"Investigation of the role of the *MID1* and *MID2* genes in the Opitz Syndrome"

By

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Contents

Abstract

To determine whether or not the *MID1* and *MID2* genes contribute to the OS phenotype, a mutation screen was undertaken. Fifteen Australasian and one British patient diagnosed as having Opitz Syndrome were screened for mutations in the *MID1* and *MID2* genes. In total, 8 unique *MID1* mutations (E115X, Δexon2, F/S 1051delC, R368X, F/S 1330insA, Q468X, R495X & L626P) were identified. The majority of these mutations resulted in the truncation of the MID1 protein, midin (6 of the 8 mutations), disrupting the C-terminal domain (CTD). E115X was the most N-terminal mutation identified, resulting in a midin protein with only the RING finger domain. Two OS patients had nucleotide changes producing a frameshift, one was a deletion of a C nucleotide (1051delC) while the other was an insertion of a single A nucleotide (1330insA). One OS patient had a small in-frame deletion encompassing exon 2 (Δexon2) and another unique mutation resulted in an amino acid change from a leucine to a proline (L626P), representing the most C-terminal mutation identified so far. In the *MID2* gene, there was a missense mutation caused by a change 1073C>A. This mutation was present in OSP#7, 9 and 13 probands, which suggested that the *MID2* gene may also have a role in the OS phenotype. The OS phenotype varies between individuals but there was no correlation between the OS phenotype and genotype in the mutations examined.

The *MID1* mutations, E115X, Δexon2, R495X and L626P, were chosen to investigate the effect of the mutations on the intracellular localisation of the MID1 protein (midin). A Green Fluorescent Protein tag (GFP) was used to visualise the intracellular localization of the wild-type and mutated midin. Wild-type midin was located only in the cytoplasm of the cell and was associated with the microtubule network, while the mutated forms of midin were found to have an altered intracellular localisation. The E115X mutated protein lost its ability to localise in the cytoplasm. The Δexon2 mutated protein remained in the cytoplasm but lost

its ability to associate with the microtubules. Intracellular localisation of R368X and L626P mutated proteins showed cytoplasmic clumping. The R368X and L626P mutated proteins also had a reduced ability to associate with microtubules. This suggests that the mutations result in a loss of function of the ability of midin to bind to the microtubules, overall resulting in the OS phenotype.

An antibody to human midin, MID1 antibody, was raised in rabbits and characterised using Western analysis. Experiments were undertaken to determine the specificity of the MID1 antibody. The MID1 antibody was found to be specific to midin and was unable to interact with the MID2 protein. In addition, the MID1 antibody was unable to interact with midin that had mutations. The MID1 antibody also was unable to interact with tissue samples from zebrafish embryos and chicken embryos although these species have the *MID1* gene present. This suggested that the C-Terminal domain appeared to facilitate the interaction between MID1 antibody and midin.

The zebrafish (*Danio rerio*) was used as a model system in which to find a *MID1* homologue in order to further analyse the function of *MID1* and how it may cause the OS phenotype. From the zebrafish genome a homologue, *zMID*, was isolated and cloned. *In situ* hybridisation experiments revealed that the zMID was expressed only in the retinal neuroepithelium cells of the developing eye in zebrafish, highlighting the boundary of the optic stalk before it differentiated into the optic nerve. This expression was very different to the MID1 pattern of expression in mice, which has been shown to be ubiquitous. The multiple banding patterns observed in Southern analysis, when various probes were hybridised to zebrafish genomic DNA, indicated that there were multiple copies of the *MID1* gene in the zebrafish genome. In addition, the banding pattern suggested there might be multiple copies of *MID2* or even the presence of *MID-like* genes in the zebrafish genome. A phylogenetic analysis using the *MID1* and *MID2* homologues revealed that the *zMID* gene and the fugu *MID* gene were a monophyletic group that excluded the other homologues. A

protein alignment of all MID sequences revealed these homologues had been highly conserved across species. The lowest conservation seen across species was in the most carboxy terminus of the MID1 protein.

In the future, all multiple copies of the *MID1* gene present in the zebrafish genome need to be isolated and sequenced to enable transgenic experiments to be are carried out in the zebrafish. Transgenic zebrafish could be used to model the mutations found in OS patients and used to determine the mechanisms involved in the loss of function of the *MID1* gene.

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Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Lillian Allen

Abbreviations

A	adenine
bp	base pair
BSA	bovine serum albumin
C	cytosine
°C	degree Celcius
cDNA	complementary deoxyribonucleic acid
CTD	C-terminal domain
dATP	2′-deoxycytosine 5′-triphosphate
dCTP	2′-deoxyguanosine 5′-triphosphate
dH_2O	distilled water
ddH_2O	de-ionised distilled water
DNA	deoxyribonucleic acid
dpc	days post-copulation
DTT	dithiothreitol
DTTP	2′-deoxythymidine 5′-triphosphate
EDTA	ethylenediaminetetraactetic acid
EtBr	ethidium bromide
FITC	fluorescein isothiocyanate
FNIII	fibronectin type III repeat
Fxy	mouse homologue of MID1
g	gram
G	guanine
GFP	green fluorescent protein

h	human
hpf	hours post-fertilisation
dpf	days post-fertilisation
IPTG	isopropyl-1-β-D-thiogalactopyranoside
kb	kilobase
kV	kilovolts
LB	Luria broth
MID1	midline 1 gene
MID2	midline 1 gene
M	molar
m	mouse
min or ′	minutes
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
NaOH	sodium hydroxide
ng	nanograms
ORF	open reading frame
OS	Opitz syndrome
OSP#	Opitz syndrome patient number
PBS	phosphate buffer saline
PBS	plasmid blue script
PCR	polymerase chain reaction
RE	restriction enzyme

RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate – Polyacrylamide Gel Electrophoresis
SSC	sodium chloride sodium citrate
SSCP	single stranded conformation polymorphism
T	thymine
T_A	annealing temperature
TAE	tris acetate ethylenediaminetertraacetic acid
TBE	tris-borate-EDTA
TE	tris ethylenediaminetetraacetic acid
TEMED	N, N, N′, N′-tetramethylethylenediamine
Tris	trizma base (tris(hydroxymethyl) amino methane
µg	microgram
µl	microliter
µM	micromolar
UV	ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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