PROFILING SERINE-THREONINE KINASE PHOSPHORYLATION IN TGFβ PATHWAY IN TAAD PATIENTS WITH TGFBR2 MUTATION

PROFIL FOSFORILASI KINASE SERINE THREONINE TGFβ PATHWAY PADA PASIEN TAAD DENGAN MUTASI TGFBR2

THESIS
Submitted to fulfill the assignment and fit-out requisite
in passing Post-Graduate Program

Faculty of Medicine
Diponegoro University Semarang

Donny Nauphar
22010111400098

FACULTY OF MEDICINE
DIPONEGORO UNIVERSITY
SEMARANG
2013

PROFILING SERINE-THREONINE KINASE PHOSPHORYLATION IN TGFβ PATHWAY IN TAAD PATIENTS WITH TGFBR2 MUTATION

PROFIL FOSFORILASI KINASE SERINE THREONINE TGFβ PATHWAY PADA PASIEN TAAD DENGAN MUTASI TGFBR2

THESIS
Submitted to fulfill the assignment and fit-out requisite in passing Post-Graduate Program

Faculty of Medicine
Diponegoro University Semarang

Donny Nauphar
22010111400098

FACULTY OF MEDICINE
DIPONEGORO UNIVERSITY
SEMARANG
2013

THESIS

PROFILING SERINE-THREONINE KINASE PHOSPHORYLATION IN TGFβ PATHWAY IN TAAD PATIENTS WITH TGFBR2 MUTATION

Arranged by
DONNY NAUPHAR, B.Sc.
22010111400098

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

The Netherlands	**Indonesia**
Principal Supervisor,	Principal Supervisor,
Gerard Pals, PhD	Prof. Sultana MH Faradz, MD, PhD NIP. 19520202 197901 2 001
Supervisor,	Supervisor,
Erik Sistermans, PhD	dr. Farmaditya E.P. Mundhofir, Msi.Med. PhD NIP. 19810425 200812 1 002

Approved by,
Head of Master Degree Program in Biomedical Science
Faculty of Medicine Diponegoro University

Prof. Dr. dr. Tri Nur Kristina, DMM, M.Kes
NIP. 19590527 1986603 2 001

DECLARATION

I hereby declare that this thesis is my own work and has not been submitted in any forms for another degree or diploma at any university or other institution of tertiary education and there are no elements belonging as Plagiarism mentioned forth in Permendiknas No. 17 of 2010. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of reference is given.

Medan, December 2013

Donny Nauphar

CURRICULUM VITAE

Personal Data

Name	: Donny Nauphar
Sex	: Male
Nationality	: Indonesian
Place & Date of Birth	: Medan, November 25th 1981
Last Degree	: Bachelor of Science (Biotechnology)
Address	: Jendral Gatot Subroto 236H, Medan, North Sumatera Indonesia 20119
Mobile Phone	: +62 81262511903
Email	: dnauphar@yahoo.com

Educational Background

1990-1995	Elementary School at SD Harapan 1 Medan
1995-1997	Junior High School at SMP Harapan 1 Medan
1997-1999	High School at SMU Negeri 4 Medan majoring in Natural Science
1999-2000	Sunway College, Canadian International Matriculation Program, Diploma in Science
2000-2003	Monash University, Bachelor of Science in Biotechnology

2012-Present Post Graduate Program Diponegoro University. Master in Biomedical Science Majoring in Genetic Counseling (Joint Degree Program with Vrije Universiteit Amsterdam, The Netherlands)

Training and Course

2006 ***BRAHMS Kryptor Classic Field Service Engineer***
Cezanne, Nimes, France

2008 ***BRAHMS Kryptor Compact Field Service Engineer***
Cezanne, Nimes, France

2009 ***BRAHMS Kryptor Classic Expert Engineer Training***
Cezanne, Nimes, France

2009 ***BRAHMS Kryptor Advanced Application Training***
Cezanne, Nimes, France

2009 ***Responsible Care and Use of Laboratory Animals***
National University of Singapore Laboratory Animals Center

2011 ***Good Clinical Practices***
Komisi Nasional Etik Penelitian Kesehatan in collaboration with Fakultas Kedokteran Universitas Sumatera Utara

2011 ***The 3rd Annual Pathobiology Course From Concept to Clinic: Leading Stem Cell Science and Medical Practice***
Ikatan Dokter Indonesia Sumatera Utara

2012 ***10th Medical Genetic Course: From Basic to Clinic***
Faculty of Medicine Diponegoro University in collaboration with Radboud University Nijmegen Medical Center, The Netherlands

2012 ***Workshop on Neurogenetics***

Ikatan Dokter Indonesia Jawa Tengah

2012 ***Ciliary Dysfunction in Developmental Abnormalities and Diseases Symposium***

Center for Biomedical Research Faculty of Medicine Diponegoro University in collaboration with Radboud University Nijmegen Medical Center, The Netherlands

Working Experience

2009-2011 Dyamed Biotech Pte. Ltd. Singapore

Senior Product Specialist

2009-2011 The Biofactory Pte. Ltd. Singapore

Equipment Development Manager

2006-2009 Dyamed Biotech Pte. Ltd. Singapore

Product Specialist

Publications

2010 Device performance testing using the Microkit Lysonator prototype or sample preparation to investigate the use of DNA intercalating dyes for the distinction of live/dead cells by PCR
Singapore : Singapore Polytechnic, School of Chemical and Life Sciences, 2010.

ACKNOWLEDGEMENT

It is a pleasure to express my gratitude to all of those who gave me the opportunity and support to complete this thesis. My heartfelt gratitude to my supervisor in Netherlands, Gerard Pals, PhD, and Erik Sistermans, PhD, for their support and encouragement in this research and for the hospitality during my stay in The Netherlands. Thank you for providing me the samples, adequate training and freedom to explore the topic that I am interested in during my research at the Center for Connective Tissue Disorders, Department of Clinical Genetics Vrije Universiteit Medisch Centrum, Amsterdam, The Netherlands.

My utmost gratitude to my teacher and supervisor, Prof. Dr. Sultana MH Faradz, PhD for her relentless support and guidance and despite her extremely busy schedule, still find time to encourage me to finish this thesis. Thank you to dr. Farmaditya EP Mundhofir, PhD for his patience and guidance in guiding me in writing this thesis.

My utmost gratitude to dr. Yahwardiah Siregar, PhD for the opportunity and all the support given to me during my research.

I wish to thank all the staff of the Center for Biomedical Research (CEBIOR), Semarang, Central Java, Indonesia, Dwi Kustiani, Intus, Lusi Suwarsi, Rita Indriati, and Wiwik Lestari for their assistance during my study.

I would also like to thank all the staff of the Department of Clinical Genetics Vrije Universiteit Medisch Centrum, Amsterdam, The Netherlands for their hospitality, help and guidance during my work in the laboratory. Special

thanks to Dimitra Micha and Youssef Moutouakil for mentoring me in Cell Culture and DNA works. My sincere thanks to great colleagues that support me during my stay, Kirsten Verheul, Mark aan t'Goor, Ans Morren, Irma Van Der Beek, Haweya Shire, Wesley Busch, Gwen Kwak, Jorrit Pals, Emily Nash and Martijn Boone.

I wanted to thank Batch 6 students if the Masters in Biomedical Science, Almira Zada, An Nisaa Utami Tihnulat, Ariestya Indah Permata Sari, Gara Samara Brajadenta, Hesty Wahyuningsih, Isna Rahmia Fara, Stefani Harum Sari, Venty Muliana Sari, Ziske Maritska, Zukhrofi Muzar and Wahid Mouhib. Your support and encouragement has been a source of inspiration for me to finish this thesis.

My sincere thanks also go to all the patients, whose fibroblast have been examined in the Department of Clinical Genetics Vrije Universiteit Medisch Centrum, Amsterdam. Without them, this research would not be possible.

My utmost gratitude to my parents, Salim Usman and Juanita Tanuwijaya and my in-laws, Sujono Djojodihardjo and Setiawati, to my wife Sesilia Susetiasih and my boys, Dito Aditama Usman and Devin Saputra Usman, who have been my strength and my support in my research.

This opportunity to join the Master's Degree, to have my research in the Netherlands would not have been possible without the fellowship from Biro Perencanaan Kerjasama Luar Negeri (BPKLN), Ministry of Education, Indonesia, especially DR. AB Susanto, MSc. as the *Beasiswa Unggulan* program coordinator.

I would like to thank all of the Master's Degree and Fellowship coordinators, especially Prof. Dr. Sultana MH Faradz, PhD, Dr. Tri Indah Winarni, MsiMed. PhD, Ms. Ardina Aprilani, and Dr. Farmaditya EP Mundhofir, PhD for all their hardwork.

CONTENTS

TITLE	i
APPROVAL SHEET	ii
DECLARATION	iii
CURRICULUM VITAE	iv
ACKNOWLEDGEMENT	vii
CONTENTS	x
GLOSSARY	xiii
ABBREVIATIONS	xiv
LIST OF TABLES	xvi
LIST OF FIGURES	xvii
LIST OF APPENDIX	xviii
ABSTRACT (ENGLISH)	xix
ABSTRAK (BAHASA INDONESIA)	xx
CHAPTER 1 INTRODUCTION	1
1.1 Background	1
1.2 Research Question	3
1.3 Research Purpose	3
1.3.1 General Research Purposes	3
1.3.2 Specific Research Purposes	3
1.4 Research Benefits	4
1.5 Originality	4

CHAPTER 2 STATE-OF-THE-ART LITERATURE REVIEW 5

2.1 Thoracic Aortic Aneurysm and Dissection (TAAD 5

2.1.1 Epidemiology of TAAD 5

2.1.2 Classification and Diagnosis of TAAD 6

2.1.2.1 Anatomical Classification of TAAD 6

2.1.2.2 Clinical Classification of TAAD 8

2.1.2.3 Diagnosis of TAAD 9

2.1.3 Pathogenesis of TAAD 12

2.1.3.1 Anatomy and Histology of Thoracic Aorta 12

2.1.3.2 Vascular Smooth Muscle Cells 13

2.1.3.3 Extracellular Matrix 14

2.1.4 Molecular Pathology of TAAD 20

2.1.4.1 Cytoskeletal Pathway TGFβ Pathway 20

2.1.4.2 MMP Pathway 24

2.1.4.3 ATII Pathway 25

2.2 Protein Kinases and Serine Threonine Kinases 26

2.3 Theoretical Framework 28

2.4 Conceptual Framework 28

CHAPTER 3 RESEARCH METHODS 29

3.1 Research Field 29

3.2 Settings, Location, and Period of Research 29

3.3 Research Design 29

3.4 Samples 29

3.5 Kinase Substrate Profiling using PamStation and PamChip 30

3.5.1 Growing Cells 30

3.5.2 Seeding to 6-well plates 31

3.5.3 Serum Starvation 33

3.5.4 Stimulation with TGF-β1 33

3.5.5 Preparing Cell Lysates 33

3.5.6 Protein Concentration Determination 34

3.5.7 PamChip Serine-Threonine Microarray 34

3.6 Research Flow 38

3.7 Data Analysis 38

3.8 Research Ethics 39

CHAPTER 4 RESULTS 40

4.1 Array Images 40

4.2 Volcano Plot with Bionavigator log p-value and log fold-change 43

CHAPTER 5 DISCUSSION 48

CHAPTER 6 CONCLUSION AND FUTURE DIRECTIONS 55

CHAPTER 7 SUMMARY 56

RINGKASAN 60

REFERENCES 64

APPENDICES 71

ARTICLE 85

SUPPLEMENTARY MATERIAL 90

GLOSSARY

Aneurysm	A localized, blood-filled balloon-like bulge in the wall of a blood vessel.
Aorta	A large blood vessel that distributes blood from the heart to the rest of the body.
Canonical	Typical pathway utilized by a signal transduction pathway.
Dissection	Tearing of layers in blood vessel that cause abnormal blood flow to occur in between the layers.
Fibroblast	Most common connective tissue in animals. Responsible for synthesis of extracellular matrix and collagen.
Kinase domain	Part of the protein that contains tyrosine, serine, threonine, and histidine that are target for phosphorylation by protein kinases.
Kinase substrate	Specific molecules target of protein kinase.
Ligand	A molecule that binds on a receptor
Microarray	Collection of DNA or protein fragments embedded on a solid surface. Often used for simultaneous analysis of large number of genes or proteins.
Non-canonical	Alternative pathway utilized by a signal transduction pathway.
Protein kinase	Enzyme that transfers high energy phosphate group for a donor molecule to a specific acceptor molecule.
Receptor	A protein molecule that receives ligands.
TGFβ pathway	A molecular pathway that controls proliferation, apoptosis, cellular differentiation, and other cellular functions.

ABBREVIATIONS

ACTA2	Actin Smooth Muscle Alpha 2
ATII	Angiotensin II
ATP	Adenosine Tri Phosphate
BAD	Bcl2 Antagonist of Cell Death
BAV	Bicuspid Aortic Valve
BCKDK	Branched-chain Ketoacid Dehydrogenase Kinase
BMP	Bone Morphogenetic Protein
COL3A1	Collagen type 3 Alpha 1
CGHB	Choriogonadotropin Subunit Beta Precursor
ECM	Extracellular Matrix
EDS	Ehler-Danlos Syndrome
EMT	Epithelial-to-Mesenchymal Transition
FBN1	Fibrillin 1
FRAP	FKBP12-rapamycin Complex-associated Protein
FTAAD	Familial Thoracic Aortic Aneurysm and Dissection
JNK	C-Jun N-terminal Kinase
KCNA	Potassium Voltage-gated Channel Subfamily A
LAP	Latency Associate Propeptide
LDS	Loeys-Dietz Syndrome
LLC	Large Latent Complex
LTBP	Latent TGFβBinding Protein
MFS	Marfan Syndrome

MMP	Matrix Metalloproteinase
MTOR	Mammalian Target of Rapamycin
MYH11	Myosin Heavy Chain 11
MYLK	Myosin Light Chain Kinase
PI3K	Phosphoinositide 3-Kinase
RAP	Ras-related Protein
ROCK	Rho-associated Protein Kinase
RYR	Ryanodine Receptor
SLC	Small Latent Complex
SMAD	Small Mothers Against Decapentaplegic
SLRP	Small Leucine Rich Proteogylcans
SMURF2	Smad Ubiquitination Regulatory Factor 2
TAA	Thoracic Aortic Aneurysms
TAD	Thoracic Aortic Dissection
TAK	TGFβ-activated Kinase
TAAD	Thoracic Aortic Aneurysms and Dissection
TGFβ	Transforming Growth Factor Beta
TGFβR	Transforming Growth Factor Beta Receptor
VASP	Vasodilator-associated Phosphoprotein
VSMC	Vascular Smooth Muscle Cell

LIST OF TABLES

Table 1	Normal adult aortic diameters	6
Table 2	Risk factors for development of thoracic aortic dissection	8
Table 3	Samples and controls	30
Table 4	Significant peptides from unstimulated controls and mutants	44
Table 5	Significant peptides from stimulated controls and mutants	45
Table 6	Function and mutation effects of the significant peptides from the unstimulated group	49
Table 7	Function and mutation effects of the significant peptides from the unstimulated group.	50

LIST OF FIGURES

Figure 1	Classification of thoracic aortic dissection	7
Figure 2	Normal anatomy of the thoracoabdominal aorta	12
Figure 3	Fibrillin formation and its relation to other proteins	17
Figure 4	The cytoskeletal pathway of TAAD	20
Figure 5	The canonical and non-canonical TGFβ pathway	23
Figure 6	Proposed Regulation of SMADs and downstream transcription	27
Figure 7	Plate 1 – Unstimulated samples	32
Figure 8	Plate 2 – Stimulated samples	32
Figure 9	The PamStation12	35
Figure 10	The Serine-Threonine Kinase PamChip and Reagents	35
Figure 11	The PamChip Array	36
Figure 12	PamChip sample layout	37
Figure 13	Array images	42
Figure 14	Volcano plot of unstimulated controls and sample	44
Figure 15	Volcano plot of stimulated controls and samples	47
Figure 16	Peptide hits on the TGFβ pathway	51
Figure 17	A schematic representation of SMAD2 and SMAD3	52
Figure 18	Possible pathways for targeted drug therapy	54

LIST OF APPENDIX

Appendix I	Informed Consent Form	59
Appendix II	Calculation Example for 6-well plate seeding	63
Appendix III	Calculation of TGF-β1 concentration for stimulation	64
Appendix IV	Kinase substrate list on Serine-Threonine Kinase PamChip	65
Appendix V	PamStation12 Protocol	71

ABSTRACT

Background: Thoracic aortic aneurysm and dissection (TAAD) is one of the top 20 most leading causes in the USA and one of the silent killers in the world. The dysregulation of TGFβ pathway has been linked with pathogenesis of the disease. TGFβ pathway is a tightly regulated pathway that is controlled by phosphorylation of their downstream secondary messenger. The kinase substrate peptide microarray is utilized to see how the phosphorylation pattern of TGFβ downstream secondary messenger is regulated in TAAD patient.

Methods: Fibroblast samples from 3 mutants with TAAD carrying TGFβRII mutations and 3 normal patients were grown and stimulated with TGF-β1after 24 hours of serum starvation. The cells were then lysed and their protein concentration determined using bicinchoninic acid (BCA) assay. The phosphorylation of kinase substrate peptides were then analyzed using Serine-Threonine Kinase Microarray Chip.

Results: Kinase substrate peptides were chosen based on the significant phosphorylation changes between controls and mutants of the stimulated and unstimulated groups. Four kinase substrate peptides were found under-phosphorylated between the controls and mutants of the TGF-β1 unstimulated group. The TGF-β1 stimulated group yields 34 significantly over-phosphorylated peptides and 1 under-phosphorylated peptide.

Conclusion: The non-canonical and canonical pathway is activated simultaneously in TAAD patients despite the absence of TGFβRII. Kinase substrate peptide has huge potential to unravel the complicated TGFβ pathway via studying the phosphorylation pattern of TGFβ downstream secondary messenger.

Keywords: Thoracic aortic aneurysm and dissection, TGF-β1, *TGFβRII*, Serine-Threonine Kinase, Microarray

ABSTRAK

Latar Belakang: *Thoracic aortic aneurysm and dissection* (TAAD) adalah salah satu dari 20 penyebab kematian tertinggi di Amerika Serikat dan merupakan salah satu *silent killer* di dunia. Disregulasi *pathway* TGFβ sering dikaitkan dengan pathogenesis aneurisma dan diseksi aorta. Komunikasi instraseluler TGFβ adalah proses yang sangat teratur dan terjaga yang dikontrol ketat dengan fosforilasi komponen intraselulernya. Microarray peptida substrat kinase dapat digunakan untuk mempelajari bagaimana pola fosforilasi komponen intraseluler TGFβ diatur apda pasien TAAD.

Metode: Sampel fibroblast dari 3 mutan TAAD dengan mutasi TGFβRII dan 3 sampel normal dikultur dan di stimulasi dengan TGF-β1 setelah puasa serum selama 24 jam. Sel-sel tersebut kemudian di lisis dan konsentrasi proteinnya dihitung dengan *bicinchoninic acid (BCA) assay*. Pola fosforilasi peptide substrat kinase dianalisa dengan menggunakan *Serine-Threonine Kinase Microarray Chip*.

Hasil: Peptida substrat kinase dipilih berdasarkan besar perubahan pola fosforilasi antara kontrol dan mutan pada grup yang distimulasi dan tidak distimulasi. Empat peptida substrat kinase memiliki tingkat fosforilasi yang sedikit lebih rendah antara mutan dan kontrol pada grup yang tidak distimulasi dengan TGF-β1. Mutan pada grup yang distimulasi dengan TGF-β1 memiliki 34 peptida yang memiliki tingkat fosforilasi yang lebih tinggi dan 1 peptida yang memiliki tingkat fosforilasi yang sedikit lebih rendah dibandingkan dengan kontrol.

Kesimpulan: *Pathway canonical* dan *non-canonica*l tetap aktif secara bersamaan pada pasien TAAD walaupun tidak memiliki TGFβRII. *Microarray* adalah metode yang berpotensi untuk mengungkap *pathway* TGFβ yang kompleks melalui analisa pola fosforilasi reseptor intraseluler *TGFβ*.

Keywords: *Thoracic aortic aneurysm and dissection*, TGF-β1, *TGFβRII*, kinase Serine-Threonine, *Microarray*