

**MUTATION ANALYSIS IN *SMAD2*, *TGFβ2*, AND  
*SMURF2* GENES IN PATIENTS WITH THORACIC  
AORTIC ANEURYSM AND DISSECTION**

***ANALISIS MUTASI GEN SMAD2, TGFβ2, DAN SMURF2 PADA  
PASIEN DENGAN THORACIC AORTIC ANEURYSM AND  
DISSECTION***



**THESIS**

**Submitted to fulfill the assignment and fit-out requisite  
in passing Post-graduate Program Majoring Genetics Counseling  
Diponegoro University Semarang**

By  
Dian Mayasari Aji Atmaja  
22010110400089

**Biomedical Science Post Graduate Program  
Majoring Genetics Counseling  
Diponegoro University Semarang  
2013**

# THESIS

MUTATION ANALYSIS IN *SMAD2*, *TGF $\beta$ 2*, AND *SMURF2* GENES IN PATIENTS WITH THORACIC AORTIC ANEURYSM AND DISSECTION

Arranged by  
DIAN MAYASARI AJI ATMAJA, MD  
22010110400089

Has been defended in front of the defense committee  
and has been approved by:

**The Netherlands**  
Principal Supervisor,

**Indonesia**  
Principal Supervisor,

Gerard Pals, PhD

Bahrudin, MD, PhD  
NIP. 19760315 200604 1 001

Supervisor,

Supervisor,

Erik Sistermans, PhD

Prof. Sultana MH Faradz, MD, PhD  
NIP. 19520202 197901 2 001

Approved by,  
Head of Master Degree Program in Biomedical Science  
Post Graduate Program Diponegoro University

Prof. Dr. dr. Tri Nur Kristina, DMM, M.Kes  
NIP. 19590527 198603 2 001

## **DECLARATION**

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgement is made in the text.

Semarang, January 2013

Dian Mayasari A.A

## **CURRICULUM VITAE**

### **Personal Data**

Name : Dian Mayasari Aji Atmaja  
Sex : Female  
Nationality : Indonesian  
Place & Date of Birth : Semarang / November 14th, 1986  
Latest Degree : Medical Doctor  
Address : Wolter Monginsidi 105, Semarang, Central Java,  
Indonesia, 50192  
Mobile phone : +62 8122833122  
Email : dianmayasari04@gmail.com

### **Educational Background**

1992-1998 Elementary School at SD PL St.Yusuf Semarang  
1998-2001 Junior High School at SMP PL Domenico Savio Semarang  
2001-2004 High School at SMU Kolese Loyola Semarang majoring  
Natural Science  
2004-2008 Diponegoro University, Medical Faculty (Bachelor Degree)  
2008-2010 Diponegoro University, Medical Faculty (Medical Doctor)  
2011-present Post Graduate Program Diponegoro University, Master in  
Biomedical Science Majoring Genetic Counseling  
(Twinning Program with Vrije Universiteit Amsterdam,

The Netherlands)

### **Training and Course**

- 2010, 24-26<sup>th</sup> Sept 14<sup>th</sup> Annual Scientific Meeting of Indonesian Internist, Semarang, Indonesia (Certificate from PAPDI/ Perhimpunan Ahli Penyakit Dalam, Cab. Semarang)
- 2010, 20<sup>th</sup> Nov Treatment of Surgery Trauma and ECG Workshop (Certificate from Indonesian Surgeons Association / IKABI)
- 2011, 25-29<sup>th</sup> Jan 9<sup>th</sup> Medical Genetic Course: From Basic to Clinic (Certificate from Medical Faculty Diponegoro University, Semarang, Indonesia – Radboud University Medical Centre, The Netherland)
- 2011, 30<sup>th</sup> Jan Recurrent Pregnancy Loss (Certificate from Obstetric Gynecology Department, Faculty of Medicine, Diponegoro University, Semarang, Indonesia)
- 2011, 4-6<sup>th</sup> Feb “Advanced Trauma Life Support” Course (Certificate from Indonesian Surgeons Association/ IKABI)
- 2011, 18-20<sup>th</sup> July Workshop on Human Genetics, with special interest on Ophthalmogenetics (Certificate from Medical Faculty Diponegoro University Semarang – Radboud University Medical Centre, The Netherland)

2012, 23<sup>rd</sup>-25<sup>th</sup> Nov 2<sup>nd</sup> International Seminar and Workshop on Stem Cell and  
Clinical Biology: Stem Cell and Disorder of Sex  
Development (Certificate from Diponegoro University,  
Semarang, Indonesia)

**Working Experience and Internship**

2006-2007 Student Assistant in Biochemistry Department Medical  
Faculty Diponegoro University

## ACKNOWLEDGEMENT

It is a pleasure to express my gratitude to all of those who gave me the possibility to complete this thesis. Thank you for my supervisor in Netherlands, Gerard Pals, Phd, for his patience and encouragement in guiding and teaching me, for his ideas that support me to explore more for this research, and for his hospitality when I was in Netherlands. Thank you for allowing me to use the sample and for giving me experience doing internship under your supervision in VUmc DNA en Diagnostiek Laboratory.

Thank you for great teacher and my supervisor, Prof. Dr. Sultana MH Faradz, PhD, for her support in guiding and teaching me. It gave me spirit to finish this thesis. Working on this thesis would not be possible without her enormous help and support. Thank you for dr. Bahrudin, PhD for his patience and precision in guiding me to write this thesis.

Thank you for Prof. Ben CJ Hamel, MD, Phd and Helger Yntema, PhD for sharing the basic genetic in medical field from wide experience abroad since the first time I was starting this study. It gave me more knowledge and spirit to study in this field.

I wish to express sincere thanks to Erik Sistermans, PhD, the Head of Genome Diagnostic VU Medisch Centrum Amsterdam, The Netherlands, for the opportunity to undertake this research in his laboratory and for his enormous help which enabled me to experience learning molecular genetics in the laboratory.

I would also like to gratefully acknowledge the guidance and tuition of all my teachers and advisors in Genetic Counseling (Master Program of Biomedical Sciences Diponegoro University). Thank you to the Rector of Diponegoro University, Prof. Sudharto P Hadi, MES, PhD; the former Head of Biomedical Science Post Graduate Program of Diponegoro University DR. dr. Winarto, Sp.MK, Sp.M(K); the present Head of Biomedical Science Post Graduate Program of Diponegoro University Prof. DR. dr. Tri Nur Kristina, DMM, M.Kes, and the Dean of Medical Faculty Diponegoro dr. Endang Ambarwati, Sp.RM for the recommendation, the opportunity and great support in this study.

Thank you to all the staf of Centre for Biomedical Research, Semarang, Indonesia, particularly to Wiwik Lestari, Lusi Suwarsi, Dwi Kustiani, Intus and Rita Indriati for laboratory assistantship when I learn my first basic in molecular genetics.

I will always be grateful to all the staf of DNA Laboratory VUMC Amsterdam for their kindly help, cooperation and discussions on lab works. Thank you especially for Dimitra Micha, Youssef Moutouakil, and Rob van Anandel for the guidance during working hours in the laboratory. My thanks also go to my colleagues in the DNA division, Renate Bijman, Lysette van Bommel, and Maxine Cooks, also Jorrit Pals for helping me with the lab working.

My sincere thank would also go to all the patients, whose the DNA have been examined in the DNA Laboratory VUMC Amsterdam. Without their participation, this research would certainly never exist.



Thank you for my parents, Djoko Kamto Atmodjo and Maria Stefanie Silvana, who have been always supporting me in any situations.

Thank you for the reviewer, Prof. DR. dr. HA Faik Heyder, SpB, SpBTV, dr. RA Kisdjamiatun, MSc, PhD, and DR.dr.Suhartono, MKes for all the correction and advice for my thesis.

This opportunity to join the master degree, to have the laboratory experience in The Netherlands, and to do the research would not have been possible without the fellowship from Biro Perencanaan Kerjasama Luar Negeri (BPKLN), Ministry of Education, Indonesia, especially for DR. AB Susanto, MSc as the program coordinator for *Beasiswa Unggulan*. My grateful to all of the master degree and fellowship coordinators, especially to Prof. Dr. Sultana MH Faradz, PhD, Dr. Tri Indah Winarni, MsiMed, Ms. Ardina Aprilani, and Dr. Farmaditya Eka Putra M, I am deeply thankful for your hardworks.

## CONTENTS

<b>TITLE</b> .....	i
<b>APPROVAL SHEET</b> .....	ii
<b>DECLARATION</b> .....	iii
<b>CURRICULUM VITAE</b> .....	iv
<b>ACKNOWLEDGEMENT</b> .....	vii
<b>CONTENTS</b> .....	x
<b>ABBREVIATIONS</b> .....	xiv
<b>LIST OF TABLES</b> .....	xvi
<b>LIST OF FIGURES</b> .....	xvii
<b>LIST OF APPENDIX</b> .....	xix
<b>ABSTRACT (ENGLISH)</b> .....	xx
<b>ABSTRAK (BAHASA INDONESIA)</b> .....	xxi
<b>CHAPTER 1 INTRODUCTION</b> .....	1
1.1 Background.....	1
1.2 Research Question .....	3
1.3 Research Purposes .....	3
1.4 Research Benefits.....	4
1.5 Originality.....	4
<b>CHAPTER 2 STATE-OF-THE-ART LITERATURE REVIEW</b> .....	6
2.1 Thoracic Aortic Aneurysm and Dissection (TAAD) .....	6
2.1.1 Epidemiology of TAAD .....	6

2.1.2 Classification and Diagnosis of TAAD .....	7
2.1.2.1 Anatomical Classification of TAAD .....	7
2.1.2.2 Clinical Classification of TAAD .....	9
2.1.2.3 Diagnosis of TAAD .....	10
2.1.3 Familial TAAD.....	13
2.1.3.1 <i>SMAD2</i> Gene.....	14
2.1.3.2 <i>TGFβ2</i> Gene .....	15
2.1.3.3 <i>SMURF2</i> Gene .....	16
2.1.4 Pathogenesis of TAAD .....	17
2.1.4.1 Anatomy and Histology of Thoracic Aortic .....	17
2.1.4.2 Vascular Smooth Muscle Cell.....	18
2.1.4.3 Extracellular Matrix .....	19
2.1.5 Molecular Pathology of TAAD.....	24
2.1.5.1 Cytoskeletal Pathway.....	24
2.1.5.2 Transforming Growth Factor Beta (TGFβ) Pathway .....	25
2.1.5.3 Matrix Metalloproteinase (MMP) Pathway .....	28
2.2 Identification of Mutation by High Resolution Melting .....	29
2.3 Theoretical Framework .....	33
2.4 Conceptual Framework .....	34
<b>CHAPTER 3 RESEARCH METHODS.....</b>	<b>35</b>
3.1 Research Field .....	35
3.2 Setting, Location, and Period of Research .....	35
3.3 Research Design .....	35

3.4 Population and Sample.....	35
3.4.1 Population .....	35
3.4.2 Sample .....	36
3.4.2.1 Inclusion Criteria.....	36
3.4.2.2 Exclusion Criteria.....	37
3.5 Research Variables.....	37
3.6 Mutation Detection .....	37
3.6.1 Preparation .....	37
3.6.2 Amplification and High Resolution Melting (HRM) .....	39
3.6.3 Curve Analysis .....	39
3.6.4 DNA Sequencing.....	39
3.6.5 Mutation Analysis .....	41
3.6.6 Confirmation of the mutation on healthy control samples.....	42
3.7 Research Flow .....	43
3.8 Data Analysis.....	44
3.9 Research Ethics.....	44
<b>CHAPTER 4 RESULTS .....</b>	<b>45</b>
4.1 Mutation Screening.....	45
4.2 Pathogenicity Prediction.....	48
4.3 Confirmation of the mutation on healthy control samples.....	51
<b>CHAPTER 5 DISCUSSION .....</b>	<b>54</b>
<b>CHAPTER 6 CONCLUSION AND FUTURE DIRECTIONS .....</b>	<b>60</b>
<b>CHAPTER 7 SUMMARY .....</b>	<b>62</b>

<b>REFERENCES</b> .....	66
<b>Appendix</b> .....	72

## ABBREVIATIONS

ACTA2	Actin Smooth Muscle Alpha 2
BAV	Bicuspid Aortic Valve
COL3A1	Collagen type 3 Alpha 1
ECM	Extracellular Matrix
EDS	Ehler-Danlos Syndrome
FBN1	Fibrillin 1
FTAAD	Familial Thoracic Aortic Aneurysm and Dissection
HRM	High Resolution Melting
LAP	Latency Associate Propeptide
LDS	Loeys-Dietz Syndrome
LLC	Large Latent Complex
LTBP	Latent TGF $\beta$ Binding Protein
MFS	Marfan Syndrome
MMP	Matrix Metalloproteinase
MYH11	Myosin Heavy Chain 11
MYLK	Myosin Light Chain Kinase
PDA	Patent Ductus Arteriosus
SLC	Small Latent Complex
SMAD	Mothers against decapentaplegic
SMURF2	Smad Ubiquitination Regulatory Factor 2
TAA	Thoracic Aortic Aneurysms

TAAD	Thoracic Aortic Aneurysms and Dissection
TGF $\beta$	Transforming Growth Factor Beta
TGF $\beta$ R	Transforming Growth Factor Beta Receptor
VSMC	Vascular Smooth Muscle Cell

## LIST OF TABLES

<b>Table 1</b>	List of previous associated studies .....	5
<b>Table 2</b>	Normal adult aortic diameters.....	7
<b>Table 3</b>	Risk factors for development of thoracic aortic dissection.....	9
<b>Table 4</b>	Genetic properties of TAAD.....	13
<b>Table 5</b>	The accession number of gene studied according to Genbank and Ensembl.....	38
<b>Table 6</b>	M13 primers.....	38
<b>Table 7</b>	List of all mutations, SIFT prediction, AGVGD and risk estimation.....	49
<b>Table 8</b>	Presentation of Gene Variant in the TAAD patients and in the control patients. ....	52



## LIST OF FIGURES

<b>Figure 1</b>	Classification of thoracic aortic dissection .....	8
<b>Figure 2</b>	Smad7 and Smurf2 regulate receptor turnover .....	16
<b>Figure 3</b>	Normal anatomy of the thoracoabdominal aorta.....	17
<b>Figure 4</b>	Fibrilin formation and its relation to other proteins .....	22
<b>Figure 5</b>	The cytoskeletal pathway of TAAD.....	25
<b>Figure 6</b>	Canonical signaling by TGF $\beta$ family members .....	27
<b>Figure 7</b>	Example of PCR and high resolution melting program .....	30
<b>Figure 8</b>	Distinguish heterozygote and homozygote allelic in the HRM curve .....	31
<b>Figure 9</b>	Example analysis of HRM curve .....	32
<b>Figure 10</b>	Align GVGD classifiers and risk estimation .....	42
<b>Figure 11</b>	Mutation at exon 3 of <i>SMAD2</i> in a TAAD patient (VD number 32868) .....	45
<b>Figure 12</b>	Mutation at exon 12 of <i>SMAD2</i> in TAAD patients. ....	46
<b>Figure 13</b>	Mutation at exon 1 of <i>TGF<math>\beta</math>2</i> in TAAD patients (VDnumber 26967 and 23496). ....	47
<b>Figure 14</b>	Mutation at exon 3 of <i>TGF<math>\beta</math>2</i> in a TAAD patient (VDnumber 21433). ....	47
<b>Figure 15</b>	Mutation at exon 4 of <i>TGF<math>\beta</math>2</i> in TAAD patients (VDnumber 24640 and 10D6295).....	48
<b>Figure 16</b>	Difference in predicted glycosylation between normal and	

	mutant form of <i>SMAD2</i> , c.6_8del, p.Ser3del.....	50
<b>Figure 17</b>	Difference in predicted phosphorylation between normal and mutant form of <i>SMAD2</i> , c.1346 T>C, p.Leu449Ser.....	50
<b>Figure 18</b>	Difference in predicted phosphorylation between normal and mutant form of <i>SMAD2</i> , c.1369 G>A, p.Gly457Arg .....	51
<b>Figure 19</b>	HRM curve analysis to confirm mutation in control healthy sample for exon 3, mutation of <i>SMAD2</i> , c.6_8del (Ser3del).....	52
<b>Figure 20</b>	HRM curve analysis to confirm mutation in control healthy sample for exon 12, mutation of <i>SMAD2</i> , c.1346T>C (Leu449Ser).....	53
<b>Figure 21</b>	HRM curve analysis to confirm mutation in control healthy sample for exon 12, mutation of <i>SMAD2</i> , c.1369G>A (Gly457Arg).....	53
<b>Figure 22</b>	<i>SMAD2</i> domain and location of the variants found.....	55
<b>Figure 23</b>	TGF $\beta$ 2 domain and location of the variants found.....	56

## LIST OF APPENDIX

<b>Appendix 1</b>	Informed consent form.....	72
<b>Appendix 2</b>	High resolution melting mixture and specification.....	76
<b>Appendix 3</b>	Primer sequence and HRM setting program .....	77

## ABSTRACT

**Background:** Thoracic aortic aneurysm and dissection (TAAD) is one of 15<sup>th</sup> most leading cause of the death in USA and of the silent killer in the world. Several genes associated with TAAD have been recognized, i.e. *FBNI*, *ACTA2*, *TGF $\beta$ 1*, *TGF $\beta$ 2*, *MYH11*, *MYLK* and *SMAD3*. However, many cases of familial TAAD have not been found for the mutation in those genes. The other genes that might be had association with TAAD are *SMAD2*, *TGF $\beta$ 2* and *SMURF2*.

**Methods:** Three hundred sixty five patients with TAAD and related disorders, who did not carry any mutation in *FBNI*, *TGF $\beta$ 1*, *TGF $\beta$ 2*, *ACTA2* and *MYH11* were included. Mutation screening of the gene variants in *SMAD2*, *TGF $\beta$ 2* and *SMURF2* were done by using high resolution melting (HRM) technique. The aberrant sample patterns on the curve analysis then were sequenced. Confirmation of the mutation was done by comparing the HRM curves between patients and the healthy control. The pathogenicity potency of the mutation was predicted by using mutation prediction software SIFT and align GVGD, which integrated in Alamut software. Phosphorylation/glycosylation site was predicted by YinOYang software.

**Results:** Three patients were found to carry *SMAD2* mutation, i.e., c.6\_8del, c.1346T>C, c.1369G>A. One mutation, c.1369G>A was predicted to increase phosphorylation, and the other mutations predicted to loss of phosphorylation/glycosylation site. All of the *SMAD2* mutations were not found in the control. One patient was found to carry nonsense mutation of *TGF $\beta$ 2*, c.547C>T (Gln183X). Two missense mutation of *TGF $\beta$ 2*, c.272G>A and c.703C>G were found in four patients and have been registered in SNP databases with frequency less than 1%. All mutations in *SMAD2* and *TGF $\beta$ 2* were predicted to be pathogenic. No mutation was found in *SMURF2* gene.

**Conclusion:** Three novel mutations were found in *SMAD2* gene and one novel mutation was found in *TGF $\beta$ 2* in TAAD patients who did not carry any mutation in *FBNI*, *TGF $\beta$ 1*, *TGF $\beta$ 2*, *ACTA2* and *MYH11*.

---

Keywords: Thoracic aortic aneurysm and dissection, mutation, *SMAD2*, *TGF $\beta$ 2*, *SMURF2*

## ABSTRAK

**Latar belakang:** *Thoracic aortic aneurysm and dissection* (TAAD) adalah satu dari 15 teratas penyebab kematian di Amerika Serikat dan merupakan *silent killer* di dunia. Beberapa gen yang berkaitan dengan TAAD sudah dikenali yaitu *FBN1*, *ACTA2*, *TGF $\beta$ 1*, *TGF $\beta$ 2*, *MYH11*, *MYLK* dan *SMAD3*. Namun, pada banyak kasus TAAD familial tidak ditemukan mutasi pada gen-gen tersebut. Gen lain yang kemungkinan berkaitan dengan TAAD adalah *SMAD2*, *TGF $\beta$ 2* dan *SMURF2*.

**Metode:** Sampel DNA diambil dari 365 pasien TAAD dan penyakit terkait, yang tidak memiliki mutasi di gen *FBN1*, *TGF $\beta$ 1*, *TGF $\beta$ 2*, *ACTA2* dan *MYH11*. Skrining mutasi gen pada *SMAD2*, *TGF $\beta$ 2* dan *SMURF2* dilakukan dengan teknik *high resolution melting* (HRM). Sampel dengan pola berbeda pada analisa kurva dilakukan sekuensing. Konfirmasi mutasi dilakukan dengan membandingkan kurva HRM antara pasien dan kontrol sehat. Potensi patogenitas mutasi diprediksi dengan software SIFT dan *align* GVG, yang tergabung dalam software Alamut. Situs fosforilasi/glikosilasi diprediksi dengan software YinOYang.

**Hasil:** Mutasi c.6<sub>del</sub>, c.1346T>C, c.1369G>A pada gen *SMAD2* ditemukan pada 3 pasien yang berbeda. Mutasi c.1369G>A diprediksi meningkatkan fosforilasi dan dua mutasi lainnya kehilangan situs fosforilasi/glikosilasi. Semua mutasi *SMAD2* tidak ditemukan pada kontrol. Mutasi c.547C>T (Gln183X) pada gen *TGF $\beta$ 2* ditemukan pada satu pasien. Dua mutasi pada gen *TGF $\beta$ 2*, c.272G>A dan c.703C>G ditemukan pada empat pasien dan telah terdaftar di database SNP dengan frekuensi kurang dari 1%. Semua mutasi pada *SMAD2* dan *TGF $\beta$ 2* tersebut diprediksikan patogenik. Tidak ditemukan mutasi pada gen *SMURF2*.

**Kesimpulan:** Tiga mutasi novel ditemukan pada gen *SMAD2* dan satu mutasi novel ditemukan pada gen *TGF $\beta$ 2* pada pasien TAAD yang tidak memiliki mutasi pada gen *FBN1*, *TGF $\beta$ 1*, *TGF $\beta$ 2*, *ACTA2* dan *MYH11*.

---

Kata kunci: *Thoracic aortic aneurysm and dissection*, mutasi, *SMAD2*, *TGF $\beta$ 2*, *SMURF2*