



**UNIVERSITI PUTRA MALAYSIA**

***MICRORNA EXPRESSION AND ASSESSMENT OF POTENTIAL  
ROLE OF miR-181a IN HEAD AND NECK CANCER***

**NURUL SYAKIMA AB MUTALIB**

**FPSK(p) 2012 25**

**MICRORNA EXPRESSION AND ASSESSMENT OF POTENTIAL ROLE OF  
miR-181a IN HEAD AND NECK CANCER**

By

**NURUL SYAKIMA AB MUTALIB**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia,  
in Fulfillment of the Requirements for the Degree of Doctor of Philosophy**

**May 2012**

*Dedicated to my parents*



© COPYRIGHT UPM

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

**MICRORNA EXPRESSION AND ASSESSMENT OF POTENTIAL ROLE OF  
miR-181a IN HEAD AND NECK CANCER**

By

**NURUL SYAKIMA BINTI AB MUTALIB**

**May 2012**

**Chair: Cheah Yoke Kqueen, PhD**

**Faculty: Medicine and Health Sciences**

MicroRNAs (miRNAs) represent a class of small non-coding RNAs that regulate gene expression by either inducing mRNA degradation or repressing mRNA translation. The involvements of miRNAs in various human cancer-related processes have been studied in recent years. The first objective of this study was to determine differentially expressed miRNAs in head and neck cancer. Global miRNA profiling was performed on 12 tissue samples from various head and neck cancers by using the microarray approach followed by real time RT-PCR validation. The microarray analyses identified 10 miRNAs that were able to distinguish malignant from normal tissues whereby seven miRNAs (hsa-miR-181a-2\*, hsa-miR-29b-1\*, hsa-miR-181a, hsa-miR-181b, hsa-miR-744, hsa-miR-1271 and hsa-miR-221\*) showed up-regulation while three miRNAs (hsa-miR-141, hsa-miR-95 and hsa-miR-101) showed down-regulation. Therefore, these miRNAs may aid in simple profiling strategies to identify individuals at higher risk of developing head and neck cancers,

as well as elucidate the molecular mechanisms involved in head and neck cancers pathogenesis.

The second objective of this study was to identify the putative targets of miRNAs differentially expressed in head and neck cancers and the pathways involved, which was achieved through *in silico* analysis aided by online databases, whereby several cancer-associated genes and pathways were found to be targeted by miR-181a. The role of miR-181a in head and neck carcinogenesis was subsequently determined through functional analyses as the third objective of this study. It was found out that miR-181a regulates the proliferation, migration, invasion and colony-forming ability of head and neck cancer cell.

Fourth objective was achieved by using pathway analysis to profile changes in the activities of 10 signaling pathways related to cancer caused by miR-181a down-regulation. Six of these pathways, namely the p53/DNA damage, TGF $\beta$ , MAPK/ERK, MAPK/JNK, Wnt and NF $\kappa$ B pathways, were found to be significantly influenced, suggesting miR-181a may act as an oncomiR, and therefore its inhibition may be a potential therapeutic target for head and neck cancer patients. The fifth and final objective of this study involved visualizing miR-181a expression and localization in head and neck tissues, for which *in situ* hybridization was utilized. miR-181a is preferentially expressed in the cytoplasm of cancer cells, and its expression is significantly increased in malignant compared to benign tumors of the head and neck. Collectively, these findings provide basis for study into the role of miR-181a as a biomarker and/or therapeutic target in head and neck tumors.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

**EKSPRESI MIKRORNA DAN PENILAIAN PERANAN POTENSI miR-181a  
DALAM KANSER KEPALA DAN LEHER**

Oleh

**NURUL SYAKIMA BINTI AB MUTALIB**

**Mei 2012**

**Pengerusi: Cheah Yoke Kqueen, PhD**

**Fakulti: Perubatan dan Sains Kesihatan**

MikroRNA (miRNA) mewakili kelas RNA bukan pengekodan kecil yang mengawal selia gen sama ada secara menginduksi degradasi mRNA atau represi translasi. Penglibatan mereka dalam proses yang berkaitan dengan kanser telah dikaji dalam pelbagai jenis kanser manusia. Objektif yang hendak dicapai dalam kajian ini adalah pertamanya untuk menentukan pembezaan ekspresi miRNA dalam kanser kepala dan leher. miRNA profil global telah dilakukan ke atas 12 tisu sampel kanser kepala dan leher menggunakan pendekatan mikroarray diikuti oleh pengesahan menggunakan qRT-PCR. Analisis mikroarray mengenal pasti 10 miRNA yang boleh membezakan lesi kanser kepala dan leher dari tisu normal; 7 miRNA (hsa-miR-181a-2 \*, hsa-miR-29b-1\*, hsa-miR-181a, hsa-miR-181b, hsa-miR-744, hsa-miR-1271 dan hsa-miR-221\*) dikawal selia naik manakala 3 miRNA (hsa-miR-141, hsa-miR-95 dan hsa-miR-101) dikawal selia turun. Kumpulan miRNA ini boleh menyumbang dalam strategi pemprofilan yang mudah untuk membantu dalam mengenal pasti individu berisiko tinggi untuk mendapat kanser kepala dan leher serta dapat membantu dalam

mengenal pasti mekanisma molekular yang terlibat dalam patogenesis kanser kepala dan leher.

Kemudian, sasaran diduga miRNA yang diekspresi secara berbeza dalam kanser kepala dan leher serta laluan kanser yang terlibat telah dikenal pasti melalui analisis silico menggunakan pangkalan data dalam talian. Beberapa gen dan laluan berkaitan dengan kanser didapati disasarkan oleh kumpulan miRNA tersebut. Fungsi miRNA yang dipilih (miR-181a) dalam karsinogenesis kepala dan leher ditentukan melalui analisis fungsi. Kajian ini mendapati bahawa miR-181a mengawal proliferasi, migrasi, pencerobohan dan keupayaan membentuk koloni dalam kanser kepala dan leher.

Analisis laluan dilakukan untuk memprofil perubahan dalam aktiviti 10 laluan isyarat kanser yang disebabkan oleh penurunan paras miR-181a. Laluan p53/kerosakan DNA, TGF $\beta$ , MAPK / ERK, MAPK / JNK, Wnt dan NF $\kappa$ B didapati banyak dipengaruhi oleh penurunan paras miR-181a. Keputusan ini mencadangkan bahawa miR-181a boleh dicalonkan sebagai oncomiR dan seterusnya boleh dijadikan sebagai sasaran potensi terapeutik untuk pesakit kanser kepala dan leher. Akhir sekali, untuk menggambarkan ekspresi miR-181a dan penyetempatan di dalam tisu kepala dan leher, hibridisasi in situ telah digunakan. miR-181a terzahir dalam sitoplasma sel-sel kanser, dan ekspresinya meningkat dengan ketara dalam tumor malignan berbanding dengan tumor benigna kepala dan leher. Secara kolektif, penemuan ini menyediakan asas kepada kajian terhadap peranan miR-181a sebagai biopenanda dan / atau sasaran terapeutik tumor di kepala dan leher.

## ACKNOWLEDGEMENT

First and foremost, I would like to extend my deepest gratitude towards my supervisor, Associate Professor Dr. Cheah Yoke Kqueen, who has been an incredible mentor, for his generous guidance, support and patience throughout my doctorate study. Without his guidance, it would be harder to complete this thesis. It is an honor to have him as my supervisor.

My heartfelt appreciations to my co-supervisors, Associate Professor Dr. Shiran Mohd Sidik, Associate Professor Dr. Sabariah Abdul Rahman and Dr. Avatar Singh Mohan Singh for their patience, precious intellectual contribution and vital enthusiasm. This study would not be perfect without their contributions.

Special thanks must go to my most respected senior, Lee Learn Han, for his knowledge, motivation and assistance throughout these few years and for always being there whenever I needed professional or personal consult. Not forgetting deepest appreciation to my other lab mates for their continuous assistance as well. I also would like to thank the laboratory staff from the Molecular Biology Laboratory, Cell Signaling Laboratory, Histopathology Laboratory and Physiology Laboratory of Faculty of Medicine and Health Sciences, Universiti Putra Malaysia for providing great support towards the completion of my research.

I would like to express my utmost appreciations to my beloved family for their encouragement, support, patience and understanding. Their endless love is the great force that has kept me going throughout these difficult years.

Again, thanks to all who have helped me in this PhD journey.



I certify that a Thesis Examination Committee has met on 7 May 2012 to conduct the final examination of Nurul Syakima binti Ab Mutalib on her thesis entitled “MicroRNA Expression and Assessment of Potential Role of miR-181a in Head and Neck Cancer” in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

Members of the Thesis Examination Committee were as follows:

**Sabrina binti Sukardi, PhD**

Associate Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Chairman)

**Noorjahan Banu binti Mohammed Alitheen, PhD**

Associate Professor  
Faculty of Biotechnology and Biomolecular Sciences  
Universiti Putra Malaysia  
(Internal Examiner)

**Tan Soon Guan, PhD**

Professor  
Faculty of Biotechnology and Biomolecular Sciences  
Universiti Putra Malaysia  
(Internal Examiner)

**Chin-Yuan Tzen, PhD**

Professor  
Fu Jen Catholic University  
Taiwan  
(External Examiner)

---

**SEOW HENG FONG, PhD**

Professor and Deputy Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date: 23 July 2012

The thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

**Cheah Yoke Kqueen, PhD**

Associate Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Chairman)

**Shiran Mohd Sidik, MBBS, MPath**

Associate Professor  
Faculty of Medicine and Health Sciences  
Universiti Putra Malaysia  
(Member)

**Sabariah Abdul Rahman, MBBS, MPath**

Professor  
Faculty of Medicine  
Universiti Institute Teknologi Mara  
(Member)

**Avatar Singh Mohan Singh, MBBS, FAGE, MS (ORL-UKM)**

Dr.  
Head of ENT Head-Neck Surgery Unit  
Taiping General Hospital  
(Member)

---

**BUJANG BIN KIM HUAT, PhD**

Professor and Dean  
School of Graduate Studies  
Universiti Putra Malaysia

Date:

## DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.



---

**NURUL SYAKIMA BINTI AB MUTALIB**

Date: 7 May 2012

## TABLE OF CONTENTS

	<b>Page</b>
<b>ABSTRACT</b>	iii
<b>ABSTRAK</b>	v
<b>ACKNOWLEDGEMENTS</b>	vii
<b>APPROVAL</b>	viii
<b>DECLARATION</b>	x
<b>LIST OF TABLES</b>	xv
<b>LIST OF FIGURES</b>	xvi
<b>LIST OF ABBREVIATIONS/ ANNOTATIONS</b>	xvii
<b>CHAPTER</b>	
<b>1 INTRODUCTION</b>	<b>1</b>
1.1 Thesis overview	1
1.2 Head and neck cancers	2
1.2.1 Classification, diagnosis and prognosis	2
1.2.2 Treatment	4
1.2.3 Risk factors	4
1.2.4 Prevalence in Malaysia	9
1.3 Importance of study	10
1.4 Specific research objectives	11
<b>2 LITERATURE REVIEW</b>	<b>13</b>
2.1 Introduction	13
2.2 Molecular aspects of head and neck cancers	13
2.3 MicroRNAs (miRNAs)	15
2.3.1 miRNA discovery	16
2.3.2 miRNA biogenesis	18
2.3.3 miRNA versus siRNA	20
2.3.4 miRNA and human diseases	21
2.3.5 miRNA in head and neck cancers	23
2.4 Roles of miRNAs in cancer	25
2.4.1 miRNAs as oncogene and tumor suppressor	26
2.4.2 miRNAs in apoptosis	27
2.4.3 miRNAs in cell proliferation	29
2.4.4 miRNAs in invasion, migration and metastasis	29
2.4.5 miRNAs in cell cycle regulation	31
2.4.6 miRNAs in diagnosis and prognosis	32
2.5 Available tools for miRNAs investigation in cancer research	34
2.5.1 miRNA detection and profiling strategies	34
2.5.1.1 miRNA microarray	36
2.5.1.2 Quantitative reverse transcription PCR (qRT-PCR)	37
2.5.1.3 Next generation sequencing (NGS)	40
2.5.2 Functional validation of miRNA	41
2.5.3 <i>In silico</i> bioinformatic tools in miRNAs research	42
2.5.4 miRNA localization in human tissue	44

2.5.5	miRNA target validation	46
2.5.6	Animal model for miRNA studies in head and neck cancers	48
2.6	miRNAs' potential cancer therapeutic properties	50
2.6.1	miRNA inhibition	50
2.6.1.1	Antisense inhibition of mature miRNA	50
2.6.1.2	Targeting miRNA processing machinery	52
2.6.2	miRNA replacement therapy	52
2.6.3	Delivery of therapeutic miRNAs	53
3	<b>MICRORNA EXPRESSION PROFILING ANALYSIS IN HEAD AND NECK CANCERS AND NORMAL TISSUE</b>	55
3.1	Introduction	55
3.2	Specific objectives	56
3.3	Methodology	57
3.3.1	Samples collection	57
3.3.2	Total RNA extraction	59
3.3.3	miRNA microarray	60
3.3.4	Validation of miRNA microarray data by qRT-PCR	62
3.4	Results	64
3.4.1	Patterns of miRNA expression in head and neck cancers and normal tissues	64
3.4.2	qRT-PCR validation	67
3.4.2.1	Validation of $2^{-\Delta\Delta Ct}$ method	67
3.4.2.2	Validation of microarray data through qRT-PCR	70
3.5	Discussion	72
3.5.1	miRNAs are expressed differently in carcinoma and normal tissues of the head and neck	72
3.5.2	New miRNAs differentially expressed in head and neck cancers are identified	75
3.6	Conclusion	79
4	<b><i>IN SILICO</i> ANALYSIS OF DYSREGULATED MICRORNAS IN HEAD AND NECK CANCERS</b>	81
4.1	Introduction	81
4.2	Specific objectives	82
4.3	Methodology	82
4.3.1	Identification of miRNAs' targets	82
4.3.2	Identification of pathways targeted by miRNAs	83
4.3.3	Identification of validated miRNA targets	84
4.4	Results	84
4.4.1	Genes and pathways targeted by single miRNA	84
4.4.2	Pathways potentially influenced by combination of multiple miRNAs	87
4.4.3	Validated targets of differentially expressed miRNAs	88
4.5	Discussion	89
4.5.1	Bioinformatics <i>in silico</i> analysis reveals cancer-related genes and pathways targeted by miRNAs	89
4.5.2	Targets of differentially expressed miRNAs in head and neck cancer require validation	92

4.6	Conclusion	93
5	<b>miR-181a FUNCTIONAL ANALYSIS IN HEAD AND NECK CANCER</b>	94
5.1	Introduction	94
5.2	Specific objectives	95
5.3	Methodology	96
5.3.1	Cell culture	96
5.3.2	miRNA transfection	96
5.3.3	Cell proliferation assay	97
5.3.4	Colony formation assay	97
5.3.5	Cell migration assay	98
5.3.6	Cell invasion assay	98
5.3.7	Cell adhesion assay	99
5.3.8	Statistical analysis	100
5.4	Results	100
5.4.1	miR-181a affects FaDu cell proliferation	100
5.4.2	Inhibition of miR-181a suppresses colony formation <i>in vitro</i>	101
5.4.3	miR-181a enhanced cell migration activity	103
5.4.4	miR-181a influenced the invasiveness of FaDu cells <i>in vitro</i>	103
5.4.5	Cell adhesion in FaDu was not significantly altered by miR-181a	104
5.5	Discussion	105
5.6	Conclusion	110
6	<b>ANALYSIS OF PATHWAYS REGULATED BY miR-181a IN HEAD AND NECK CANCER</b>	111
6.1	Introduction	111
6.2	Specific objective	112
6.3	Methodology	113
6.3.1	Cell line and culture condition	113
6.3.2	Dual luciferase pathway reporter transfection	113
6.3.3	Luciferase assay	114
6.3.4	Statistical analysis	115
6.4	Results	115
6.5	Discussions	117
6.6	Conclusion	122
7	<b>LOCALIZATION OF miR-181a IN HEAD AND NECK TISSUE ARRAY</b>	124
7.1	Introduction	124
7.2	Specific objectives	125
7.3	Methodology	126
7.3.1	Head and neck tissue array	126
7.3.2	Hematoxylin and Eosin staining	126
7.3.3	LNA-modified oligonucleotide probes	127
7.3.4	miRNA LNA-ISH	127
7.3.5	Image acquisition and analysis	129
7.4	Results	130
7.4.1	Clinicopathologic characteristics	130

7.4.2	Expression patterns of miR-181a in intact head and neck tissue array	130
7.4.3	Localization of miR-181a in head and neck tissue array	136
7.5	Discussion	138
7.6	Conclusion	142
<b>8</b>	<b>GENERAL CONCLUSIONS, LIMITATIONS OF STUDY, FUTURE DIRECTIONS AND RECOMMENDATIONS</b>	<b>143</b>
8.1	General conclusions	143
8.2	Limitations of study	145
8.3	Future directions and recommendations	146
	<b>REFERENCES</b>	<b>147</b>
	<b>APPENDICES</b>	<b>182</b>
	<b>BIODATA OF STUDENT</b>	<b>238</b>
	<b>LIST OF PUBLICATIONS</b>	<b>239</b>