)	-
Total volume (µl)	20	20
Incubation time	O/N	1 hr. or O/N

*Final concentration per reaction in each DNA digestion reaction

Note: Keep the enzymes in the cold block and add last to the master mix.

3.2.5 DNA precipitation

1. Add a half volume of 10 M NH_4OAc to 1 volume of the DNA sample.

2. Add an equal volume of chilled complete 100% EtOH to the DNA sample, add 5 µl of glycogen, and mix thoroughly by gently inverting.

3. Incubate at –20 °C overnight or at –80 °C for 1 hour.

4. Centrifuge at 14,000 rpm for 15 minutes at 4 ^{0}C and remove the ethanol and salt.

5. Add 1 ml of chilled complete 70% EtOH, mix thoroughly by gently inverting, and centrifuge at 14,000 rpm for 5 minutes at 4 ^{0}C.

6. Remove the ethanol with care and dry the pellet in 50 °C oven for 5 minutes or dry the pellet at room temperature overnight.

7. Resuspend the dried DNA in appropriate amount of sterile TE (pH 8.0), or water, and store at 4 °C for further manipulation or at –20 °C for long-term storage.

3.4 Transfection assay

To test for an effect of mutations in the *PGDGFRa* 3' UTR, we performed co-transient transfection-based luciferase reporter gene assays as described below.

Day 1: Plating cells

This step was prepared for seeding cells in 24-well culture plates with DMEM containing 10%FBS (GIBCO) without antibiotics for 24 hours before transfection by Lipofectamine^{TM} 2000 (Invitrogen). The number of cells for seeding were 5x10^{4} cells/well.

1. Inspect COS7 cells (simian virus 40-transformed African green monkey kidney fibroblasts) in the T-75 flask by using an inverted microscope to ensure that they were 60-70% confluent..

2. Remove the growth medium from T-75 flask, cells were washed with 10 ml of 1X phosphate buffer saline (PBS), and remove the rinse solution.

3. Add 2 ml of trypsin-EDTA and incubate cells in a 5% CO_2 incubator for 5 minutes.

4. Inhibit trypsinization with growth medium (DMEM+10% FCS) and subculture cells at ratio 1:3. Adjust solution volume to 10 ml of growth medium (DMEM+10% FCS).

5. Pipette up and down to mix cells, then transfer cells to a 15-ml centrifuge tube.

6. Using micropipette (size 1,000 µl) to mix cells again and sampling cells by pipetting 1 ml of suspension cells to a 1.5-ml microcentrifuge tube. Stain cells 50 µl with trypan blue 450 µl (Ratio cells:dye = 1:10).

7. Count cells using the hemocytometer by transferring cell solution into a counting chamber.

8. Calculate the number of cells by using formula as described below:

N (cell number per ml) = the average count per square x dilution factor x 10^4

9. Seed cells with the number calculated in DMEM containing 10% FBS (Hyclone) and incubate cells for 24 h in the CO_2 incubator.

Note: In this step, don't add antibiotics to growth medium as this causes cell death.

Day 2: Transfection

1. Prepare DNA for transfection

pMIR (wild-type and mutants)	48	ng/well
pRL-TK	12	ng/well
pcDNA™ 3.1/myc-His B	40	µg/well
Pre-miR™ miRNA Precursor *miR-140*	20	µM/well
Pre-miR™ miRNA Precursor Molecules-Negative Control #1	20	µM/well

2. Dilute the plasmid DNA into 100 µl of Opti-MEM I reduced serum Medium without serum (GIBCO; Invitrogen). See table 24

Table 24 Mixture of transfection reaction for *PGDGFRa* 3' UTR

	pMIR-*PDGFRa* 3'UTR wt (48ng/ µl)		pMIR-*PDGFRa* 3'UTR mut (48ng/ µl)					
			34G>A		51G>A		480C>A	
	Pre-*miR* *140*	Pre-miR Neg. 1	Pre-*miR* *140*	Pre-miR Neg. 1	Pre-*miR* *140*	Pre-miR Neg. 1	Pre-*miR* *140*	Pre-miR Neg. 1
pcDNA3.1 (40ng/ µl)	1µl	1µl	1µl	1µl	1µl	1µl	1µl	1µl
pRL-TK (12ng/ µl)	1µl	1µl	1µl	1µl	1µl	1µl	1µl	1µl
Opti-MEMI	46µl		46µl		46µl		46µl	
Total	50 µl		50 µl		50 µl		50 µl	

3. Mix Lipofectamine™2000 gently before use, then dilute 4.0 µl of Lipofectamine™ 2000 in 100 µl of Opti-MEM I reduced serum medium. Incubate for 5 minutes at room temperature (table 25).

Table 25 Dilute LF™ 2000 in OptimemI-Reduced Serum Medium without Serum for *PGDGFRa* 3' UTR activity

Components	Volume
LF™ 2000 reagent	0.5 µl
Opti-MEMI	49.5 µl
Total Volume	50 µl

4. Combine the diluted DNA (from step 2) with the diluted LF ™2000 reagent (from step 3) (total volume = 100µl). Incubate at room temperature for 20 minutes to allow DNA- LF ™2000 reagent complexes to form.

Note: Complexes are stable for 6 hours at room temperature.

5. Remove the growth medium from the cells and add 0.4 ml of DMEM (without serum) and 200 µl of DNA-LF™2000 complexes to each well. Add the DNA- LF™2000 reagent complexes (100 µl) directly to each well and mix gently by rocking the plate back and forth.

6. Incubate the cells at 37°C in a CO_2 incubator for 24 hours.

Day 3: harvest cells

1. Remove the growth medium from the 24-culture well plates, and wash with 0.5 ml per well of phosphate buffer saline (PBS). Completely remove the rinse solution before applying the passive lysis buffer (PLB) reagent.

2. Add 150 µl of 1X PLB to each well.

3. Place plates on the orbital shaker and gently shake for 20 minutes at room temperature.

4. Transfer the lysate to a labeled 1.5-ml microcentrifuge tube.

5. Inspect cells to ensure that they are lysed and freeze the samples overnight at -80 °C.

Day 4: Detection of dual luciferase activities by using luminometer.

1. Thaw the frozen lysate and vortex for 15 seconds.

2. Centrifuge for 30 seconds at 4 °C and transfer supernatant into a new labeled 1.5-ml microcentrifuge tube.

3. Aliquot 20 µl of cell lysate and mix with 100 µl of LARII reagent in 1.5-ml microcentrifuge tube and measure the activity by GloMax 20/20 Luminometer with Single Auto-Injector. The number represents the firefly luciferase activity.

4. Add 100 µl of Stop and Glo reagent and read the luciferase activity again. This number represents the renilla luciferase activity.

5. Record all readings.

6. Data analysis (using excel on the lab bench computers)

a. Calculate the ratio of firely to renilla luciferase activities

b. Relative luciferase activity was calculated and shown as fold induction relative to the luciferase activity of Pre-miR™ miRNA Precursor Molecules-Negative Control #1. All experiments were performed in triplicate and repeated two times. The results were reported as fold induction ± SD. Statistical analyses were performed using ANOVA

CHARTER IV
RESULTS

SMALL UBIQUITIN-LIKE MODIFIER 1 (*SUMO1*)

The entire coding sequences of the *SUMO1* gene were analyzed by PCRsequencing in 123 and 104 Thai patients with nonsyndromic CL/P and nonsyndromic CP, respectively. One sequence variant was identified in 75 individuals with nonsyndromic CL/P. The variant was a single nucleotide change in intron 3 (Table 26). The observed distribution of genotypes among controls was compared with that expected according to the Hardy–Weinberg equilibrium: no difference was found (χ^2 = 0.028, *df* = 2, *P* = 0.99). Genotype frequencies of the patients also followed the Hardy–Weinberg equilibrium (χ^2 = 0.772, *df* = 2, *P* = 0.68). The distribution of alleles and genotypes among patients were compared with those among controls: no differences of either allelic (P = 0.052) or genotypic (P = 0.170) distributions between patients and controls were found. In addition, no association was found with the recessive inheritance mode (AG+AA vs. GG, odds ratio 1.58, 95% CI 0.90 - 2.77; Table 26) and dominant inheritance mode (AA vs. GG+AG odds ratio 2.62, 95% CI 0.54-17.46; Table 26).

Table 26 Genotypic and allelic distribution and comparison of *SUMO1* c.88-150G>A in patients with CL/P and controls.

Alleles	Patients (*n* = 227)	Controls (*n* = 105)
G	368 (0.81)	183 (0.87)
A	86 (0.19)	27 (0.13)
χ^2 (*P* value, *df* = 1)	3.765 (0.0523)	*ref.*
Genotypes	**Patients (*n* = 227)**	**Controls (*n* = 105)**
AG	64 (0.282)	23 (0.219)
AA	11 (0.048)	2 (0.019)
χ^2(*P* value, *df* = 2)	3.545 (0.170)	*ref.*
	Odds ratio (*P* value)	**95% CI**
AG+AA vs. GG	1.58 (0.088)	0.90 - 2.77

AA vs. GG+AG	2.62 (0.199)	0.54 - 17.46

PLATELET-DERIVED GROWTH FACTOR RECEPTOR, ALPHA (*PDGFRa*)

1. Sequence analysis in the *PDGFRa* gene

From 104 of nonsyndromic CP by PCR-sequencing of the *PDGFRa* gene, 4 sequence variants were identified. PCR-RFLP was also used to screen for their presence in the unaffected controls (Table 27). The multiple sequence alignments revealed that the p.V544A and p.T1052D were conserved (Figure 5).

Table 27 Sequence variants in the *PDGFRa* gene.

Location	Variant	Polarity	Case (Allele)	Control (Allele)
Exon 8	A401D	nonpolar>polar	3/204	1/1000
Exon 10	T474M	polar>nonpolar	1/204	1/1000
Exon 11	V544A	nonpolar>nonpolar	1/204	0/1000
Exon 23	T1052M	polar>nonpolar	1/204	1/204

a) *PDGFRa* A401

b) *PDGFRa* T474

c) *PDGFRa* V544

d) *PDGFRa* T1052

Figure 6 Multiple alignments of the identified *PDGFRa* sequence variants

Homo = *Homo sapiens* Bos = *Bos Taurus* Mus = *Mus musculus*

Gallus = *Gallus gallua* Xenopus = *Xenopus laevis* Danio = *Danio rerio*

1. Sequence analysis in the 3'UTR *PDGFRa* gene

Four noncoding sequence variants were identified in a cohort of 104 patients with nonsyndromic CP by PCR-sequencing (Table 27). All these variants map to the 3' untranslated region (UTR) of the transcript and correspond to the non conserved nucleotides (Figure 6) within the predicted binding site for the human microRNA (miRNA) hsa-miR-140 (Figure 7). The PCR-RFLP result comparing the presence of these variants between the patients and unaffected controls was shown in Table 27.

Table 28 Sequence variants in the 3' UTR *PDGFRa* gene.

Location	Variant	Case (Allele)	Control (Allele)
3'UTR *PDGFRa*	c.*34G>A	1/208	0/1000
	c.*51G>A	1/208	1/1000
	c.*455A>C	40/208	44/200
	c.*479C>A	2/208	2/1000

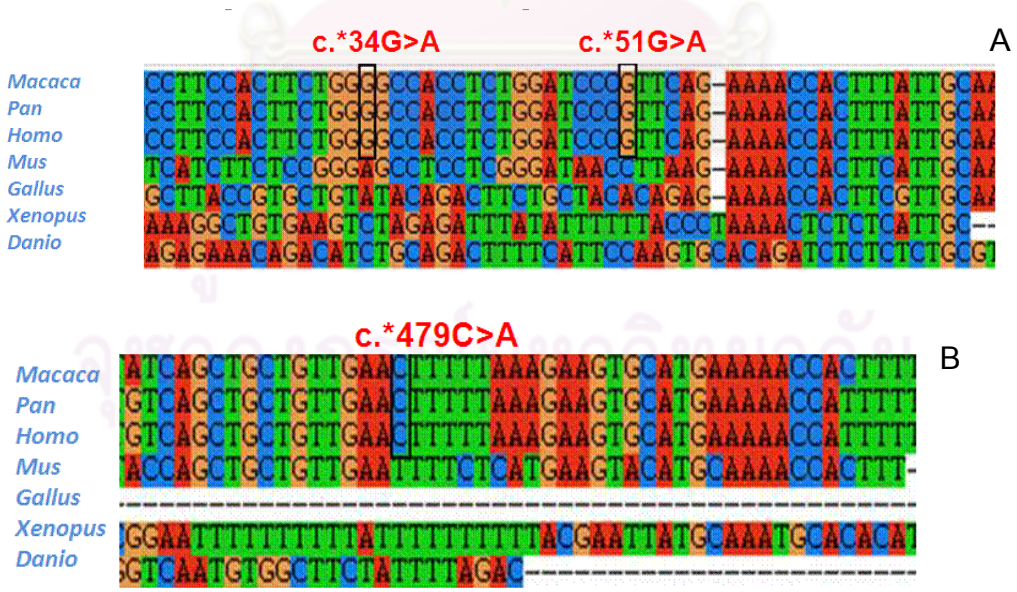


Figure 7 Multiple alignments of the sequence variants identified in the 3' UTR *PDGFRa*

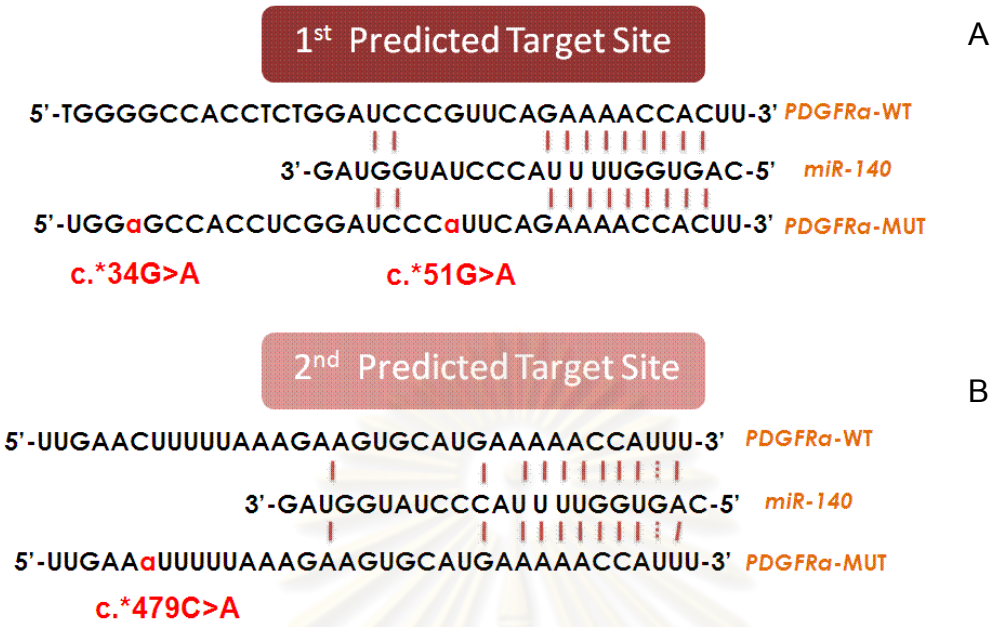


Figure 8 The predicted *miR-140* binding site of the 3' UTR *PDGFRa*

Functional analysis of the mutant 3' UTR *PDGFRa* gene.

The objective of this experiment is to determine the effect of the mutant 3'UTR *PDGFRa* on the miRNA-mediated repression using the luciferase reporter system. In the presence of Pre-miR™ *miR140* can mediate protein repression through its binding at the miRNA binding site in the 3'UTR *PDGFRa*. In transient co-transfection studies, the wild-type or mutant 3'UTR *PDGFRa* constructs were cotransfected with the Pre-miR™ miRNA Precursor *miR-140* into COS-7 cells. 20 μM of the Pre-miR™ miRNA Precursor *miR-140* were added to the culture media. All experiments were performed in triplicate. Cotransfection with the renilla luciferase reporter was used as the internal control to minimize the variability of the obtained results caused by differences in the transfection efficiency between different samples of the transfected cells. The data were represented as relative luciferase activity (firefly luciferase activity was normalized with renilla luciferase activity).

Statistical analysis

All data of the luciferase activity were calculated as relative luciferase activity. All experiments were performed in triplicate. Statistical analyses were performed using ANOVA (independent samples *t-test*).

We found that, with the presence of miR-140, the relative luciferase activity of the construct with the c.*34G>A mutation was statistically significantly lower than that of the wild type (*P*=0.014). The relative luciferase activities of the construct with the other two variants were not different from that of the wild type. (Figure 8).

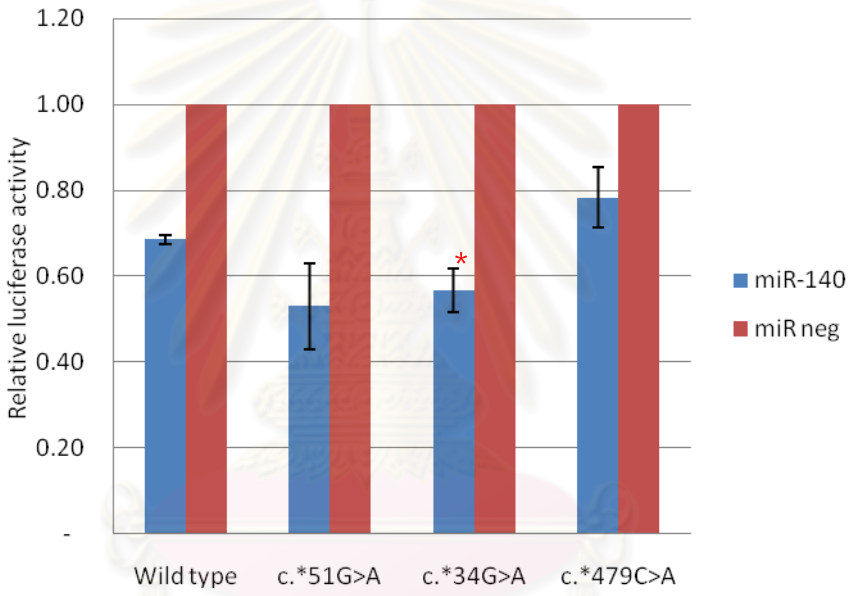


Figure 9 Relative luciferase activity

MICRO RNA 140(*miR-140*)

The *ha miR-140* was analyzed in 104 CP patients by by PCR sequencing. One variant was identified in 7 out of the 104 patients. The variant was a single nucleotide change, +10G>A. This variant was also identified by RFLP in 8 out of 100 unaffected controls (Table 28).

Table 29 Mutation screening of the *miR-140* gene.

Location	Variant	Case(Allele)	Control(Allele)
Intron	+10G>A	7/204	8/200

CHAPTER V
DISCUSSION AND CONCLUSION

Common birth defects such as neural tube defects, congenital heart disease, and cleft lip and palate can occur in both syndromic (which include structural abnormalities, developmental delay, or dysmorphic features), and non-syndromic forms. While both genes and environment play a role in the non-syndromic forms, the specific causes of these have remained largely undiscovered until recently. For neural tube defects, a strong environmental component is clearly shown by the ability of prenatal folic acid to prevent a proportion of these cases[57]. Genetic factors, perhaps including predispositions to the effects of folate or relative folate deficiency, seem likely also to play a role. For congenital heart disease, endophenotypes such as tetralogy of Fallot in apparent non-syndromic forms has been reported associated with NKX2.5 mutations, which suggests a specific gene component to this formerly complex trait in 4% of families[58]. Thus, searches for mutations in single genes whose defects include CL/P or CP may also disclose specific abnormalities in non-syndromic forms.

Samples included in this study were comprised primarily of subjects who have isolated cleft lip as well as cleft lip and palate. In *SUMO1* previous study reported a balanced translocation disrupting the *SUMO1* gene in a girl patient with a unilateral cleft lip and palate. Additional studies in animal models confirmed the role of SUMO1 in palate formation[12]. Recently, it has been demonstrated that *SUMO1* is able to regulate *MSX1* [51]. Genetic studies in both humans and mice have indicated that the Msx1 transcription factor is associated with specific disorders, including cleft palate. This suggests that *SUMO1* might control the activity of a repertoire of downstream effectors involved in palatogenesis, accounting for the sensitivity of palatal development to *SUMO1* gene dosage. Even though there was no significant sequence variants identified in this study, it was not surprising as the numerous genetic and environmental factors in distinct populations would probably contribute differently to the formation of CL/P. Ourstudy demonstrates an absence of *SUMO1* mutations in Thai patients with isolated cleft lip with or without cleft palate and cleft palate only. Other genetic or

environmental factors might contribute to the occurrence of nonsyndromic CL/P and CP in the Thai population.

Sequencing analysis of the coding regions of *PDGFRa* gene revealed four nonsynonymous variants, A401D, T474M, V544A, T1052M. All four have not been previously reported. Only one non-synonymous variant, the V544A, is not present in 500 control individuals of Thai ethnic background.

We compared human sequence with other species to search for evolutionary conservation. While the A401 and T474 are not conserved, the V544 is conserved in four of the five available species and the T1052 is conserved in all five. All four variants are not in the known functional domains of the *PDGFRa* (Figure 9). Whether these variants play a role in the pathogenesis of the oral clefts require further studies.

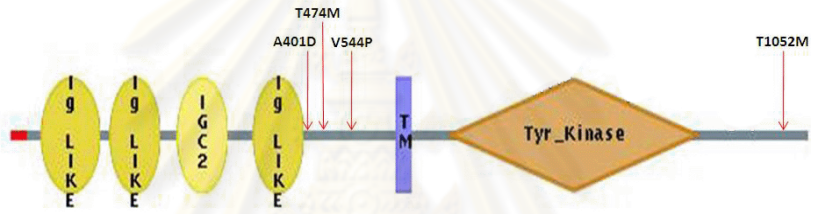


Figure9 The functional domain of *PDGFRa*

In addition to the non-synonymous variants in coding regions, we identified four variants in the 3' untranslated region (UTR) of the transcript; c.*34G>A, c.*51G>A, c.*455A>C, c.*480C>A. The 3'UTR Interestingly, one, c.*51G>A, corresponds to the conserved nucleotide within the predicted binding sites (c.*45-66 and c.*488-509) for the human microRNA (miRNA) hsa-miR-140 and the other three variants are located near the miRNA binding sites.

MiRNAs are known to inhibit gene expression by binding to the 3'UTR of the target transcript. It is currently estimated that miRNAs account for ~1% of predicted genes in higher eukaryotic genomes and that up to 30% of genes might be regulated by miRNAs. 3'UTR are reported to be associated with various diseases that include hereditary thrombophilia [59], α-thalassemia [60], insulin sensitivity [61], human papilloma virus infection[62] , increased sensitivity to 5-fluorouracil chemotherapy[63; 64], G-to-A transition in the 3'UTR of the myostatin gene in Texel sheep creates a potential

illegitimate miRNA target site, which recruits miR1 and miR206 binding and leads to translational inhibition of the gene, resulting in muscular hypertrophy[63]and SNPs residing in miRNA-binding sites were shown to affect the expression of miRNA targets and contribute to the susceptibility to complex disorders such as cancer, asthma, cardiovascular disease and Tourette syndrome[65]. Recently, it has been shown that SNP-829C>T leads to a decrease in microRNA binding leading to overexpression of its target and results in resistance to methotrexate. Studies have demonstrated that a naturally occurring miRSNP (a SNP located at or near a microRNA binding site in 3' UTR of the target gene or in a microRNA) is associated with enzyme overproduction and drug resistance. However, only very few miRNAs have been functionally characterized in details.

Of the four variants, one (the c.*455A>C) has similar prevalence between cases and controls. The other three have a significant difference or a trend to be different between cases and controls. We, therefore, performed a functional assay to determine the effects of the three variants found in 3'UTR of *PDGFRa*. Luciferase assays revealed that the c.*34G>A variant in the 3'UTR of *PDGFRa*, with the presence of a *miR-140*, significantly reduces expression of the luciferase. This provides a strong evidence that the c.*34G>A plays a role in pathogenesis of oral clefts.

Sequencing analysis of the *hsa-miR-140* identified one variant. This variant was also found in controls with similar prevalence. We therefore conclude that it is unlikely to play a role in oral cleft pathogenesis in our population.

In conclusion, our data represent an important step in understanding the complex mechanisms of expression regulation. We found cis-regulatory mechanisms to fine-tune expression of the *PDGFRa* by translational repression. We hypothesize that the repression expression of *PDGFRa* may affect interaction of *PDGFRa* mRNA with *miR-140* contributed to CL/P in the patients carrying c.*34G>A. For many complex disorders, the discovery of rare mutations in small subsets of patients has had a major impact in the identification of fundamental pathways that underlie disease pathogenesis. Further study of this new candidate gene, *PDGFRa*, may serve a similar role in the effort to better understand CL/P at the molecular and cellular level and may open novel therapeutic options in the treatment of this disease.

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APPENDICES

APPENDIX A
BUFFERS AND REAGENT

1. Lysis Buffer I

Sucrose	109.54	g
1.0 M Tris – HCl (pH 7.5)	10	ml
1.0 M $MgCl_2$	5	ml
Triton X – 100 (pure)	10	ml
Distilled water to	1,000	ml

Sterilize the solution by autoclaving and store in a refrigerator (at 4^0C).

2. Lysis Buffer II

5.0 M NaCl	15	ml
0.5 M EDTA (pH 8.0)	48	ml
Distilled water to	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

3. 10% SDS solution

Sodium dodecyl sulfate	10	g
Distilled water to	100	ml

Mix the solution and store at room temperature.

4. 20 mg/ml Proteinase K

Proteinase K	2	mg
Distilled water to	1	ml

Mix the solution and store in a refrigerator (at -20^{0}C).

5. 1.0 M Tris – HCl

Tris base	12.11	g

Dissolve in distilled water and adjusted pH to 7.5 with HCl

Distilled water to	100	ml

Sterilize the solution by autoclaving and store at room temperature.

6. 0.5 M EDTA (pH 8.0)

Disodium ethylenediamine tetraacetate.2H_2O	186.6	g

Dissolve in distilled water and adjusted pH to 8.0 with NaOH

Distilled water to	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

7. 1.0 M $MgCl_2$ solution

Magnesium chloride.6H_2O	20.33	g
Distilled water to	100	ml

Dispense the solution into aliquots and sterilize by autoclaving.

8. 5 M NaCl solution

Sodium chloride	29.25	g

Distilled water to	100	ml

Dispense the solution into aliquot and sterilize by autoclaving.

9. 10X Tris borate buffer (10X TBE buffer)

Tris – base	100	g
Boric acid	55	g
0.5 M EDTA (pH 8.0)	40	ml

Adjust volume to 1,000 ml with distilled water. The solution was mixed and store at room temperature.

10. 6X loading dye

Bromphenol blue	0.25	g
Xylene cyanol	0.25	g
Glycerol	50	ml
1M Tris (pH 8.0)	1	ml
Distilled water until	100	ml

Mixed and stored at 4^0C

11. 7.5 M Ammonium acetate (CH_3COONH_4)

Ammonium acetate	57.81	g
Distilled water	80	ml

Adjust volume to 100 ml with distilled water and sterilize by autoclaving.

12. 25:24:1 (v/v) Phenol-chloroform-isoamyl alcohol

Phenol	25	volume
Chloroform	24	volume
Isoamyl alcohol	1	volume

Mix the reagent and store in a sterile bottle kept in a refrigerator.

13. 2% Agarose gel (w/v)

Agarose	1.6	g
1X TBE	80	ml

Dissolve by heating in microwave oven and occasional mix until no granules of agarose are visible.

14. Ethidium bromide

Ethidium bromide	10	mg
Distilled water	1	ml

Mix the solution and store at 4^{0}C

15. LB agar

Agar	1.5	g
Peptone	1.0	g
Yeast extract	0.5	g
NaCl	0.5	g
Distilled water	100	ml

16. LB broth

Peptone	1.0	g
Yeast extract	0.5	g
NaCl	0.5	g
Distilled water	100	ml

17. SOC medium (100 ml)

Peptone		2.0	g
Yeast extract		0.5	g
1M NaCl	(1 ml)	0.06	g
1M KCl	(0.25 ml)	0.02	g
2M Mg^{2+}		1.0	ml
2M Glucose		1.0	ml
Distilled water		97.0	ml

18. Cell culture medium:

DMEM+10%(v/v) FCS+1%PenStrep

DMEM	45	ml
10% (v/v) FCS	5	ml
1%PenStrep	0.5	ml
Total Volume	50	ml

19. Cell culture medium:

DMEM+10%(v/v) charcoal-stripped FBS +1%PenStrep

DMEM	45	ml
10%(v/v)charcoal-stripped FBS	5	ml
1%PenStrep	0.5	ml
Total Volume	50	ml

APPENDIX B

Raw data of luiferase reporter assays

miR-140											
wt -*PDGFRa* 3'UTR			c.*51G>A -*PDGFRa* 3'UTR			c.*34G>A -*PDGFRa* 3'UTR			c.*480C>A -*PDGFRa* 3'UTR		
Renilla	Firefly	R/F	Renilla	Firefly	R/F	Renilla	Firefly	R/F	Renilla	Firefly	R/F
2,398,019	127,080	18.87	23,665,746	1,773,552	13.34	10,951,642	901,621	12.15	11,791,513	613,222	19.23
5,574,421	304,593	18.30	8,022,784	741,811	10.82	13,166,955	1,125,943	11.69	12,099,783	735,335	16.45
28,971,712	1,543,706	18.77	4,714,271	521,663	9.04	14,958,707	1,092,948	13.69	9,105,385	556,371	16.37
Ctrl miR											
wt -*PDGFRa* 3'UTR			c.*51G>A -*PDGFRa* 3'UTR			c.*34G>A -*PDGFRa* 3'UTR			c.*480C>A -*PDGFRa* 3'UTR		
Renilla	Firefly	R/F	Renilla	Firefly	R/F	Renilla	Firefly	R/F	Renilla	Firefly	R/F
7,536,114	235,676	31.98	24,867,676	1,173,048	21.20	3,037,141	150,032	20.24	15,669,074	653,771	23.97
8,349,205	365,815	22.82	28,413,148	1,440,732	19.72	14,571,493	563,766	25.85	16,756,442	730,704	22.93
3,919,973	146,196	26.81	31,345,510	1,444,282	21.70	14,777,337	733,235	20.15	7,311,114	373,953	19.55

Relative luciferase activity is expressed as the Renilla to Firefly ratio (R/F).

APPENDIX C

RAW DATA OF STATISTIC ANALYSIS

1. wt -*PDGFRa* 3'UTR(1) and c.*51G>A -*PDGFRa* 3'UTR(2)

Descriptives

luciferase

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.00	3	.6833	.01155	.00667	.6546	.7120	.67	.69
2.00	3	.5300	.10536	.06083	.2683	.7917	.43	.64
Total	6	.6067	.10746	.04387	.4939	.7194	.43	.69

Test of Homogeneity of Variances

luciferase

Levene Statistic	df1	df2	Sig.
4.087	1	4	.113

ANOVA

luciferase

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.035	1	.035	6.279	.066
Within Groups	.022	4	.006		
Total	.058	5			

2. wt -*PDGFRa* 3'UTR(1) and c.*34G>A -*PDGFRa* 3'UTR(2)

Descriptives

luciferase

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.00	3	.6833	.01155	.00667	.6546	.7120	.67	.69
2.00	3	.5667	.04726	.02728	.4493	.6841	.53	.62
Total	6	.6250	.07092	.02895	.5506	.6994	.53	.69

Test of Homogeneity of Variances

luciferase

Levene Statistic	df1	df2	Sig.
6.063	1	4	.070

ANOVA

luciferase

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.020	1	.020	17.254	.014
Within Groups	.005	4	.001		
Total	.025	5			

3. wt -*PDGFRa* 3'UTR(1) and c.*34G>A -*PDGFRa* 3'UTR(2)

Descriptives

luciferase

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.00	3	.6833	.01155	.00667	.6546	.7120	.67	.69
2.00	3	.7833	.07506	.04333	.5969	.9698	.74	.87
Total	6	.7333	.07285	.02974	.6569	.8098	.67	.87

Test of Homogeneity of Variances

luciferase

Levene Statistic	df1	df2	Sig.
11.191	1	4	.029

ANOVA

luciferase

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.015	1	.015	5.202	.085
Within Groups	.012	4	.003		
Total	.027	5			

BIOGRAPHY

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