

**CIRCULATING AND SALIVARY MICRORNA  
EXPRESSION ANALYSIS IN NASOPHARYNGEAL  
CARCINOMA: POTENTIAL FOR DIAGNOSTIC  
BIOMARKER**

**BY**

**AZMIR BIN AHMAD**

**A thesis submitted in fulfilment of the requirement for the  
degree of Doctor of Philosophy in Health Sciences**

**Kulliyyah of Allied Health Sciences  
International Islamic University Malaysia**

**APRIL 2020**

## ABSTRACT

Nasopharyngeal carcinoma (NPC) is among the most frequently reported cancer in Malaysia where it is usually diagnosed at late stages. The inconvenient and painful contemporary diagnostic methods for NPC discourage the population at risk from being screened at early stages. Thus, the discovery on non-invasive biomarkers for early detection of NPC is warranted. MicroRNAs (miRNAs), a class of RNAs that have been found to circulate stably in body fluids, are widely studied as potential biomarkers for NPC. While many countries with high NPC prevalence are actively studying extracellular miRNAs as diagnostic biomarkers for NPC, such studies in Malaysia were scarce. Thus, this study aimed to identify the circulating and salivary miRNAs as early detection biomarkers of the disease by analysing their differential expression. Blood and saliva samples were collected from 37 NPC and 37 control subjects in the states of Pahang and Kelantan, and were subjected to miRNA extraction. miRNA extracts from NPC (n=10) and control (n=11) plasma samples were used to screen the differentially expressed miRNAs using Taqman® Low Density Array card A and B. The significant differentially expressed ( $p < 0.05$ ) miRNAs in plasma of NPC subjects were selected for validation using 36 plasma and 37 saliva samples of NPC and control subjects. The consistent differential expression of miRNAs in plasma, as well as their corresponding miRNAs in saliva, were further analysed to evaluate their diagnostic performance. The selected miRNAs were cross-validated to select the best combination of miRNA model in predicting NPC. ROC curve analysis was used to evaluate the diagnostic performance of miRNA models. The result on circulating miRNA screening showed that eleven miRNAs were significantly differentially expressed ( $p < 0.05$ ) in NPC as compared to control subjects. The validation on eight selected miRNAs revealed that four miRNAs, namely hsa-miR-150, hsa-miR-205, hsa-miR-639 and hsa-miR-889, were consistently differentially expressed in plasma of NPC as compared to control subjects with significant result. The cross-validation showed three similar circulating and salivary miRNAs were selected as the best model in predicting NPC. Additionally, two circulating miRNAs, namely hsa-miR-150 and hsa-miR-205, and one salivary miRNA, namely hsa-miR-144#, were also proposed as miRNA models for diagnostic performance analysis due to their significant expression. The ROC curve analysis demonstrated that the model with two circulating miRNAs, namely hsa-miR-150 and hsa-miR-205, with adjustment for known risk factors of NPC was the best model in predicting NPC (AUC = 0.865) and early stage NPC (AUC = 0.860). Therefore, the present study proposed the two miRNAs, namely hsa-miR-150 and hsa-miR-205, as minimal invasive diagnostic biomarkers for early detection of NPC. The findings from this study is expected to provide more evidences for the gaps in discovery phase of biomarker development pipelines for NPC.

## خلاصة البحث

يعتبر سرطان البلعوم الأنفي (NPC) من أكثر أنواع السرطان إنتشارا في ماليزيا حيث يتم تشخيصه في مراحل متأخرة. طرق التشخيص الحديثة غير مريحة ومؤلمة وتثني أولئك المعرضين للخطر من الفحص في المراحل الأولى. وبالتالي فإن اكتشاف مؤشرات حيوية غير توغلية للكشف المبكر عن NPC أمر مطلوب. الحمض الريبوزي النووي الميكروي (miRNA) هي فئة من الأحماض الريبوزية النووية واكتشف أنها تسير بثبات في سوائل الجسم، حيث أنها درست بشكل واسع كمؤشرات حيوية واعدة للNPC. لدى العديد من البلدان ذات الانتشار المرتفع ل NPC جهود موثقة في دراسة miRNA خارج الخلية لاستعمالها كمؤشر تشخيصي، ولكن الدراسات المماثلة على المايزيين كانت نادرة. ولذلك هدفت هذه الدراسة إلى تحديد miRNA السائر في الدم واللعاب والذي بإمكانها أن تكون مؤشرات حيوية للكشف المبكر عن المرض عن طريق تحليل تعبيرها الجيني التفاضلي. تم جمع عينات الدم واللعاب من 37 مرضى NPC و 37 مشاركا ضابطا في ولايتي باهانغ وكلنتن ومن ثم تم استخلاص miRNA منها. تم استخدام مستخلصات miRNA من عينات ال NPC (n=10) والعينات الضابطة في البلازما (n=11) لفحص ال miRNA المعبرة تفاضليا باستخدام بطاقات Taqman® A و B ذي المنظومة المخفضة الكثافة. تم اختيار ال miRNA المعبرة عنها تفاضليا بشكل ملحوظ ( $p > 0.05$ ) في بلازما مرضى ال NPC لغرض التحقق من الصحة واستخدمت 36 عينة من البلازما و 37 عينة لعاب من مرضى ال NPC والمجموعة الضابطة. تم القيام بالمزيد من التحليل على التعبير التفاضلي الثابت لل miRNA في البلازما وال miRNA المقابلة في اللعاب لتقييم أدائها التشخيصي. تم التحقق من صحة ال miRNAs المختارة استعراضيا لتحديد أفضل مزيج من نموذج ال miRNA في التنبؤ ب NPC. تم استخدام تحليل منحنى ROC لتقييم الأداء التشخيصي لنماذج miRNA. أظهرت نتائج فحص ال miRNA السائرة أنه تم التعبير عن أحد عشر miRNA تفاضليا بشكل ملحوظ ( $p > 0.05$ ) في مرضى ال NPC مقارنة بالمجموعة الضابطة. كشف التحقق من صحة ثمانية miRNA المختارة أن أربعة من ال miRNA وهي: hsa-miR-150 و hsa-miR-205 و hsa-miR-639 و hsa-miR-889 تم التعبير عنها بشكل مختلف في بلازما ال NPC مقارنة بالمجموعة الضابطة وبنائج ملحوظة. أظهرت عملية التحقق الاستعراضية لثلاثة miRNA سائرة وشائعة تم اختيارها كأفضل نموذج للتنبؤ بال NPC. وفي النهاية اثنتان من ال miRNA السائرة وهما: hsa-miR-150 و hsa-miR-205 و hsa-miR-144# لعايي واحد وهو hsa-miR-144# قد تم اقتراحها كنماذج miRNA لتحليل أداءها التشخيصي نظرا لتعبيرها الملحوظ. أظهر تحليل منحنى ROC أن النموذج المحتوي على اثنين من ال miRNA السائرة وهي: hsa-miR-150 و hsa-miR-205، مع تعديل عوامل الخطر المعروفة لل NPC، قد كانا أفضل نموذجين للتنبؤ بال NPC (AUC=0.865) والمرحلة المبكرة من ال NPC (AUC=0.865). ولذلك اقترحت هذه الدراسة اثنين من ال miRNA وهما hsa-miR-150 و hsa-miR-205 كمؤشرات حيوية تشخيصية عديمة التوغل للكشف المبكر عن ال NPC. من المتوقع أن تقدم نتائج هذه الدراسة مزيداً من الأدلة على الفجوات الموجودة في مرحلة الاستكشاف في خطة تطوير المؤشرات الحيوية لل NPC.

## APPROVAL PAGE

The thesis of Student's Name has been approved by the following:

---

Asst. Prof. Dr. Mohd. Arifin Kaderi  
Supervisor

---

Asst. Prof. Dr. Siti Marponga Tolos  
Co-Supervisor

---

Assoc. Prof. Dr. Mohammad Syaiful Bahari Abdul Rasad  
Internal Examiner

---

Prof. Dr. Teh Lay Kek  
External Examiner

---

Assoc. Prof. Dr. Muhammad Farid Johan  
External Examiner

---

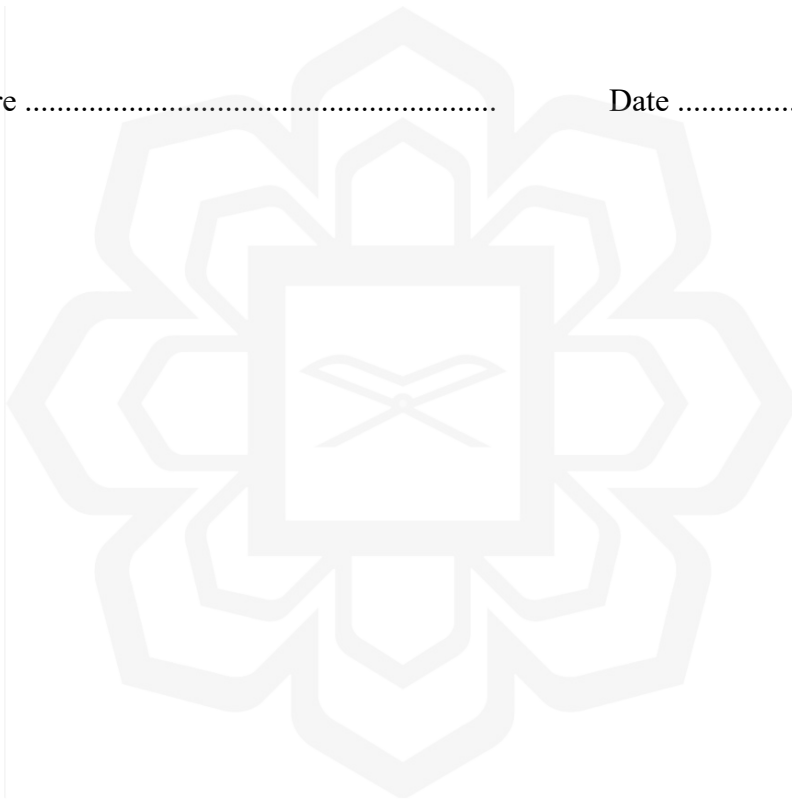
Assoc. Prof. Dr. Zarina Zainuddin  
Chairman

## DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Azmir Bin Ahmad

Signature ..... Date .....



**INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA**

**DECLARATION OF COPYRIGHT AND AFFIRMATION OF  
FAIR USE OF UNPUBLISHED RESEARCH**

**CIRCULATING AND SALIVARY MICRORNA EXPRESSION  
ANALYSIS IN NASOPHARYNGEAL CARCINOMA:  
POTENTIAL FOR DIAGNOSTIC BIOMARKER**

I declare that the copyright holders of this dissertation are jointly owned by the student and IIUM.

Copyright © 2020 Azmir Bin Ahmad and International Islamic University Malaysia. All rights reserved.

No part of this unpublished research may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording or otherwise without prior written permission of the copyright holder except as provided below

١. Any material contained in or derived from this unpublished research may be used by others in their writing with due acknowledgement.
٢. IIUM or its library will have the right to make and transmit copies (print or electronic) for institutional and academic purposes.
٣. The IIUM library will have the right to make, store in a retrieved system and supply copies of this unpublished research if requested by other universities and research libraries.

By signing this form, I acknowledged that I have read and understand the IIUM Intellectual Property Right and Commercialization policy.

Affirmed by Azmir Bin Ahmad

.....  
Signature

.....  
Date

## ACKNOWLEDGEMENTS

First and foremost, praises and thanks to Allah the Almighty, for His showers of blessing and guidance, I able to complete the research successfully. I would like to express my gratitude to Him for giving me strength and patience to work through all these years to explore the knowledge and finish the study to the best of my efforts.

A special thanks to my research supervisor, Dr. Mohd. Arifin b. Kaderi and my co-supervisors, Dr. Siti Marponga bt. Tolos and Dr. Afidalina bt. Tumian, for providing me their valuable guidance, suggestions, continuous support and encouragement. It was a great honour to work and study under their supervision.

I wish to express my appreciation and thanks to Ministry of Higher Education and International Islamic University Malaysia for funding this research. I extend my thanks to all the respondents that agreed to contribute their information, and also to all doctors and nurses in Hospital Tengku Ampuan Afzan (HTAA), Hospital Sultan Haji Ahmad Shah (HOSHAS), Hospital Universiti Sains Malaysia (HUSM) and Hospital Raja Perempuan Zainab II (HRPZ), whom helped my team research to collect the biological samples. Without their contributions, the study would not have started.

I am also thankful to all my friends who provided their time, effort and support directly or indirectly for this research. For that, I will never forget your kindness and encouragement.

Finally, I owe more than thanks to my beloved family members. I acknowledge the people who mean a lot to me, my parents, Ahmad b. Mohd. Nor and Wan Hasnah bt. Wan Daud, as well as my parents-in-law, Mohd Yassin b. Amin and Siti Mureseh bt. Md. Yatim for their unconditional love, prayers, caring and sacrifices for educating and preparing me for my future. I am very much grateful to my dear wife, Wardah bt. Mohd. Yassin for her endless love, understanding and dedicated efforts which contributed a lot for the completion of my thesis. Also, I extend my thanks to my parents in law and siblings for their encouragement and valuable prayers. May Allah bless all of them and reward with goodness.

# TABLE OF CONTENTS

Abstract in English.....	ii
Abstract in Arabic .....	iii
Approval Page.....	iv
Declaration .....	v
Copyright Page.....	vi
Acknowledgements.....	vii
List of Tables .....	xii
List of Figures .....	xv
List of Abbreviations .....	xvii
List of Symbols .....	xxi
<b>CHAPTER ONE: INTRODUCTION</b>	
1.1 Background of the study.....	1
1.2 Research Problem.....	4
1.3 Research Questions .....	10
1.4 Research Objectives.....	10
1.4.1 General Objective .....	10
1.4.2 Specific Objectives.....	11
1.5 Hypotheses .....	11
<b>CHAPTER TWO: LITERATURE REVIEW</b> .....	12
2.1 Nasopharyngeal Carcinoma.....	12
2.1.1 Histological Classification of NPC .....	15
2.1.2 NPC Staging .....	17
2.1.3 Aetiology .....	22
2.1.4 Pathogenesis .....	24
2.1.5 Detection of NPC .....	26
2.1.5.1 Clincial Presentation of NPC.....	26
2.1.5.2 Late Detection of NPC in Malaysia.....	28
2.1.5.3 Current Methods of NPC Diagnosis.....	31
2.2 MicroRNA .....	32
2.2.1 Canonical Pathway of miRNAs Biogenesis.....	33
2.2.2 Mechanisms of miRNA-mediated Gene Regulation.....	36
2.2.3 Circulating miRNAs.....	38
2.2.4 Salivary miRNAs.....	39
2.2.5 Circulating and Salivary miRNAs as Biomarker for Early Detection of Cancers.....	42
2.3 Interplay of miRNAs in NPC .....	45
2.3.1 Carcinogenic Roles of miRNAs in NPC .....	45
2.3.2 Potential Utiliy of Circulating and Salivary miRNAs In Diagnosis of NPC.....	47
<b>CHAPTER THREE: METHODOLOGY</b> .....	50
3.1 Materials.....	50

3.1.1 Consumables .....	50
3.1.2 Chemicals and Reagents.....	51
3.1.3 Instruments and Apparatus.....	52
<i>3.2 Overview on Methodology of the Study .....</i>	<i>52</i>
<i>3.3 Pre-analytical Phase.....</i>	<i>54</i>
<i>3.3.1 Sample Size Calculation .....</i>	<i>54</i>
<i>3.3.2 Subject Recruitment.....</i>	<i>56</i>
<i>3.3.3 Biological Sample Collection.....</i>	<i>58</i>
<i>3.3.4 miRNA Isolation .....</i>	<i>58</i>
3.3.4.1 miRNA Isolation from Plasma .....	59
3.3.4.2 miRNA Isolation from Saliva.....	62
<i>3.3.5 Quality Control for Plasma and Saliva miRNA Extracts .....</i>	<i>63</i>
3.3.5.1 Reverse Transcription Reaction .....	64
3.3.5.2 Preamplification Reaction .....	66
3.3.5.3 Quantitative Polymerase Chain Reaction .....	67
<i>3.4 Screening Phase.....</i>	<i>70</i>
3.4.1 Reverse Transcription Reaction.....	72
3.4.2 Preamplification Reaction .....	73
3.4.3 Quantitative Polymerase Chain Reaction.....	74
3.4.4 Pre-processing of Raw Cq Data .....	77
3.4.5 Calculation of Differential Expression for Circulating miRNAs .....	78
3.4.6 Selection of Reference Genes for Validation .....	79
<i>3.5 Validation Phase .....</i>	<i>80</i>
3.5.1 Reverse Transcription Reaction.....	82
3.5.2 Preamplification Reaction .....	84
3.5.3 Quantitative Polymerase Chain Reaction.....	86
3.5.4 Pre-processing of Raw Cq Data .....	87
3.5.5 Calculation of Differential Expression for Circulating and Salivary miRNAs.....	88
<i>3.6 Diagnostic Analysis Phase.....</i>	<i>88</i>
3.6.1 General Statistical Analysis.....	88
3.6.2 Statistical Analysis on Diagnostic Performance of Circulating and Salivary miRNAs Models.....	89
<b>CHAPTER FOUR: RESULTS .....</b>	<b>91</b>
<i>4.1 Pre-analytical Phase .....</i>	<i>91</i>
4.1.1 Demographical Data .....	91
4.1.1.1 Demographical Data for Plasma Samples in Validation Phase .....	91
4.1.1.2 Demographical Data for Plasma and Saliva Samples in Validation Phase .....	93
4.1.2 Quality Control of miRNA Extracts for Plasma and Saliva Samples.....	95
4.1.3 Overall Results in Pre-analytical Phase.....	99
<i>4.2 Screening Phase.....</i>	<i>100</i>
4.2.1 Pre-processing for Raw Cq Data .....	100

4.2.2 Identification of the Best Normalization Method Using Box Plots Assessment .....	103
4.2.3 Identifying Significant Differentially Expressed Circulating miRNAs in NPC as Compared to Control Subjects .....	109
4.2.4 Selection of Reference Genes for miRNA Expression Validation .....	113
4.2.5 Overall Results in Screening Phase .....	115
<b>4.3 Validation Phase .....</b>	<b>115</b>
4.3.1 Pre-processing for Raw Cq Data .....	116
4.3.2 Identification of the Best Normalization Method Using Box Plots Assessment .....	118
4.3.3 Validation on Differential Expression of Eight Circulating miRNAs in Larger Size of Plasma and Saliva Samples .....	124
4.3.4 Overall Results in Validation Phase .....	130
<b>4.4 Diagnostic Analysis Phase.....</b>	<b>130</b>
4.4.1 Modelling of Diagnostic Panels .....	131
4.4.2 Assessment of Diagnostic Models to Discriminate NPC from Control Group .....	137
4.4.3 Assessment of Diagnostic Models to Discriminate Early Stage NPC from Control Group.....	140
4.4.4 Overall Results in Diagnostic Analysis Phase.....	141
<b>CHAPTER FIVE: DISCUSSION.....</b>	<b>143</b>
<b>5.1 Pre-analytical Phase.....</b>	<b>144</b>
5.1.1 Quality Control of miRNA Extracts for Plasma and Saliva Samples.....	144
<b>5.2 Screening Phase.....</b>	<b>151</b>
5.2.1 Pre-processing of Raw Cq Data Prior to Statistical Analysis.....	152
5.2.2 Selection of Normalization Method .....	154
5.2.3 Screening of Differentially Expressed miRNAs in Plasma of NPC.....	159
<b>5.3 Validation Phase.....</b>	<b>162</b>
5.3.1 Differential Expression Validation in Plasma Samples.....	162
5.3.2 Differential Expression Validation in Saliva Samples .....	164
<b>5.4 Diagnostic Analysis Phase.....</b>	<b>166</b>
5.4.1 Modelling of Circulating and Salivary Diagnostic Panel of miRNAs.....	166
5.4.2 ROC Curve Analysis on the Developed Diagnostic miRNA Models .....	170
5.4.2.1 Diagnostic Performance in Discriminating NPC from Control Group .....	171
5.4.2.2 Diagnostic Performance in Discriminating Early Stage NPC from Control Group.....	173
5.4.2.3 Overall Diagnostic Performance of miRNA Models .....	175
<b>5.5 Limitations of Study .....</b>	<b>176</b>
<b>CHAPTER SIX: CONCLUSION .....</b>	<b>178</b>
<b>6.1 Conclusion.....</b>	<b>178</b>
<b>6.2 Future Recommendations .....</b>	<b>180</b>

**REFERENCES..... 182**

**APPENDIX A: COMPARISON OF BH CRITICAL VALUE FOR  
DIFFERENT FDR IN RESULT OF MANN-WHITNEY U  
TEST ON CARD A .....224**

**APPENDIX B: ETHIC APPROVAL NMRR .....227**

**APPENDIX C: ETHIC APPROVAL IREC.....228**

**APPENDIX D: ETHIC APPROVAL JEPeM.....229**

**APPENDIX E: INFORMED CONSENT FORM .....230**

**APPENDIX F: LIST OF PUBLICATIONS AND PRESENTIONS.....237**

**APPENDIX G: ABSTRACT OF PUBLICATIONS .....238**



## LIST OF TABLES

<u>Table No.</u>		<u>Page No.</u>
2.1	Description on TNM system of NPC.	19
2.2	Description on group staging of NPC.	20
3.1	List of consumables.	50
3.2	List of chemicals and reagents.	51
3.3	List of instruments and apparatus.	52
3.4	RT mixture for individual assay.	65
3.5	RT reaction set up.	66
3.6	Preamplification mixture for individual assay.	66
3.7	Preamplification reaction set up.	67
3.8	qPCR mixture for individual assay.	68
3.9	qPCR set up.	69
3.10	RT master mix for screening.	72
3.11	RT reaction set up for screening.	73
3.12	Preamplification master mix for screening.	73
3.13	Preamplification reaction set up for screening.	74
3.14	qPCR mix for screening.	74
3.15	qPCR reaction set up for screening.	76
3.16	List of primers used in validation phase.	83
3.17	RT master mix for validation.	83
3.18	RT reaction set up for validation.	84
3.19	Preamplification mixture for validation.	85
3.20	Preamplification reaction set up for validation.	85

3.21	Sample pre-mix mixture for validation.	86
3.22	Assay mixture for validation.	87
4.1	Normality test on plasma demographical data for screening phase.	92
4.2	Demographical characteristics for screening phase.	93
4.3	Normality test on demographical data for plasma and saliva samples in validation phase.	94
4.4	Demographical data on plasma and saliva samples for validation phase.	95
4.5	Purity and miRNA RNA yield of plasma samples for screening phase.	96
4.6	Spectrophotometric and fluorometric results of six plasma miRNA extracts.	98
4.7	Spectrophotometric and fluorometric results of six saliva miRNA extracts.	98
4.8	Significant differentially expressed circulating miRNAs in NPC patients as compared to control subjects and the most least significant circulating miRNA in card A and B.	110
4.9	Scores of candidate reference genes from geNorm, NormFinder and CV.	114
4.10	Candidate miRNAs for validation phase.	116
4.11	Scores of validated five candidate reference genes in plasma and saliva samples.	118
4.12	The <i>p</i> -values for normality test on Cq data of plasma and saliva samples for validation phase.	125
4.13	Validation of six significant differentially expressed circulating miRNAs in NPC patients as compared to control subjects.	126
4.14	Validation of seven significant differentially expressed salivary miRNAs in NPC patients as compared to control subjects.	129

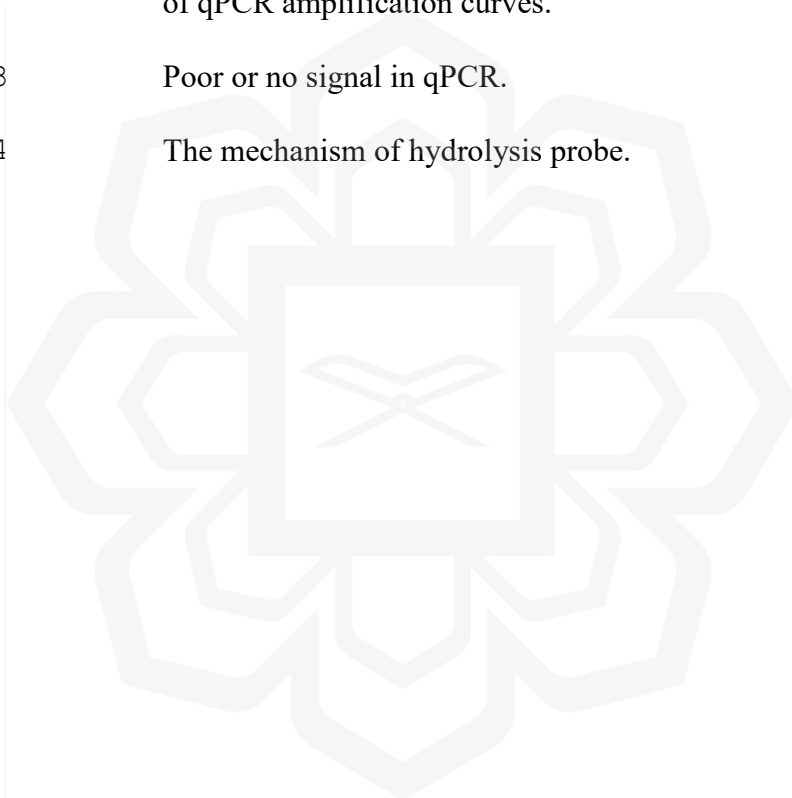
4.15	OR and $p$ -value of selected circulating miRNAs and risk factor variables from modelling of diagnostic panel.	133
4.16	OR and $p$ -value of selected salivary miRNAs and risk factor variables from modelling of diagnostic panel.	135
4.17	Developed and suggested models for diagnostic performance analysis.	136
4.18	Diagnostic performance of four circulating miRNA models.	138
4.19	Diagnostic performance of four salivary miRNA models.	139
4.20	Diagnostic performance of circulating and salivary miRNA models for early detection of NPC in discriminating early stage NPC from controls.	140
5.1	Example on the adjustment for multiple comparisons using BH method.	162

## LIST OF FIGURES

<u>Figure No.</u>		<u>Page No.</u>
2.1	Location of nasopharynx.	14
2.2	Canonical miRNA biogenesis.	36
3.1	Overview on methodology of the study.	54
3.2	PS: Power and Sample Size Calculation.	55
3.3	Coloured chart for visual inspection of haemolyzed samples by A) Kirschner et al. (2011) and B) Myklebust et al. (2019).	60
3.4	Plate set up for individual assay.	68
3.5	Overview on procedures for pre-analytical phase.	70
3.6	Taqman® Low Density Array card.	71
3.7	Bucket for TLDA cards.	75
3.8	Sealer for TLDA cards.	76
3.9	Parts of 96.96 Dynamic Array™ IFC chip.	81
4.1	Amplification curves of ath-miR-159a and hsa-miR-16 in six plasma and six saliva miRNA extracts.	99
4.2	Flow chart on pre-processing of raw Cq data.	102
4.3	Numbers of miRNAs in card A and B before and after filtering the miRNAs with undetermined and unreliable Cq.	103
4.4	Cq and CV box plots for normalization methods in HTqPCR package for card A.	105
4.5	Cq and CV box plots for normalization methods in HTqPCR package for card B.	107
4.6	Bar charts of eleven significant differentially expressed and the two most least significant	110

	circulating miRNAs (the last two from right) in card A and B.	
4.7	Distribution of miRNAs based on $p$ -value of 0.05 in card A (A) and B (B).	112
4.8	Unsupervised hierarchical clustering of eleven significant differentially expressed ( $p < 0.05$ ) circulating miRNAs in NPC patients as compared to control subjects.	113
4.9	Overview on raw Cq of eight A) circulating and B) salivary miRNAs in validation phase.	117
4.10	Evaluation of normalization methods for plasma samples in validation phase.	119
4.11	Evaluation of normalization methods for saliva samples in validation phase.	121
4.12	Overview on quantile-normalized Cq of eight circulating miRNAs in validation phase.	123
4.13	Overview on quantile-normalized Cq of eight salivary miRNAs in validation phase.	123
4.14	Bar charts on validation of differential expression of six circulating miRNAs, where * for $p < 0.05$ ; ** for $p < 0.01$ and *** for $p < 0.001$ based on Mann-Whitney U test.	126
4.15	Comparison of differential expression result of circulating miRNAs in screening and validation phase.	127
4.16	Bar charts on validation of differential expression of seven salivary miRNAs, where * for $p < 0.05$ based on Mann-Whitney U test.	128
4.17	Comparison of differential expression results between miRNAs in saliva and plasma samples.	130
4.18	Cross-validation plot on selection of best fitted model for circulating miRNAs by experimental groups.	132
4.19	Cross-validation plot on selection of best fitted model for salivary miRNAs by experimental groups.	135

4.20	ROC curve plots of four circulating miRNA models.	139
4.21	ROC curve plots of four salivary miRNA models.	140
4.22	ROC curve plots of circulating and salivary miRNA models for early detection of NPC in discriminating early stage from controls.	142
5.1	Examples of graphs produced by NanoDrop for miRNA extracts of plasma.	146
5.2	Normal (black) and abnormal (red) sigmoid shape of qPCR amplification curves.	148
5.3	Poor or no signal in qPCR.	148
5.4	The mechanism of hydrolysis probe.	151



## LIST OF ABBREVIATIONS

AGO	Argonaute
AUC	Area under curve
BH	Benjamini-Hochberg
DNA	Deoxyribonucleic acid
CI	Confidence interval
Cq	Cycle quantification
CTNNB1	Catenin beta-1
CT	Computed tomography
CV	Coefficient of variation
EBER	Epstein Barr virus-encoded early ribonucleic acid
EBNA	Epstein Barr virus nuclear antigen
EBV	Epstein-Barr virus
EDRN	Early Detection Research Network
ENT	Ear, nose and throat
et al.	(et alia); and others
FC	Fold change
FDR	False discovery rate
FNAC	Fine needle aspiration cytological
HPE	Histopathological examination
HNSCC	Head and neck squamous cell carcinoma
IARC	International Agency for Research on Cancer
IFC	Integrated fluidic circuit

IIUM	International Islamic University Malaysia
IQR	Interquartile range
LMP	Latent membrane protein
MAPK	Mitogen-activated protein kinase
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiment
MRE	Micro ribonucleic acid response elements
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
miRNA	Micro ribonucleic acid
MOH	Ministry of Health
NF- $\kappa$ $\beta$	Nuclear factor kappa beta
NPC	Nasopharyngeal carcinoma
NSCLC	Non-small cell lung cancer
NTC	No template control
OR	Odds ratio
P2-miR	Plasma 2 miRNAs
P3-miR	Plasma 3 miRNAs
PCR	Polymerase chain reaction
pH	Potential hydrogen
Pre-miRNA	Precursor micro ribonucleic acid
Pri-miRNA	Primary micro ribonucleic acid
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cells
RIN	Ribonucleic acid integrity number

RISC	Ribonucleic acid-induced silencing complex
ROC	Receiver operating characteristics
RNA	Ribonucleic acid
RT	Reverse transcription
S1-miR	Saliva 1 miRNA
S3-miR	Saliva 3 miRNAs
SD	Standard deviation
SSS	Summarized stability score
TLDA	Taqman® Low Density Array
UICC	Union for International Cancer Control
UTR	Untranslated region
WHO	World Health Organization

## LIST OF SYMBOLS

%	Per cent
kDa	Kilo Dalton
°C	Degree Celsius
x g	Times gravity
μL	Microlitre
pmol	Picomole
μg	Microgram
mL	Millilitre
U	Micromole per minute
U/μL	Unit per microlitre
mM	Millimolar
rpm	Revolutions per minute
∞	Infinity
ng/μL	Nanogram per microlitre
ΔCq	Delta Cq
ΔΔCq	Delta delta Cq
nm	Nanometre

# CHAPTER ONE

## INTRODUCTION

### 1.1 BACKGROUND OF THE STUDY

Cancers are among the leading causes of morbidity and mortality worldwide. Global health observatory data 2015 from World Health Organization (WHO) (WHO, 2017a) showed that, cancers, specifically trachea, bronchus and lung cancers, were among causes of death worldwide. Among the non-communicable diseases, cancers were the second cause of death after cardiovascular diseases (WHO, 2017b). International Agency for Research on Cancer (IARC) of WHO (Stewart & Wild, 2014) has reported that in 2012 there were approximately 14.1 million new cases and 8.2 million cancer-related deaths worldwide where more than half occurred in less developed countries. This figure was expected to increase to 22 million in next two decades. Meanwhile, in Malaysia, cancers were the fourth leading cause of death in government hospitals and the first in private hospitals (Ministry of Health [MOH], 2017). A report by Manan, Tamin, Abdullah, Abidin and Wahab (2016) indicated that death due to cancers was increasing from 2007 until 2011. Ten leading cancers that were frequently reported in Malaysia are breast cancer followed by colorectal, lung, lymphoma, nasopharynx, leukaemia, cervix, liver, ovary and stomach cancer (Manan et al., 2016).

Nasopharyngeal carcinoma (NPC) was an uncommon cancer globally unlike other cancers but the prevalence of NPC was significantly high in certain countries in South-East Asia, Micronesia/Polynesia and Eastern Asia and in certain ethnicities such as Chinese and Bidayuh in Malaysia (Devi, Pisani, Tang & Parkin 2004; Torre et al., 2015; Manan et al., 2016). In Malaysia, NPC was among frequently reported cancer where it was the third leading cancer in Malaysian males (Manan et al., 2016). In a

report by Manan et al. (2016) revealed that NPC was most common cancer among Chinese males followed by Malay and Indian males. The authors also reported that Malaysian Chinese was the third race with high prevalence of NPC after China and Singaporean Chinese. In the previous years, Devi et al. (2004) have reported a surprisingly high prevalence of NPC among Bidayuh native group in Sarawak where the prevalence was the highest rate recorded by worldwide population-based registry between years of 1996 until 1998. Additionally, it was reported that NPC in Malaysia was most commonly detected at late stage (Devi et al. 2004; Manan et al., 2016). Survival rate of a cancer patient is improved when the cancer is detected and treated at early stage (Cancer Research UK, 2015). Unfortunately, about 80% of NPC cases in Malaysia were diagnosed at late stage (Prasad & Pua, 2000; Devi et al., 2004). The remote anatomic location of NPC, asymptomatic and its rich lymphatic supply may contribute to the late detection of NPC (Ayadi et al., 2009; M. F. Ji et al., 2011). The body of evidence showed that more studies must be conducted to improve the performance of diagnostic programmes for NPC in Malaysia.

MicroRNA (miRNA) is a class of non-coding RNAs that post-transcriptionally inhibits or degrades messenger RNA (mRNA) (Breving & Esquela-Krescher, 2010). Normally, it involves in the regulation of cellular development such as cell growth, proliferation, differentiation and apoptosis (Schickel, Boyerinas, Park, & Peter, 2008). In cancers, miRNAs can deregulate the normal cellular physiology by being a tumour suppressor, oncogenic or viral miRNAs, depending on its function in the mechanisms of oncogenesis or tumour progression (Esquela-Krescher & Slack, 2006; Pfeffer & Voinnet, 2006; Shenouda & Alahari, 2009). A few miRNAs have been reviewed comprehensively by Kent and Mendell (2006) on their causative roles as tumour suppressor and oncogene in oncogenesis across diverse cancers. Among the tumour

suppressor miRNAs were miR-15a and miR-16-1, which involve in apoptosis and oncogenic transcript decay (Calin et al., 2002; Calin et al., 2005; Cimmino et al., 2005); let-7, which involves in cell-cycle regulation (Pasquinelli et al. 2000); and miR-143 and miR-145, which were postulated to loss of function in malignancies that due to defect at Dicer processing (Michael, O'Connor, van Holst Pellekaan, Young, & James 2003). Several studies have been performed to profile and determine the role of miRNAs in NPC. A review on miRNAs in NPC has listed the miRNAs encoded in human and Epstein-Barr virus (EBV) that involve in the development and progression of NPC (Bruce & Liu, 2014). EBV was found to encode certain miRNAs that target and suppress host pro-apoptotic genes and genes that regulate host immune response (Choy et al., 2008; Dölken et al., 2010). Preliminary studies in NPC found that human miR-2c and miR-375 have tumour suppressive role by targeting mRNA that encoded extracellular matrix protein and causing up-regulation of *metadherin* oncogene, respectively (Sengupta et al., 2008; A. B. Y. Hui et al., 2011).

Other than their presence inside the tissues, miRNAs have been found to be present in extracellular environment such as in plasma, serum, saliva and urine (Zubakov et al., 2010). The expression level of these miRNAs seems to change according to disease state (Patel et al., 2011; Ren, Dong, Tsoi, & Yu, 2015). Tissue, blood and salivary miRNA have been found to share similar expression profile, which indicates that interchange of miRNAs may occurred between tissue, blood and saliva (Weber et al., 2010; Wiklund et al., 2011). These findings have improved the understanding in the physiological correlation between miRNAs in tissue, plasma and saliva, while at the same time seem to reveal that miRNAs in saliva have potential to be the non-invasive diagnostic biomarkers. Nowadays, extracellular miRNAs have been widely studied for their potential as novel biomarkers in NPC, especially in blood.