# Association of *TFAP2B* and *KLHL20* Mutation in Patients with Patent Ductus Arteriosus

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**ABSTRACT**

Patent ductus arteriosus (PDA) is the second most common congenital heart disease in Suan Dok Hospital. Recent studies have reported mutations of the *TFAP2B* gene in patients with PDA. Recently, in a yet-to-be published study, the author's research team discovered a novel mutation of the *KLHL20* gene in a sibship with PDA. This study investigated whether PDA in Thai patients are associated with *TFAP2B* or *KLHL20* mutations. Two milliliters of saliva were collected from patients, DNA was extracted and amplified by PCR. Direct sequencing of all coding regions in the *TFAP2B* gene revealed a variance of a single base substitution in exons 6 and 7 and intron 2 in four patients. The DNA variances which were found have previously been described as single nucleotide polymorphisms. The author suggests that this finding is not a pathogenic cause of PDA, but may increase the risk of this disease.

**บทคัดย่อ**

ภาวะหลอดเลือดดักตัสอาร์เตอริโอซัสไม่ปิด เป็นโรคหัวใจพิการแต่กำเนิดที่พบได้บ่อย การศึกษาที่ผ่านมารายงานการกลายพันธุ์ของยีน *ทีแฟบ2บี* ในผู้ป่วยภาวะดังกล่าว การศึกษาล่าสุดของทีมผู้วิจัย พบการกลายพันธุ์ของยีน*เคแอลเอชแอล 20* ในพี่น้องซึ่งมีภาวะหลอดเลือดดักตัสอาร์เตอริโอซัสไม่ปิด การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาว่าการกลายพันธุ์ของยีน*ทีแฟบ2บี* และ*เคแอลเอชแอล20* สัมพันธ์กับภาวะหลอดเลือดดักตัสอาร์เตอริโอซัสไม่ปิดในผู้ป่วยไทยหรือไม่ ศึกษาโดยเก็บน้ำลายผู้ป่วย เพื่อนำไปสกัดดีเอ็นเอและดูความผันแปรของรหัสนิวคลีโอไทด์ที่พบในผู้ป่วย การถอดลำดับสารพันธุกรรมที่ครอบคลุมส่วนที่จะถอดรหัสเป็นโปรตีนของยีนทีแฟบ2บี พบการเปลี่ยนลำดับเบสในเอกซอนที่ 6, 7 และในอินตรอน2 ในผู้ป่วย 4 ราย อย่างไรก็ตามความผันแปรดังกล่าวถูกรายงานว่าเป็นซิงเกิล นิวคลีโอไทด์โพลีมอร์ฟิซึม จึงอาจไม่ก่อให้เกิดภาวะดักตัสอาร์เตอริโอซัสได้โดยตรง แต่อาจเพิ่มความเสี่ยงต่อการเกิดโรคได้

**Key Words:** Patent ductus arteriosus, *TFAP2B*, *KLHL20*

**คำสำคัญ:** ภาวะหลอดเลือดดักตัสอาร์เตอริโอซัสไม่ปิด *ยีนทีแฟบ2บี ยีนเคแอลเอชแอล20*

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## Introduction

Patent ductus arteriosus (PDA) is a congenital heart disease, which has been reported to be the second most common congenital heart disease in Chiang Mai University Hospital in the year 2012. PDA can be an isolated disease or can present as part of syndromes. Transcription AP 2 beta (TFAP2B), which is a neural-crest-related transcription factor, plays a vital role in ductal development during embryogenesis (Zhao et al., 2011). TFAP2B, together with endothelin-1 (ET-1) and hypoxia-induced transcription factor (HIF2*a*), acts in a transcriptional network, which results in genes encoding proteins that are important for the oxygen-sensing mechanism during ductus arteriosus closure (Ivey et al., 2008) Mutation in the gene encoding TFAP2B can cause syndromic PDA, Char Syndrome, which is characterized by patent ductus arteriosus, facial dysmorphic appearances and abnomalities of the middle phalanx of the fifth finger (Veetil,Gelb, 2008). Recent studies also suggest the association of *TFAP2B* mutation in isolated patent ductus arteriosus (Chen et al., 2011; Khetyar et al., 2008). Until now, ten mutations have been reported, of which six are missense mutations, one is a deletion, and three other mutations, which are found in splice site junctions (Figure 1) (Chen et al., 2011; Khetyar et al., 2008; Mani et al., 2005; Satoda et al., 1999; Zhao et al., 2001). Pathological mechanisms are due to the dominant-negative effect and happloinsufficiency (Chen et al., 2011; Mani et al., 2005; Zhao et al., 2001).

Recently, in a yet-to-be published study, the author's research team discovered a novel mutation of the *KLHL20* gene in a sibship of a Thai ethnic minority tribe with syndromic PDA. Therefore, PDA may be associated with *TFAP2B* or *KLHL20* mutations

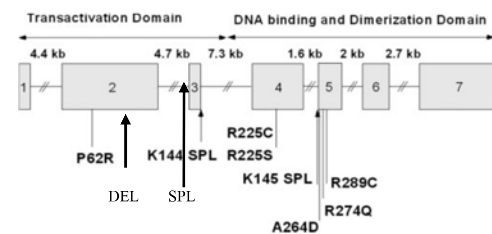


**Figure 1** Structure of *TFAP2B*. Exons are presented as boxes. Location and sequence of mutations are demonstrated. SPL is a splice site mutation, DEL is deletion. (Mani et al., 2005)

## Objective of the study

This study investigated whether isolated and syndromic patent ductus arteriosus in Thai patients are associated with *TFAP2B* or *KLHL20* mutations.

## Methodology

### Sample selection

Thirty saliva specimens were obtained from patients with PDA at the Department of Pediatric Cardiology, Faculty of Medicine, Chiang Mai University. All patients in this study were selected based on these conditions: patients with full term birth who were from four weeks to 15 years old, patients with syndromic or isolated PDA and patients with past a medical history of PDA but who had been treated by any proper treatment. Patients were excluded from the study if any one of the following conditions was found: silent PDA, any history of low birth weight, immature gestation, antenatal indomethacin administration, maternal rubella or neonatal infection. Medical history was obtained from interviewing. Clinical history taking and physical

examination were conducted to confirm the medical status of the patient. Informed consent was obtained from the parents of participating individuals.

**Control group**

Stored DNA samples of one hundred healthy, unrelated, consenting adults were used as a normal control group.

Two milliliters of saliva were collected from all participants using an Oragene® Saliva collecting kit (OG-575) (DNA Genotek Inc., Ottawa, ON, Canada).

This study was approved by the Human Experimentation Committees of the Faculty of Medicine and Faculty of Dentistry, Chiang Mai University.

**DNA preparation and Polymerase chain reaction (PCR)**

Genomic DNA was extracted from the saliva according to the prepIT® L2P protocol for the purification of genomic DNA from the Oragene® collecting kit (DNA Genotek Inc.). For polymerase chain reactions, the primers were designed such that each exon was flanked by a part of the corresponding introns. The DNA sample was diluted to 25 ng./ml. Seven exons of *TFAP2B* and 12 exons of *KLHL20* were amplified by PCR using a GENEAMP® PCR Instrument System 9700 (Applied Biosystems, Carlsbad, California, USA) with specific primers and conditions.

Primers of the *TFAP2B* and the *KLHL20* are demonstrated in Tables 1 and 2, respectively.

PCR reactions were carried out in a 25 µl. volume containing 25 ng./µl. genomic DNA, 10X PCR buffer, 10mM dNTPs, 10µM. of each of primer and 2.5 unit/µl. of HelixAmp™ *Hot-Taq* polymerse, $H_2O$ and DMSO. For the *TFAP2B* gene, samples were activated at 95°C for 15 minutes and submitted to 35 cycles of denaturation at 95°C for 30 seconds, annealing at appropriate temperatures (60°C for exons 1-3 for 40 seconds; 58°C for exons 4-7 for 40 seconds), and extension at 72°C for one minute. In the final cycle, a temperature of 72°C was extended to 10 minutes to ensure complete extension. For the *KLHL20* gene, samples were activated at 95°C for 15 minutes and submitted to 35 cycles of denaturation at 95°C for 30 seconds, annealing at appropriate temperatures (54°C for exons 2, 5, 6, 7, 9, 10 and 11 for 40 seconds, 55°C for exons 3-2, 8 and 12 for 40 seconds, 56°C for exon 4 for 40 seconds, 57°C for exon 3-1 for 40 seconds) and extension at 72°C for

**Table 1** Primers for amplifying the *TFAP2B* gene.

| Primer Name | Forward primer (5'-3') | Reverse primer (5'-3') | Ta. Use |
|---|---|---|---|
| TFAP2B 1F ,1R | TTTGGTGTGTATCCCCCATT | GAGAAAACCGACCGGAAATC | 60 |
| TFAP2B 2F, 2R | TCAGATCCTTGCTTCCCTTG | ACCTCTGGAAATCGCCACTA | 60 |
| TFAP2B 3F, 3R | GCATCCCAGATGTCTCTCAAA | GCCCTTTCCCCTAATCTGAC | 60 |
| TFAP2B 4F, 4R | GGCTTTGCCATCTGTTTGTT | AAATCTGCGGTAGGACTTGC | 58 |
| TFAP2B 5F, 5R | AAGGAAGGGGGAAAAATGTG | TTGTCTTGGAGGCTGGACCT | 58 |
| TFAP2B 6F, 6R | AAATGTCCAGCCAAATGTCC | ACTCTGCCTACCTTGCTTGC | 58 |
| TFAP2B 7F, 7R | CATTAGCCTCGCTCTTCGGT | AAATCCGGACGGTGGTCTCC | 58 |

one minute. In the final cycle, a temperature of 72°C was extended to 10 minutes to ensure complete extension.

**Gel electrophoresis**

PCR products were run on 1% agarose gel in TBE buffer using Sub-cell® GT (Bio-Rad, Hercules, California, USA) under the electrophoresis condition of 120 mA for 20 minutes and visualized by UV illumination from a Gel Doc XR+® machine (Bio-Rad).

**DNA sequence analysis**

Direct sequencing was performed by Functional Biosciences, Madison, Wisconsin, USA to detect mutations and polymorphisms in all patients and controls. All mutations were confirmed by repeat

**Table 2** Primers for amplifying the *KLHL 20* gene

| Primer Name | Forward primer (5'-3') | Reverse primer (5'-3') | Ta. Use |
| --- | --- | --- | --- |
| KLHL20 2F ,2R | TCTGCAGAAGGCCTGTGGTA | GCACTACATACAATCCACAGCAC | 54 |
| KLHL20 3F1, 3R1 | ACAGGATCACTTGAGCCCA | ACCTCTGGAAATCGCCACTA | 57 |
| KLHL20 3F2, 3R2 | TCTCTCTCCCCCATCATTCC | CTGGTAGAACACAAAATTGCCCT | 55 |
| KLHL20 4F, 4R | ACAGCTTTGTCCCATAACCC | CAAAGGAGCAAAAAGCCCACA | 56 |
| KLHL20 5F, 5R | GGTGCAGTGGTACAAGCC | TCTACACCAGTAAGCACCACAC | 54 |
| KLHL20 6F, 6R | TTATGCTTGCTTCCCTTTGCC | GGAATCATGGGTTATGAGTCAAGC | 54 |
| KLHL20 7F, 7R | TGGTATCATTGCCTTCCTTTGT | GGGAGTGGTAGTGTGTCATG | 54 |
| KLHL20 8F, 8R | TCAGCCCATACTTAGGGGAAA | AGCCTATGGGAAATCCACTGA | 55 |
| KLHL20 9F, 9R | AATAGCTTAATAGCTGGCACAGA | GCACTCTGCTGGAGGGAAAA | 54 |
| KLHL20 10F, 10R | ATGAGAGTATATGTAGTATTGTCCAGAGG | TACAACAGTTTGGAGGTTCCTC | 54 |
| KLHL20 11F, 11R | TCCCTGTGTTTCAGTAAACCAGAA | CCTCAGCTGAACATGATGGGA | 54 |
| KLHL20 12F, 12R | GTCCTGCTACTCAACAGTGCT | AGCTCCAAGGTCAAAGCTCC | 55 |

direct sequencing. The sequencing data was compared with the coding sequence of the GenBank accession number NM_003221.3 for *TFAP2B* gene and NM_014458.3 for *KLHL20* gene using Sequencher 4.8 Sequence analysis software (Genecodes, Ann Arbor, Michigan, USA). The changed amino acids were analyzed using Homo sapiens *TFAP2B* accession number NP_003212.2 and Homo sapiens KLHL20 accession number NP_055273.2 as the reference sequence.

**Results**

This study included 30 unrelated patients with a diagnosis of patent ductus arteriosus, none of whom showed the presence of facial dysmorphisms. The sequencing analysis of the *TFAP2B* gene showed DNA variances, which have been reported to be single-nucleotide polymorphisms (SNPs), in four patients. DNA variances are: a heterozygous transition (C*10A>T) at 3'UTR of exon 7 in two patients, a single base substitution in intron 2 (c.540+62G>C) in one patient, and a heterozygous transition (c.1006G>A) in exon 6 in the other patient (Table 3). DNA sequencing did not show any of the mutations of the *TFAP2B* or *KLHL20* genes, indicating that *TFAP2B* and *KLHL20* mutations were not present in the investigated patients with PDA.

**Discussion**

*TFAP2B* gene, the causative gene of Char syndrome, was selected to be the strongest candidate gene in our study for many reasons. First, this gene is expressed in cells derived from neural crest cells in the sixth aortic arch, which then develops into ductus arteriosus (Zhao et al., 2011). Second, this gene expressed during the entire process of ductus arteriosus development (Zhao et al., 2011) and has high intensity of expression in ductus smooth muscle cells (Ivey et al., 2008). Third, *TFAP2B* has an important role in ductus arteriosus closure (Ivey et al., 2008). Fourth, Zhao et al. found that rats and pups with *tfap2b*-/- died from heart failure. Histologic examination of *Tfap2b*-/- ductus arteriosus showed the ductal patency (Zhao et al., 2011). Fifth, Two studies have reported mutations of *TFAP2B* in patients with isolated patent ductus arteriosus (Chen et al., 2011; Khetyar et al., 2008). Although most of mutations they reported were familial, one mutation in 5'UTR (c.1-34G>A) was detected in an unrelated patient with isolated PDA (Chen et al., 2011; Khetyar et al., 2008). Until now, ten mutations have been identified in *TFAP2B* associated with

**Table 3** DNA sequencing results for *TFAP2B* gene

| Patient code | Phenotype | Exon/Intron Position | Nucleotide position and change | Amino acid change | Variation ID |
|---|---|---|---|---|---|
| 1396 | Isolated PDA | Exon7(3'UTR) | C*10A>T | - | rs2857513 |
| 1398 | Isolated PDA | Exon7(3'UTR) | C*10A>T | - | rs2857513 |
| 1414 | Isolated PDA | Intron2 | c.540+62G>C | - | rs2076308 |
| 1531 | Isolated PDA | Exon6 | c.1006G>A | p.Val336Ile | rs139339332 |

PDA, seven of which are associated with Char syndrome; two of which are found in isolated PDA and one of which is found in both conditions (Chen et al., 2011; Khetyar et al., 2008; Mani et al., 2005; Satoda et al., 1999; Zhao et al., 2001). In this study, the author did not find any mutations. Since patent ductus arteriosus is caused by multifactorial inheritance, which means that both genetic factors and environmental factors play a role in the pathogenesis of the disease (Nora, 1968), other uncontrolled environmental factors, such as the altitude of each individual's habitat, may play a role in the pathogenesis of PDA in patients without any DNA variance. The characteristic of heart disease inheritance is a threshold character, which means that whether or not the disease develops depends on the individual's threshold against the combination of all factors (Nora, 1968). Four patients with PDA with SNPs in this study may have had a combination of other environmental risk factors with their genetic factor sufficient to reach the threshold of disease and thus showed the disease character. On the other hand, normal people who have the same DNA variance do not develop the disease because either they do not have any environmental factors or they may have environmental risk factors but the combined disease-causing factors do not reach the threshold of the disease.

*KLHL20* is the other gene investigated in the study, due to its important role in the promotion of endothelial cell migration during the hypoxic stage of the endothelial cell of the human heart (Nacak et al., 2007; Higashimura et al., 2011). This migration is found during the ductus arteriosus closure mechanism (Coceani, Baragatti, 2012; Gournay, 2011; Weir et al., 2005). Although *KLHL20* may have a role in the development of ductus arteriosus, it also has many roles in other organs (Lee et al., 2010; Nacak et al., 2007). Two patients, in whom the author's research team found identical mutations, had many symptoms in many systems besides heart disease, so it may be possible that *KLHL20* may be associated with other symptoms. In previous studies, the amount of mutation detection varied depending on the technique of DNA analysis and sample selection used in each study. Direct sequencing results in the highest DNA mutation detection rate. Furthermore, DNA variation may be caused by abnormalities in any part of the genes, which are not detectable by direct sequencing.

**Conclusions**

In this study, the author reports *TFAP2B* variations in four Thai patients with isolated patent ductus arteriosus. The variances are a heterozygous transition (C*10A>T) at 3'UTR of exon7 in two patients, a single base substitution in intron 2 (c.540+62G>C) in one patient, and a heterozygous transition (c.1006G>A) in exon 6 in one patient. All of these DNA variances have previously been described as single nucleotide polymorphisms. The author suggests that these findings is not a pathogenic cause of patent ductus arteriosus, but may increase the risk of this disease.

**Acknowledgments**

We are grateful to the patients and their families for their cooperation. This study was supported by the Faculty of Dentistry, Chiang Mai University. We are grateful to Professor M. Kevin O Carroll, Professor Emeritus of the University of Mississippi School of Dentistry, USA, and Faculty

Consultant at the Faculty of Dentistry, Chiang Mai University for his assistance in the preparation of the manuscript.

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