UNIVERSITI PUTRA MALAYSIA

STRUCTURAL AND BIOPHYSICAL CHARACTERISATIONS OF MeCP2 MBD MUTANTS THAT CORRELATE WITH RETT SYNDROME

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FPSK(M) 2016 47

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

January 2016

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Master of Science

STRUCTURAL AND BIOPHYSICAL CHARACTERISATIONS OF MeCP2 MBD MUTANTS THAT CORRELATE WITH RETT SYNDROME

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January 2016

Chairman : Ho Kok Lian, PhD
Faculty : Medicine and Health Sciences

Methyl-CpG binding protein (MBD) family consists of Methyl-CpG Binding Protein 2 (MeCP2), Methyl-CpG Binding Domain Protein 1 (MBD1), MBD2, MBD3 and MBD4 where MeCP2 is the prototype of the family. MeCP2 contains several domains: (a) a methyl-CpG binding domain (MBD), (b) a transcriptional repression domain (TRD), (c) two AT hooks and (d) a nuclear localisation signal (NLS). MeCP2 binds to methylated DNA and represses the transcription of the associated genes. Mutations in *MECP2* lead to Rett syndrome (RTT), which is characterised by progressive neuro-developmental disorder in early childhood of females. Previous studies revealed that most RTT missense mutations alter the protein conformation and subsequently interferes the methyl-CpG recognition. To understand how the structural changes contribute to RTT, the 3-dimensional structure of these mutants need to be elucidated. Therefore, it is of interest to study the structure of RTT related MBD in complex with methylated DNA using X-ray crystallography and characterised the DNA-protein binding with some biophysical assays. Since more than 50% of the missense mutations occur within the MBD domain. Out of the 8 hot RTT spots within this domain, RTT mutants D97E, A140V, Y141C, P152R and D156E were included in this study. In band shift assay, wild-type MBD complexed with DNA was significantly shifted compared to A140V and D97E while other mutants were not significantly shifted. In SPR, wild-type MBD showed the highest affinity towards the DNA followed by A140V (K_D: 0.28 µM). Circular dichrosim (CD) analysis revealed that the secondary structures of A140V, Y141C, P152R and D156E are highly similar to wild-type MBD (14.7 % α-helix, 25.2 % β-strand, and 60.1 % turns and unordered) except for D97E which showed 31 % α-helix, 6.5 % β-strand, 62.5 % turns and unordered. The complex of MBD^{140V} with methylated DNA was crystallised and diffracted X-ray to 2.2 Å resolution. The co-crystal belongs to monoclinic space group *C2*, with unit cell parameters of a=78.66 Å, b=53.49 Å, c=62.78 Å, α=γ=90 ° and β=132.47 °. X-ray analysis revealed that the MBD domain was not altered by mutation of Ala-140 to Val (A140V). However, additional water molecules were identified at the DNA-protein contact interface and around the DNA molecule. A narrow minor groove of A/T run was observed as a result of additional bifurcated hydrogen bonds and vertical stacking of bases results from high degree of propeller twist and heavy purine-purine stacking. Two hydration spines were observed running down the wall of the minor groove. Each hydration spine is well

arranged into two shells adopting a zig-zag arrangement. Hence, this finding provides insights for the DNA geometry where the A/T run is geometrically stabilized by extensive water network and is independent of the flanking nucleotide sequence, DNA methylation and the bound MBD domain. The finding explores characteristics of the methylated DNA containing A/T run, which provide the nucleotide sequence preferences to MeCP2. In general, these additional molecular details could provide fundamental knowledge in RTT therapeutic approaches.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

KAJIAN STRUKTUR DAN BIOFIZIKAL MUTAN MeCP2 MBD YANG BERKAITAN DENGAN SINDROM RETT

Oleh

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Keluarga protein pengikat metil-CpG (MBD) terdiri daripada protein Pengikat Metil-CpG 2 (MeCP2), pengikat metil-CpG 1 (MBD1), MBD2, MBD3 dan MBD4 di mana MeCP2 adalah prototaip keluarga. MeCP2 mengandungi beberapa domain: (a) domain pengikat metil-CpG (MBD), (b) domain penindasan transkripsi (TRD), (c) dua pencangkuk AT dan (d) isyarat penempatan nuklear (NLS). Protein Pengikat Metil-CpG 2 (MeCP2) mengikat DNA bermetil dan menindas transkripsi gen yang berkaitan. Mutasi dalam *MECP2* membawa kepada sindrom Rett (RTT), yang mempunyai ciri-ciri progresif gangguan neuron dalam perkembangan awal kanak-kanak perempuan. Kajian sebelum ini menunjukkan bahawa kebanyakan mutasi missense RTT mengubah komformasi protein dan seterusnya mengganggu pengenalan metil-CpG. Untuk memahami bagaimana perubahan struktur membawa kepada RTT, struktur 3-dimensi mutan perlu dijelaskan. Oleh itu, adalah penting untuk mengkaji struktur RTT berkaitan dengan MBD kompleks dengan DNA bermetil menggunakan sinaran-X penghabluran dan mencirikan dengan beberapa analisis DNA biofizikal metilasi. Lebih daripada 50% daripada mutasi missense berkelompok dalam domain MBD. Daripada 8 lokasi penting RTT dalam domain ini, mutan RTT termasuk D97E, A140V, Y141C, P152R dan D156E telah dimasukkan dalam kajian ini. Dalam essei anjakan jalur, Kompleks DNA-protein MBD jenis liar meranjak dengan ketara berbanding dengan mutan A140V dan D97E manakala anjakan jalur mutan lain tidak ketara. Dalam SPR, MBD jenis liar mempunyai daya afiniti ke arah DNA yang paling tinggi dan diikuti oleh mutan A140V (K_D: 0.28 μM). Analisis dikreisme membulat (CD) menunjukkan bahawa struktur sekundur mutan RTT (A140V, Y141C, P152R dan D156E) adalah menyerupai MBD jenis liar (14.7 % α-heliks, 25.2 % β-helai, dan 60.1 % tidak tersusun) kecuali D97E yang menunjukkan 31 % α -heliks, 6.5 % β-helai, 62.5 % tidak tersusun. Kompleks MBD^{140V} dengan DNA bermetil telah dihablurkan dengan menggunakan 30 % (w/v) PEG 2000 dan hablur telah dibelau oleh sinaran-X kepada resolusi 2.2 Å. Hablur ini tergolong dalam kumpulan ruang monoklinik *C2*, dengan parameter unit sel = 78.66 Å, b = 53.49 Å, c = 62.78 Å, α = γ = 90 ° dan β = 132.47 °. Analisis sinaran-X mendedahkan bahawa domain MBD tidak berubah akibat mutasi Ala-140 kepada Val (A140V). Walau bagaimanapun, lebih banyak molekul air telah dikenalpasti di antara muka DNA-protein dan di sekitar molekul DNA. Lurah minor dengan urutan A/T yang berulang telah dikenalpasti dengan tambahan ikatan hidrogen bercabang dan penindihan nukleotida secara menegak yang berpunca daripada pusingan baling-baling

yang berdarjah tinggi dan penindihan purina. Dua spina hidrasi didapati menuruni dinding lurah minor. Setiap spina hidrasi tersusun dalam dua lapisan dengan penyusunan zig-zag. Oleh itu, kajian ini dapat meningkatkan pemahaman dalam geometri DNA di mana urutan A/T yang berulang adalah stabil akibat daripada rangkaian molekul air dan bebas daripada mengapitnya urutan nukleotida, metilasi DNA dan domain MBD yang terikat. Penemuan ini membongkarkan ciri-ciri DNA bermetil yang mengandungi urutan A/T berulang bagi membekalkan kegemaran jujukan nukleotida kepada MeCP2. Secara umum, Informasi molekular yang didapati dapat menawarkan perkembangan dalam permahaman asas dalam terapeutik RTT.

ACKNOWLEDGEMENTS

With God's warmest blessing, there are some people who made my journey easier and I would like to extend my greatest appreciation to those who helped me in completing my journey. First and foremost, I would like to express my heartfelt gratitude to the chairperson of the supervisory committee, Dr. Ho Kok Lian, for his endless support and invaluable guidance throughout this study. He is always in an amiable manner with his friendly gesture. All the gracious personalities of him have motivated me to become a more down to earth and heart-warming person in the near future. In addition, his impressive cognitive ability and intellectual maturity have also thought me the importance of critical and analytical reasoning, not only as a student point of view but also as a researcher.

I would also like to extend my sincere appreciation to the members of the supervisory committee, Prof. Dr. Tan Wen Siang and Assoc. Prof. Dr. Foo Hooi Ling, for their wise opinions and constructive suggestions. Their endurance and boundless enthusiasm in research are some of the characteristics that inspired me in the attempt to become a diligent, persistent and successful researcher. I am also grateful to all my labmates, especially Yoon Kam Yee; for their stimulating discussions, advice, their kind hospitality, friendship and technical assistance. A million thanks goes to all of you who have brightened and amused me in one way or another for making my long exhausting hours in lab a happy and pleasant one. Finally, my special thanks and deepest gratitude are extended to my beloved family members for their understanding, perseverance, support and endless love throughout my study.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

α	Alpha
Å	Ångström
β	Beta
µg	microgram (10^{-6} g)
µL	microliter (10^{-6} L)
µM	micromolar (10^{-6} M)
BDNF	Brain-derived neurotrophic factor
bp	Basepair
BSA	bovine serum albumin
ccc	covalently closed circular
CCP4	Collaborative Computational Project Number 4
C-terminal	carboxyl terminal
DNA	Deoxy-ribonucleic acid
dNTP	deoxynucleoside triphosphate
dsDNA	double stranded DNA
DTT	1,4-dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EMSA	Electrophoretic mobility shift assay
FPLC	fast protein liquid chromatography
HDAC	Histone deacetylase
HPLC	high performance liquid chromatography
IMAC	immobilised metal ion affinity chromatography
IPTG	isopropyl-β-d-thiogalactopyranoside

K	Kelvin
Kb	kilobase
K_d	dissociation constant
kDA	kilo Dalton
LB	Luria broth
m5C	5'methyl cytosine
MBD	Methyl-CpG Binding Domain
MBD1	Methyl-CpG Binding Domain Protein 1
MBD2	Methyl-CpG Binding Domain Protein 2
MBD3	Methyl-CpG Binding Domain Protein 3
MBD4	Methyl-CpG Binding Domain Protein 4
MeCP2	Methyl-CpG Binding Protein 2
MBP	Methyl-CpG Binding Protein
Mg	milligram (10^{-3} g)
Min	minute
NDB	Nucleic Acid Database
NLS	Nuclear localisation Signal
nM	nanomolar (10^{-9} M)
NTA	Nitrilotriacetic acid
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCM1	Protein containing MBD 1
PCR	Polymerase chain reaction
PDB	Protein Database Bank
PEG	Polyethylene glycol

pI	Isoelectric point
RMSD	root mean square deviation
rpm	revolutions per minute
RTT	Rett Syndrome
s	Second
SDS	sodium dodecyl sulphate
SEC	size exclusion chromatography
TBE	Tris-buffered EDTA solution
TEMED	Tetramethyl ethylenediamine
TRD	Transcriptional repression domain
U	Unit
UV	Ultraviolet
v	Volt
V_m	Matthew's coefficient
v/v	volume/volume
w/v	weight/volume
x *g*	centrifugal force

LIST OF AMINO ACIDS ABBREVIATIONS

	One letter code	Three letter code
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic acid	D	Asp
Cysteine	C	Cys
Glutamic acid	E	Glu
Glutamine	Q	Gln
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val

CHAPTER 1

INTRODUCTION

1.1 General Introduction

DNA methylation is an epigenetic signal that affects gene regulation, genomic stability and chromatin structure in mammalian cells (Bird, 2002; Du *et al.*, 2015). In most cases, this signal can be read by a family of proteins that contains a common methyl-CpG binding domain (MBD) (Hendrich and Bird, 1998). To date, five family members, namely MeCP2, MBD1, MBD2, MBD3 and MBD4 have been identified, in which, MeCP2 is the prototype of this family (Hendrich and Bird, 1998). MeCP2, MBD1 and MBD2 are able to recruit co-repressor complexes that can inhibit transcription in concert with chromatin modifiers (Scarsdale *et al.*, 2011). Mammalian MBD3 does not bind to methylated DNA *in vitro* and *in vivo* due to replacement of amino acid (K43H and Y47F) which is critical for DNA binding (Fraga *et al.*, 2003). MBD4 contains a thymine DNA-glycosylase at the C-terminal region that can repair G-T mismatches via hydrolytic deamination [(Refer Section 2.3.4 and Figure 2.3) (Neddermann *et al.*, 1996)].

MeCP2 is a transcriptional repressor that contains several domains: (a) a methyl-CpG binding domain (MBD), (b) a transcriptional repression domain (TRD), (c) AT hooks and (d) a nuclear localisation signal (NLS) (Lewis *et al.*, 1992; Nan and Bird, 2001; Xu and Pozzo-Miller, 2013). MBD domain of MeCP2 is able to recognise methyl-CpG containing DNA. TRD domain involves in recruitment of transcriptional co-repressors such as mSin3A and histone deacetylases (HDACs) (Bienvenu *et al.*, 2000). Two putative NLSs facilitate nuclear localisation, which targets the protein into the cell nucleus (Weaving *et al.*, 2004) and the AT hooks are believed to interact with AT rich region of the DNA (Klose *et al.*, 2005). In addition, the AT hooks of MeCP2 bearing amino acid sequences ^{185}GRGRGRP^{191} and ^{265}PKKRGRKP^{272} (superscript indicates amino acid number) which are highly similar to the high mobility group with the AT hook I chromosomal protein (HMCG-I) that is capable to bind to the minor groove of the AT stretches (A/T run) of DNA and functionally (Aravind and Landsman, 1998; Lewis *et al.*, 1992; Nan *et al.*, 1993; Reeves and Nissen, 1990). Baker *et al.* (2013) demonstrated that the disruption of second conserved AT hook at the C-terminal region of MeCP2 by truncation at R270X of MeCP2 led to failure in chromatin compaction and localization of pericentric heterochromatin domain of α-thalassemia mental retardation syndrome X-linked (ATRX); a chromatin remodelling protein, with MeCP2, and caused the R270X mice to exhibit Rett syndrome (RTT) phenotypes which is similar to MeCP2 knock-out mice (Baker *et al.*, 2013; Xu and Pozzo-Miller, 2013).

The MBD domain of MeCP2 is able to recognise single methyl-CpG dinucleotide (Lewis *et al.*, 1992). The MBD domain alone is ample for the methylated DNA binding and mutations in the MBD domain intercept its binding to methylated sequence (Baubec *et al.*, 2013; Yusufzai and Wolffe, 2000). MeCP2 mutation causes Rett syndrome (RTT); a progressive neurodevelopmental disorder in early childhood, which leads to mental

retardation in females, with a prevalence of 1 in 10,000-15,000 female births (Hagberg, 1985). RTT is caused by an X-linked mutation dominant inheritance with normally lethality in males due to severe encephalopathy (Bianciardi *et al.*, 2015; Bienvenu *et al.*, 2000; Zhao *et al.*, 2015). Studies revealed that most RTT related missense mutations alter the structure of the MBD domain and subsequently interrupt DNA recognition properties (Kriaucionis and Bird, 2003; Kucukkal *et al.*, 2015). Klose et al. (2005) showed that an A/T run adjacent to the methyl-CpG is required to enhance the MeCP2 binding (Klose *et al.*, 2005). Identified endogenous MeCP2 targeting genes such as *brain derived neurotropic factor (BDNF)* promoter region contains high occurrences of A/T runs closed to the methyl-CpGs (Chen *et al.*, 2003; Martinowich *et al.*, 2003). The A/T run in the methylated DNA facilitated the co-crystallisation of MeCP2 MBD domain in complex with methylated DNA used in this study. Due to the presence of AT hooks in MeCP2 and the requirement of A/T run for maximal binding, it has been speculated that the A/T run could interact with the AT hooks of the MeCP2. However, the characteristics of A/T run which provide specificity for the MeCP2 to recognise the methyl groups remained unclear. Therefore, it is of interest to elucidate the 3-dimensional structure of RTT mutants in order to understand how the structural changes contribute to RTT and the A/T run characteristics with MBD domain bound to its adjacent methyl-CpG dinucleotide.

X-ray analysis of previous report on a MeCP2 MBD-methylated DNA complex revealed that only a few residues are involved in direct contact with the DNA bases (Ho *et al.*, 2008). The methyl groups are recognised by the Arginine fingers of R111 and R133 while D121 is critical in maintaining the unique hydration pattern at the DNA-protein interface. The unique water molecules distribution pattern is crucial to mediate methyl group recognition (Ho *et al.*, 2008). RTT mutations within the MBD domain of MeCP2, however, are believed to alter the 3-dimensional structure of the protein and subsequently affects DNA binding. Several critical mutations such as R111G, R133C, T158M and D121G, which close to the DNA-protein contact region have been investigated (Free *et al.*, 2001; Meehan *et al.*, 1992; Nan *et al.*, 1993; Yusufzai and Wolffe, 2000). In this study, other RTT mutations which are located distance from the DNA-protein contact region have been studied. According to Wakefield and colleagues (1999), missense mutations found in RTT usually do not specifically interrupt DNA recognition but may result in structural changes in the domain (Bianciardi *et al.*, 2015; Wakefield *et al.*, 1999). In order to further investigate the details on the structural changes and molecular functional role, several mutants (D97E, A140V, Y141C, P152R and D156E) were constructed and the DNA-MBD interactions were characterised with various biophysical assays. A co-crystal structure was also elucidated, in which, more molecular details about the DNA-protein complex have been revealed compared with previous reported structure. In addition, the atomic details of the DNA geometry of the MBD bound A/T run are highlighted in comparison with the A/T run of the free DNA double helices. In general, these additional molecular details could provide fundamental knowledge in RTT therapeutic approaches.

1.2 Objectives

The general objective of this study was to explore and understand the atomic details of Rett mutants in complex with methylated DNA. The specific objectives were:

1. To construct MBD mutants

2. To characterise the interactions of MBD mutants and methylated DNA

3. To crystallise the MBD mutants in complex with methylated DNA

4. To solve the X-ray structure of MBD mutants in complex with methylated DNA

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