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

BY

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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 studied extracellular miRNAs as diagnostic biomarker for NPC, such studies in Malaysia were scarce. Thus, this study aimed to identify the circulating and salivary miRNAs as early detection biomarker 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 إنشارا 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 الNPC 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 لـ NPC جهود موثقة في دراسة الmiRNA خارج الخلية لاستعمالها كمؤشر تشخيصي، ولكن الدراسات المماثلة على الماليزيين كانت نادرة. ولذلك هدفت هذه الدراسة إلى تحديد الmiRNA السائر في الدم واللعابي والذي بإمكانها أن تكون مؤشرات حيوية للكشف المبكر عن المرض عن طريق تحليل تعبيرها الجيني التفاضلي. تم جمع عينات الدم واللعاب من ٣٧ مرضى NPC و ٣٧ مشاركا ضابطا في ولايتي باهانغ وكلنتن ومن ثم تم استخلاص الmiRNA منها. تم استخدام مستخلصات الmiRNA من عينات الـ NPC (n=10) والعينات الضابطة في البلازما (n=11) لفحص الـ miRNA المعبرة تفاضليا باستخدام بطاقات TaqMan® A و B ذي المنظومة المخفضة الكثافة. تم اختيار الmiRNA المعبرة عنها تفاضليا بشكل ملحوظ (p<٠,٠٥) في بلازما مرضى الـ NPC لغرض التحقق من الصحة واستخدمت ٣٦ عينة من البلازما و ٣٧ عينة لعاب من مرضى الـ NPC والمجموعة الضابطة. تم القيام بالمزيد من التحليل على التعبير التفاضلي الثابت للmiRNA في البلازما والmiRNA المقابلة في اللعاب لتقييم أدائها التشخيصي. تم التحقق من صحة الmiRNAs المختارة استعراضيا لتحديد أفضل مزيج من نموذج الmiRNA في التنبؤ بNPC. تم استخدام تحليل منحنى ROC لتقييم الأداء التشخيصي لنماذج miRNA. أظهرت نتائج فحص الmiRNA السائرة أنه تم التعبير عن أحد عشر miRNA تفاضليا بشكل ملحوظ (p<٠,٠٥) في مرضى الـ NPC مقارنة بالمجموعة الضابطة. كشف التحقق من صحة ثمانية miRNA المختارة أن أربعة من الmiRNA وهي: hsa-miR-150 و hsa-miR-205 و hsa-miR-639 و hsa-miR-889 تم التعبير عنها بشكل مختلف في بلازما الـ NPC مقارنة بالمجموعة الضابطة وبنتائج ملحوظة. أظهرت عملية التحقق الاستعراضي ثلاثة miRNA سائرة وشائعة تم اختيارها كأفضل نموذج للتنبؤ بالـ NPC. وفي النهاية اثنتان من الmiRNA السائرة وهما: hsa-miR-١٥٠ و hsa-miR-205 و miRNA لعابي 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 الmiRNA السائرة وهي: hsa-miR-150 و hsa-miR-205، مع تعديل عوامل الخطر المعروفة للNPC، قد كانا أفضل نموذجين للتنبؤ بالـ NPC (AUC=٠,٨٦٥) والمرحلة المبكرة من الNPC (AUC=٠,٨٦٠). ولذلك اقترحت هذه الدراسة اثنين من الmiRNA وهما hsa-miR-150 و hsa-miR-205 كمؤشرات حيوية تشخيصية عديمة التوغل للكشف المبكر عن الNPC. من المتوقع أن تقدم نتائج هذه الدراسة مزيدًا من الأدلة على الفجوات الموجودة في مرحلة الاستكتشاف في خطة تطوير المؤشرات الحيوية للNPC.

APPROVAL PAGE

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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.

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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-κβ	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 Intenational 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

١,١ 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.