The Role of MicroRNAs in Human Nasopharyngeal Carcinoma

by

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Abstract

Despite significant improvement in locoregional control in the contemporary era, nasopharyngeal carcinoma (NPC) patients still suffer from a significant risk of distant metastasis (DM). Thus, developing methods to better identify and treat NPC patients at risk of DM is of paramount importance. MicroRNAs (miRNAs) have been shown to provide insight into both the biology and clinical behaviour of human cancers; hence, this thesis aims to characterize both the biological function and the prognostic utility of miRNAs in NPC.

First, miR-375 was identified as the most consistently and significantly down-regulated miRNA in head and neck cancer samples (including NPC) analyzed. Functional experiments confirmed a tumour suppressor role for miR-375. A novel target of miR-375, metadherin, was identified as a likely mediator of these effects; possibly through activation of the PI3K/Akt pathway. In addition, a clinical relationship was observed wherein NPC patients whose tumours expressed high metadherin transcript levels had an increased risk of developing distant metastases.

Next, a 4-miRNA expression signature associated with risk of DM was identified in a training set of 125 NPC patients (HR 8.25; $p < 0.001$). This signature was subsequently confirmed to be predictive of DM in an independent validation set ($n=121$, HR 3.2; $p=0.01$). Moreover, the 4-miRNA signature was observed to add to the prognostic value of the currently utilized clinical staging system. In addition, pathway enrichment analysis identified a potential role for these miRNAs in cell cycle regulation.

Finally, a pilot project was undertaken which established the feasibility and utility of molecular inversion probe (MIP) technology for the detection of copy-number alterations (CNAs) in small archival NPC biopsy samples.

In conclusion, the insights presented herein provide both clinical and biological insights into NPC, which will advance us closer to the goal of personalized management of this disease and improve treatment and outcome for future NPC patients.

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List of Abbreviations

5-FU	5-Fluorouracil
AEG-1	Astrocyte Elevated Gene 1
AGO	Argonaute
AJCC	American Joint Committee On Cancer
AML	Acute Myeloid Leukemia
ANOVA	Analysis Of Variance
AP-1	Activator Protein 1
APC	Anaphase Promoting Complex
ARF	ADP-Ribosylation Factor
ARSL	Affymetrix Research Service Laboratory
ASPG	Asparaginase Homolog
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia And Rad3 Related
AUC	Area Under The Curve
BARF1	Bam H1 Reading Frame 1
BART	Bam H1-A Region Rightward Transcript
BCL-2	B-Cell CLL/Lymphoma 2
BCR	B-Cell Receptor
Bim	Bcl-2 interacting mediator of cell death
BSA	Bovine Serum Albumin
CCNK	Cyclin K
CDC20	Cell-Division Cycle Protein 20
CGH	Comparative Genomic Hybridization
CI	Confidence Interval
CLL	Chronic Lymphoid Leukemia
CN	Copy-Number
CNV	Copy-Number Variant
COSMIC	Catalogue Of Somatic Mutations In Cancer
CRT	Chemoradiotherapy
CT	Chemotherapy
DAPK	Death-Associated Protein Kinase
DAVID	Database For Annotation, Visualization and Integrated Discovery
DGCR8	DiGeorge Syndrome Critical Region Gene 8
DM	Distant Metastasis
DMFS	Distant Metastasis Free Survival
DNA	Deoxyribonucleic Acid
DMNT	DNA Methyltransferase
EBER	Epstein-Barr Encoded small RNA
EBNA1	Epstein-Barr Nuclear Antigen 1
EBV	Epstein-Barr Virus
EGFR	Epidermal Growth Factor Receptor

EMT	Epithelial To Mesenchymal Transition
EVP	Events Per Variable
FACS	Fluorescence-Activated Cell Sorting
FAS	Fas Cell Surface Death Receptor
FC	Fold Change
FDR	False Discovery Rate
FFPE	Formalin Fixed Paraffin Embedded
FLT3	Fms-Related Tyrosine Kinase 3
FMRP	Fragile X Mental Retardation 1 Protein
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GHSR	Growth Hormone Secretagogue Receptor
GMFB	Glia Maturation Factor, Beta
GTP	Guanosine Triphosphate
HCC	Hepatocellular Carcinoma
HDAC	Histone Deacetylase
HIF1 α	Hypoxia-Inducible Factor 1A
HMM	Hidden Markov Model
HNC	Head And Neck Cancer
HNSCC	Head And Neck Squamous Cell Carcinoma
HR	Hazard Ratio
HT	High-Throughput
HTS	High-Throughput Sequencing
IFN	Interferon
IGF1	Insulin-like Growth Factor 1
IHC	Immunohistochemistry
IMRT	Intensity Modulated Radiation Therapy
INF	Interferon
IPO7	Importin 7
KEGG	Kyoto Encyclopedia Of Genes And Genomes
KM	Kaplan–Meier
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
LCM	Laser-Capture Microdissected
LMP	Latent Membrane Protein
LNA	Locked-Nucleic Acid
LOH	Loss of Heterozygosity
LRC	Locoregional Control
LRR	Locoregional Relapse
LTBR	Lymphotoxin-Beta Receptor
MAF	Minor Allele Frequency
MAPD	Median Absolute Pairwise Difference
MAPK	Mitogen-Activated Protein Kinases
MHC	Major Histocompatibility Complex
MICB	MHC class I-related chain B

miRNA	MicroRNA
MIP	Molecular Inversion Probe
miRISC	MiRNA Inducible Silencing Complex
MMP	Matrix Metalloproteinases
mRNA	Messenger RNA
MTDH	Metadherin
MTHFSD	Methenyltetrahydrofolate Synthetase Domain Containing
MTPN	Myotrophin
NCI	National Cancer Institute
NF- κ B	Nuclear Factor Kappa-Light-Chain-Enhancer Of Activated B Cells
NOE	Normal Oral Epithelium
NOX2	NADPH Oxidase 2
NP	Nasopharynx
NPC	Nasopharyngeal Carcinoma
NuA	Nucleic Acid
OD	Optical Density
OS	Overall Survival
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PET	Polyethylene Terephthalate
PI	Propidium Iodide
PKR	Protein Kinase RNA-Dependent
PLEK	Pleckstrin
PM	Princess Margaret Cancer Center
PNA	Peptide-Nucleic Acid
Pol	Polymerase
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
PTEN	Phosphatase And Tensin Homolog
PUMA	p53 Up-Regulated Modulator of Apoptosis
QC	Quality Control
RASSF1A	Ras Association Domain Family Member 1A
RCT	Randomized Controlled Trial
REB	Research Ethics Board
RELA	V-Rel Avian Reticuloendotheliosis Viral Oncogene Homolog A
RIG1	Retinoic Acid-Inducible Gene-Like Receptor 1
RNA	Ribonucleic Acid
ROC	Receiver Operating Characteristic
RPM	Reads Per Million
RS	Risk-Score
RT	Reverse Transcription
SCID	Severe Combined Immunodeficient

SE	Standard Error
siRNA	Small Interfering RNA
SNP	Single Nucleotide Polymorphism
SP-1	Specificity Protein 1
STAC	Significance Testing For Aberrant Copy-Number
STR	Short Tandem Repeat
TAPBPL	Tap Binding Protein-Like
TCGA	The Cancer Genome Atlas
TGF β	Transforming Growth Factor β
TLD	Tumour plus Leg Diameter
TLDA	Taqman Low Density Array
TLR3	Toll-Like Receptor 3
TMP1	T-Cell Lymphoma Invasion And Metastasis 1
TNF α	Tumour Necrosis Factor A
TNM	Tumour Node Metastasis
TOMM22	Translocase Of Outer Mitochondrial Membrane 22 Homolog
TRADD	TNFR-Associated Death Domain
TRAF	TNFR-Associated Factor
TRBP	Trans-Activation-Responsive RNA-Binding Protein
UTR	Un-Translated Region
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

Chapter 1

Introduction

1. Introduction

1.1 Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma (NPC) is an epithelial malignancy of the uppermost portion of the pharynx, located posterior to the nasal cavity, extending from the upper surface of the soft palate to the base of the skull (Figure 1-1). NPC is characterized by a unique set of geographical, etiological, and biological features that distinguish it from other cancers of the head and neck region. The prevalence of NPC is highest in distinct endemic regions such as Southeast Asia, where the annual incidence can reach up to 1/4000¹. In contrast, NPC is uncommon in Canada, although in populous and ethnically diverse cities such as Toronto, up to ~150 new cases of NPC are diagnosed each year.

The World Health Organization (WHO) defines the following histological subtypes of NPC:

1. Keratinizing squamous cell carcinoma
2. Non-keratinizing carcinoma
 - A. Differentiated type
 - B. Undifferentiated type
3. Basaloid squamous cell carcinoma

Subtypes 2A&B are by far the most prevalent, accounting for nearly all NPC cases diagnosed in endemic regions¹.

1.1.1 Environmental Risk Factors for NPC

The most common causal agent in NPC is the Epstein-Barr Virus (EBV). The EBV genome is present in ~90% of NPCs observed in endemic regions, and is thought to play an

important role in many aspects of NPC development². How EBV contributes to NPC development will be covered in more depth in section 1.1.3.2.

Other environmental risk factors associated with NPC have also been identified; largely through prospective epidemiologic studies. Like other cancers of the upper aerodigestive tract, risk of NPC is significantly increased by alcohol consumption and tobacco smoking¹. Notably, a stronger association between tobacco smoking and NPC is observed in populations *outside* endemic regions than those *within* these high-risk populations (OR: 1.76 vs. 1.29, respectively)³. Relatedly, NPC patients with differentiated-type disease (the predominant type in non-endemic populations) have a greater association with tobacco smoking than those with undifferentiated type disease (OR: 2.2 vs. 1.27, respectively)³.

A variety of dietary habits are also positively and negatively correlated with NPC. For instance, consumption of salt preserved foods has consistently been confirmed to be associated with an increased risk of NPC^{1,4,5}. The process used to preserve such foods is thought to increase levels of carcinogenic nitrosamines⁶, thus leading to the ~2-fold increased incidence of NPC in frequent consumers of these preserved foods⁵. Additional environmental risk factors that may have a link to NPC include alcohol drinking, wood-fire smoke, occupational exposures and some herbal medicines¹.

In contrast, high consumption of fresh fruits and vegetables has been shown to decrease an individual's risk of NPC^{1,5}; which could be attributed to many components within fruits and vegetables that may prevent cancer such as fiber, vitamins, folate, and carotenoids⁷.

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Figure 1-1. Anatomical structures from which head and neck cancers can arise.
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1.1.2 Clinical characteristics of NPC

NPC patients who present with early stage disease (T1-2aN0M0) are commonly treated with curative radiation therapy (RT) alone⁸. Prognosis is usually excellent in these patients with 5-year local control rates of up to 95%⁸. The majority of NPC patients, however, present with locally advanced disease and thus have a far worse prognosis. These patients have been shown to benefit significantly from concomitant chemo-radiotherapy (CRT) using Cisplatin or 5-fluorouracil (5-FU)⁹. Indeed, studies comparing CRT vs. RT alone report 3-year overall survival rates of 78-84% vs. 47-65%, respectively, in favour of the combinatorial strategy¹⁰⁻¹². Despite the obvious survival benefit, which has been documented in randomized clinical trials (RCTs), CRT also results in a higher incidence of acute and late toxicities compared with RT alone^{13,14}. This highlights the importance of selecting patients with advanced disease who could be cured with RT alone, and thereby spared the potential morbidities of combined therapy, as well as the development of novel, less toxic therapies. Moreover, while the implementation of CRT for advanced NPC patients has shown a marked improvement in local control, distant control has not improved substantially, with ~17% of advanced-stage patients treated with modern modalities still developing distant metastases within 3 years¹⁵.

1.1.3 Molecular biology of NPC

The majority of NPCs arise as a result of EBV infection combined with additional genetic and epigenetic aberrations, which collaboratively promote the proliferation, survival, and motility of the malignant epithelial cells¹⁶. While EBV is likely an early event in nasopharyngeal tumourigenesis, there is mounting evidence to suggest that pre-existing genetic abnormalities are required to support the persistent infection of pre-malignant and malignant

epithelial cells of the nasopharynx as well^{17,18}. This complex interplay between EBV infection and host alterations is summarized in **Figure 1-2**.

Figure 1-2. Summary of the host-EBV relationship promoting oncogenic phenotypes in NPC cells.

1.1.3.1 Genetic alterations in NPC

The majority of genomic studies on NPC have focused on gross chromosomal abnormalities utilizing cytogenetic techniques such as G-banding, comparative genomic hybridization (CGH), and array CGH (aCGH) to detect copy number events and translocations. The first studies examined cell lines and xenografts, identifying multiple aberrations on chromosome 3, including duplications, translocations and deletions¹⁹⁻²¹. Subsequent studies in primary NPC samples led to the identification of a number of frequently altered genomic regions now known to harbour oncogenes and tumour suppressors important in NPC development and progression.

Important examples of recurrent chromosomal events in NPC include focal losses on the p arm of chromosomes 3 and 9, and the q arm of chromosomes 11, 13, 14, and 16. These have been identified and confirmed in multiple studies²²⁻²⁸, corresponding to the genetic loci of multiple tumour suppressors critical in NPC. One important region is the 9p21.3 cytoband, which was originally observed to exhibit copy number losses in a majority (61%), and a homozygous deletion in one of the primary NPC samples tested²⁸. Contained within this region are two genes which encode three tumour suppressor proteins: p16 (INK4a), ARF (gene: CDKN2A) and p15 (INK4b) (gene: CDKN2B). Hypermethylation of the p16 promoter was also frequently detected in NPC samples, indicating that this was the target of copy number loss in this region^{29,30}. In subsequent studies, the functional implication of p16 loss in NPC was examined. Wang *et al.*³¹ demonstrated that re-expression of p16 in NPC cell lines led to G0/G1 arrest along with reduced *in vivo* tumourigenicity, most likely mediated *via* its canonical role as an inhibitor of the interaction between cdk4/cdk6 and cyclin D1 (CCND1). Moreover, it was also observed that deletion of p16 was a critical event in hTERT (telomerase) immortalization

of normal nasopharyngeal epithelial cells³², providing support for p16 inactivation as an early event in the development of NPC³¹.

Similar to p16, Ras Association Domain Family Member 1A (RASSF1A) was identified as an important tumour suppressor in NPC due to the observation of frequent deletion of its genomic locus at 3p21.3, and methylation of its promoter^{22,33,34}. In addition to copy number and methylation aberrations, somatic mutations in RASSF1A have also been detected in a small proportion of NPC samples (9.5%)³⁴. The *RASSF1* gene was first cloned in 2000 by Dammann *et al.*³⁵, where they reported the expression of three transcripts (RASSF1A/B/C) through alternative splicing and promoter usage. Since its discovery, multiple putative functions have been suggested for RASSF1A, although its precise role remains controversial³⁶. Only a few studies have explored the role of RASSF1A in NPC specifically. Chow *et al.*³⁷ examined the tumour suppressive function of RASSF1A by stably expressing the gene in the EBV-positive NPC cell line, C666-1, which has lost expression of *RASSF1A* through promoter hypermethylation³⁴. This study demonstrated that re-expression of *RASSF1A* resulted in growth inhibition both *in vitro* and *in vivo*³⁷. This group went on to show that RASSF1A regulated the expression of genes involved in the tumour growth factor β (TGF- β) signaling pathway (activin β E, Id2)³⁸. RASSF1A is also known to play an important role in regulating mitotic progression through direct inhibition of the APC (anaphase promoting complex)-CDC20 (cell-division cycle protein 20) complex, which functions to promote microtubule stability³⁹⁻⁴¹. Hence, when RASSF1A expression was suppressed in immortalized nasopharyngeal epithelial cells, microtubule structures were disrupted, and chromosomal instability ensued, thereby enhancing tumourigenicity⁴².

A number of oncogenes are also activated in NPC *via* genetic and epigenetic mechanisms. Frequent amplification of 11q13 was found in multiple cytogenetic studies, with CCND1 identified as the likely target of these copy number gains^{22,43}. Moreover, CCND1 was observed to be over-expressed in >90% of primary NPC samples by immunohistochemical (IHC) staining, and its knockdown in NPC cell lines resulted in a significant decrease in cellular proliferation⁴³. Similarly, recurrent amplification of 12p13 was found to target lymphotoxin-beta receptor (LTBR), overexpression of which was confirmed to contribute to cell survival and tumour growth in NPC models *via* activation of the NF- κ B pathway⁴⁴. Likewise, an increase in copy number of PIK3CA at 3q26.1 has been observed in 70-75% of primary NPC samples^{26,45}, and IHC evidence indicated that the PI3K-Akt pathway was activated in ~85% of NPC samples⁴⁶. Hence, given that PIK3CA point mutations are only rarely detected in NPC, amplification is likely the major mechanism of constitutive activation of this pathway⁴⁵.

While no comprehensive sequencing studies have yet been reported in NPC, a small number of point mutations have been identified through targeted sequencing of specific tumour suppressor and oncogenes. TP53, the most widely mutated gene in human cancers, is rarely mutated in NPC (0-8%)⁴⁷⁻⁴⁹. Although the major mechanisms of RASSF1A deactivation are copy number loss and promoter hypermethylation, mutations have been detected in ~10% of NPC samples³⁴. Finally, infrequent mutations in PIK3CA, and three NF- κ B pathway regulators (TRAF2, TRAF3, and A20) have been reported; all in less than 10% of samples examined^{50,51}. The small number of identified point mutations in NPC is likely due in part to a lack of high-throughput sequencing studies in this disease. It is also probable that because EBV infection functions to activate and suppress many of the necessary signaling pathways in NPC cells, somatic mutations are of less importance than in other, non-virally driven cancers.

1.1.3.2 The role of EBV in NP tumourigenesis

EBV is an enveloped, double stranded DNA, human herpesvirus that is associated with a number of human malignancies, including Burkitt's lymphoma, Hodgkin's lymphoma, gastric carcinoma and NPC. NPC cells exhibit a type II EBV latency profile, resulting in the expression of several protein-coding (Epstein-Barr nuclear antigen 1 (EBNA1), Bam H1 Reading Frame 1 (BARF1), latent membrane proteins (LMPs) 1, 2A, and 2B)), and non-protein coding (Epstein-Barr encoded small RNAs (EBERs) 1 and 2, Bam H1-A region rightward transcript (BART) miRNAs) EBV genes with a variety of oncogenic properties^{46,52-54}.

1.1.3.2.1 LMP1

Perhaps the most well characterized EBV encoded protein in the context of NPC is LMP1. LMP1 is thought to act, at least in part, as a viral mimic of the TNF- α receptor family members TNFR1 and CD40⁵⁵. Although LMP1 shares little sequence homolog to these receptors, it is capable of modulating a number of TNF- responsive signaling proteins including TNFR-associated factors (TRAFs) and the TNFR-associated death domain protein (TRADD)^{55,56}. Unlike TNFRs however, LMP1 does not depend on ligand activation, but is constitutively activated and consequently promotes tumour development and progression *via* a number of signaling pathways^{57,58}.

Early studies showed that expression of LMP1 transformed immortalized rodent cells, and inhibited differentiation in human epithelial cells^{59,60}. Subsequent studies provided evidence that LMP1 is capable of promoting all of the “hallmarks of cancer”⁶¹. Specifically, LMP1 has been shown to increase NPC cell proliferation and survival by inducing the expression of mitogenic receptors such as EGFR^{62,63} and c-Met⁶⁴, as well as activating the pro-growth and anti-apoptotic NF- κ B⁶⁵, PI3K/Akt⁶⁶, and MAPK kinase pathways⁶⁷. LMP1 has also

been found to increase the metastatic potential of NPC cells through induction of epithelial to mesenchymal transition (EMT)^{68,69}, as well as increased expression and the release of matrix metalloproteinases (MMPs)^{70,71} through activation of NF- κ B, SP-1 (specificity protein 1), AP-1 (activator protein 1), and ERK-MAPK pathways^{70,72-74}. Evidence also indicates that LMP1 promotes angiogenesis in the NPC microenvironment through the induction of Hypoxia-Inducible Factor 1 α (HIF-1 α), and Vascular Endothelial Growth Factor (VEGF)⁷⁵⁻⁷⁸. Notably, high LMP1 expression in primary NPC samples has also been shown to be associated with regional and distant metastasis⁷⁹. Another mechanism of action of LMP1 is modulation of epigenetic marks on the host genome⁵⁸. Specifically, LMP1 has been demonstrated to induce the expression and activity of DNA methyltransferases (DMNTs) 1, 3a, and 3b, resulting in promoter hypermethylation of the tumour suppressor gene E-cadherin, and possibly others^{68,80}.

Despite its many functions in NPC development and progression, LMP1 expression is heterogeneous within and amongst NP tumours⁸¹. Indeed, LMP1 expression is undetectable by current techniques in as much as 50% of NPC specimens examined⁷⁹. As such, additional EBV encoded genes also function in concert with LMP1 to promote NP oncogenesis through both overlapping and novel mechanisms.

1.1.3.2.2 LMP2A/B

In contrast with LMP1, LMP2A is consistently expressed in up to 100% of NPC tumours while LMP2B expression mirrors LMP1 more closely with expression observed in ~40% of NPC biopsies⁸¹. While LMP2A and B do not appear to transform normal cells alone, they do function to regulate several cellular pathways resulting in a variety of oncogenic changes in NPC cells⁸².

LMP2A transfected epithelial cells exhibit increased proliferation, EMT, invasion and migration through the activation of a variety of signaling pathways including Notch, ERK, Syk, PI3K/Akt, and Wnt/beta-catenin⁸³⁻⁹⁰. LMP2A has also been demonstrated to negatively regulate the B-cell receptor (BCR) in epithelial cells, resulting in inhibition of a number of BCR regulated processes including intracellular calcium release, phospho-tyrosine signaling, and the switch from latency to lytic replication⁹¹. Although slightly less well understood, LMP2B has also been found to activate PI3K/Akt signaling, and can promote cell motility through disruption of cellular adhesion⁸⁶. Finally, both LMP2A and B have been shown to impart resistance to anti-viral IFN signaling in NPC cells through the increased turnover and degradation of INF responsive receptors, IFNAR and IFNGR⁹².

1.1.3.2.3 EBNA1

EBNA1 is the only EBV encoded protein expressed in all EBV-associated tumours, and its expression is both necessary and sufficient for the stable maintenance of EBV episomes in dividing cells through its interactions with EBV oriP^{93,94}. EBNA1 is also capable of inducing the expression of other EBV encoded genes through transcriptional activation of the Cp and LMP promoters^{95,96}. In addition, evidence suggests that EBNA1 directly promotes oncogenesis in NPC cells, aside from its role in the maintenance of the EBV genome and modulation of other EBV genes⁵⁴. Indeed, knockdown of EBNA1 *via* RNA interference resulted in reduced cell proliferation and survival in an NPC derived cell line⁹⁷, and its ectopic expression promoted tumour growth and metastasis in NPC xenografts⁹⁸.

It has been demonstrated that a variety of EBNA1-host interactions are at play in NPC cells resulting in modulation of numerous cellular pathways and alteration of cellular homeostasis⁵⁴. Interestingly, EBNA1 has been shown to induce reactive oxygen species (ROS)

in NPC cells, possibly through induction of expression of the ROS producing NADPH oxidase 2 (NOX2)^{99,100}. This increase in oxidative stress was also demonstrated to lead to increased DNA damage in B-cells⁹⁹, which if extended to NPC cells could presumably result in additional oncogenic mutations. EBNA1 has also been found to directly modulate the activity of a number of signaling pathways including TGF- β , STAT1, and NF- κ B, leading to a variety of phenotypic outcomes including increased growth, survival, metastasis and angiogenesis¹⁰⁰⁻¹⁰⁴.

EBNA1 is capable of reducing p53 levels by interacting with the ubiquitin-specific protease, USP7, to prevent the deubiquitination and consequent stabilization of p53^{105,106}. This relationship was subsequently shown to result in decreased p53 accumulation in response to DNA damage, with a resultant reduction in apoptosis and increased radioresistance *in vitro*¹⁰⁶⁻¹⁰⁸. Relatedly, EBNA1 has been shown in NPC and gastric carcinoma cell lines to disrupt Promyelocytic leukemia (PML) nuclear bodies^{107,109}, which are also involved in p53 activation, apoptosis and DNA repair¹¹⁰⁻¹¹². Moreover, PML loss has also been demonstrated *in vivo*, wherein EBV-positive gastric carcinoma biopsies were found to have significantly decreased PML as compared to EBV-negative gastric carcinomas¹⁰⁹.

Finally, a series of Chromatin immune-precipitation (ChIP) experiments have also identified a number of putative EBNA1-binding sites on cellular DNA¹¹³⁻¹¹⁵. However, whether EBNA1 functions to directly bind and promote transcription at these sites remains to be shown^{113,115}.

1.1.3.2.4 EBERs

EBER 1 and 2 are small (167nt, and 172nt long, respectively), highly expressed in NPC cells, with up to 1 million copies detected per cell¹¹⁶⁻¹¹⁸. Evidence suggests that EBERs can function within NPC cells to promote growth, survival and modulation of host-immunity

through a variety of mechanisms¹¹⁶. Studies in the EBV-positive NPC cell line, C666-1, revealed that EBERs can promote cell growth through an autocrine mechanism by inducing the expression and release of insulin like growth factor-1 (IGF-1); possibly through activation of the IGF-1 promoter¹¹⁹. Moreover, IGF-1 expression was frequently detected in NPC biopsies, indicating that the EBER/IGF-1 axis may contribute to the development of NPC *in vivo* as well¹¹⁹. In addition, preliminary data suggests that this induction of IGF-1 occurs through the interaction of the EBERs with the double-stranded RNA (dsRNA) sensing Toll-like receptor 3 (TLR3)¹²⁰⁻¹²². EBV also encodes a number of miRNAs (25 precursors, 44 mature) whose expression and function will be explored in **section 1.2.6**.

1.1.4 Prognostic/diagnostic biomarkers in NPC

While there is no ubiquitously utilized clinical biomarker for NPC, a number of diagnostic and prognostic molecular markers have been reported, and validated to varying degrees. The two most well established biomarkers in NPC are circulating antibodies against EBV (serology), and circulating EBV DNA. Indeed, both have been found to be significantly elevated in NPC patients *vs.* healthy controls in numerous studies¹²³⁻¹²⁸. While a role as an early diagnostic screening technique has been suggested for EBV serology¹²⁹⁻¹³², the utility of circulating EBV DNA for early detection remains unclear¹³³. Conversely, while EBV serology appears to lack a relationship with post-treatment tumour burden^{134,135}, post-treatment EBV DNA load effectively identifies recurrent or residual disease^{136,137}. This feature has been implemented clinically in a recent feasibility study where serial circulating EBV DNA measurement showed promising results as a screen for early detection of distant failure¹³⁸. A number of groups have also shown a role for pre-treatment concentration and post-treatment

clearance rate of circulating EBV DNA as prognostic biomarkers capable of predicting relapse and survival¹³⁹⁻¹⁴¹. While pre-treatment concentration of EBV DNA is likely a reflection of initial tumour burden, post-treatment clearance might well provide additional information regarding residual disease and occult metastases^{136,139,140}. Notably, Le *et al.*¹⁴² and the WHO (NIBSC code: 09/260) have recently proposed strategies to standardize the analytical methodologies used to measure plasma EBV DNA from NPC patients. Standard operating procedures such as these will enable the robust implementation plasma EBV DNA within future randomized trials; which often accrue patients from multiple institutions and even continents¹⁴².

In addition to EBV DNA and anti-EBV antibodies, several EBV proteins themselves, as well as EBV encoded RNAs have been identified in circulation for NPC patients. Preliminary results indicated that EBV proteins LMP1 and BARF1 could be detected in the plasma of NPC patients; not in healthy controls¹⁴³. In addition, two classes of non-coding RNA molecules have been detected by several groups in the plasma and/or serum of NPC patients: 1) EBV-encoded small RNAs (EBER1/EBER2)¹⁴⁴, and 2) several EBV encoded miRNAs from the BART genomic region¹⁴⁵⁻¹⁴⁷. However, the clinical implications of measuring EBV RNA molecules in circulation of NPC patients remain to be determined¹⁴⁸.

A number of human nucleic acid, protein and metabolite biomarkers have also been proposed for diagnostic and prognostic use in NPC patients as well. These include DNA fragments containing hypermethylated promoter regions for several known tumour suppressor genes (e.g. *CDHI*, *DAPK*, and *CDKN2A*)^{149,150}, hemoglobin and lactate dehydrogenase¹⁵¹, as well as multiple circulating cytokines including TGF- β ¹⁵², interferon- γ ¹⁵³, and CD40-ligand¹⁵⁴. Moreover, the cytokines MIP-3 α ¹⁵⁵, and CD23¹⁵⁶ have also been shown to have prognostic potential.

Finally, a number of molecular entities within the tumour and surrounding stroma themselves have been explored as potential indicators of clinical outcome. Some examples of cellular proteins observed to have putative prognostic ability include caspase-3, nuclear survivin¹⁵⁷ as well as c-met¹⁵⁸. However, much like their circulating counterparts, none of these cellular biomarkers have been widely implemented in the clinic.

1.2 MicroRNAs

MicroRNAs (miRNAs) are endogenous small (18-25nt) non-protein coding RNA molecules^{159,7}. Originally discovered in *C. Elegans* but have since then been identified in over 200 different species (miRBase, Release 20). MiRNAs primarily function to decrease the expression of protein coding genes by binding their transcripts in a sequence specific manner, however miRNA-target interactions resulting in *increased* protein expression have also been noted^{160,161}. Since their discovery, miRNAs have risen to the status of arguably one of the most important genetic regulators in the mammalian system¹⁵⁹. Moreover, numerous miRNAs have been reported to function as both oncogenes and tumour suppressors, regulating tumour initiation and progression at all levels¹⁶².

1.2.1 MiRNA Biogenesis and Function

MiRNA biogenesis is a multistep process, which is tightly regulated within the cell. Figure 1-3 depicts the steps in miRNA processing from transcription in the nucleus, to their interaction with mRNAs in the cytoplasm. MiRNA genes can be located in intergenic regions, or within the exons or introns of other genes. Transcription of these miR-genes is performed predominantly by RNA polymerase II (Pol II), and Pol III in some cases^{163,164}. A variety of Pol

II-associated transcription factors have been found to direct miRNA transcription, providing regulation of miRNA expression at the level of transcription¹⁶⁵. The RNA product that is transcribed is called a primary-miRNA (or pri-miRNA). These pri-miRNAs vary in length from ~100 to 1000s of base pairs, and exist in diverse stem-loop structures¹⁶³. Following transcription, the pri-miRNA is cleaved within the nucleus by the type III RNase Drosha, in association with DGCR8 (DiGeorge syndrome critical region gene 8) at the stem of the loop structure to release a ~70 nt precursor miRNA (pre-miRNA)¹⁶³. This pre-miRNA is then exported out of the nucleus by the dsRNA specific transporting complex composed of Exportin-5 and RAN-GTP¹⁶⁶.

In the cytoplasm, the pre-miRNA is further processed by Dicer and its cofactor Trans-activation-Responsive RNA-binding protein (TRBP) to release the ~22 nt mature miRNA duplex¹⁶³. One strand of this duplex, termed the 'guide strand', is then preferentially incorporated into the miRNA inducible silencing complex (miRISC)¹⁶³. Although this process is not completely understood, the guide strand is almost always the strand with the thermodynamically least stable 5' terminus¹⁶⁷. The miRISC is a multipart entity with several potential members. The key functional element of the miRISC is the Argonaute protein (in mammals, one of AGO1-4)¹⁶³. In addition to Argonaute, several other proteins may be involved in the miRISC such as the P-body marker, FMRP (Fragile X mental retardation 1 protein)¹⁶⁸, and the de-capping activator RCK/p54^{169,170}.

Once a mature miRNA is incorporated, the miRISC can then bind to a target mRNA at a sequence specific binding site. A particular miRNA could bind to hundreds of different genes; sometimes even onto multiple sites for a given target mRNA. MiRNA binding could occur with either perfect complementarity; or more often, with imperfect complementarity¹⁶². In

these instances of imperfect complementarity, there is often a short, ~6-8 nt “seed” region, located near the 5’ end of the miRNA, that appears to be of paramount importance in terms of dictating binding to specific target mRNAs¹⁵⁹. These seed regions are often conserved among species, and form the basis for many of the current *in silico* target prediction algorithms^{163,171-173}.

MiRNAs can regulate expression of their targets either through mRNA degradation or translational inhibition. In instances of perfect or near-perfect miRNA-mRNA complementarity, degradation of target mRNAs can be mediated by AGO2; the only Argonaute protein which possesses “slicer” activity¹⁶³. In cases of imperfect complementarity, all 4 Argonaute proteins are capable of inhibiting protein translation as part of the miRISC¹⁶³. In addition, miRNA-target relationships with imperfect binding can also result in mRNA degradation through a non-sequence specific mechanism within cytoplasmic processing (P) bodies¹⁷⁴. In most cases, the net effect of miRISC binding to a target mRNA is a decrease in its protein levels; however recent reports have demonstrated a few instances wherein protein expression is actually increased¹⁶³. For example, miR-10A can increase the expression of several ribosomal proteins through binding to the 5’ UTR (untranslated region) of their mRNA transcripts¹⁶⁰.

Figure 1-3. miRNA Biogenesis.

See text of **section 1.2.1** for full description. RNA Pol II; RNA Polymerase type II, DGCR8; Drosha–DiGeorge syndrome critical region gene 8, XPO5; nuclear export factor exportin 5, RAN; Ras-related nuclear protein, GTP; guanosine tri-phosphate, AGO; Argonaute, TRBP; TAR (HIV-1) RNA binding protein 2, PACT; protein kinase, interferon-inducible double stranded RNA. Dicer; Dicer 1, Ribonuclease Type III, miR*; passenger strand from mature miRNA duplex. miRISC; microRNA RNA-induced silencing complex, P-body; processing body.

1.2.2 MicroRNAs in Cancer

As the list of miRNAs has grown, so has our knowledge regarding their biological functions. Indeed, miRNAs have been found to play a role in most, if not all, cellular processes including many pathways related to cancer development and progression. MiRNAs are known to function as tumour suppressor genes or oncogenes, with some miRNAs mediating contradictory roles in different diseases¹⁶². Aberrant miRNA expression and function has been described in a wide variety of human malignancies, with chromosomal amplifications/deletions, point mutations, or epigenetic alterations as potential causes¹⁶².

1.2.3 MicroRNA Expression Signatures in Cancer

Comprehensive miRNA expression profiling has been performed in a variety of human cancers, yielding a number of interesting observations. Expression signatures have been described which are capable of distinguishing cancerous from normal cells¹⁶², one cancer from another¹⁷⁵, predicting response to a particular drug¹⁷⁶, or predicting patient outcome^{177,178}. Indeed, miRNA signatures capable of predicting patient outcome have been developed for a number of human cancers including, but not limited to lung^{179,180}, breast¹⁸¹, brain¹⁸², and chronic lymphocytic leukaemia¹⁸³. While only our 4-miRNA prognostic signature, described in detail in Chapter 3, and one other 5-miRNA signature¹⁸⁴ exist for NPC, several studies have already reported alterations in expression, and potential biological functions of miRNAs in this disease.

1.2.4 Human MicroRNA Expression in Nasopharyngeal Carcinoma

The first study on the global profiling of miRNAs in NPC was published in 2008 by Paul Ahlquist's group at the National Cancer Institute (NCI)¹⁸⁵. Using a micro-array based approach to profile 31 laser-capture microdissected (LCM) NPCs and 10 normal nasopharyngeal epithelial samples, they discovered several miRNAs to be deregulated in NPC¹⁸⁵. In particular, miR-29c was observed to be significantly down-regulated in NPC, and several miR-29c targets involved in extracellular matrix synthesis and function were identified and validated¹⁸⁵. Subsequently, other groups have identified a number of dysregulated miRNAs in both NP tumour and blood samples from NPC patients; summarized in Table 1-1.

Table 1-1. Summary of miRNA expression in studies using primary NPC samples.

NPC Patients vs. Healthy Controls		Prognostic Association	
Up-regulated	Down-regulated	Positive	Negative
*miR-16 ¹⁸⁶	let-7g ¹⁸⁷	miR-18a ¹⁸⁸	*miR-9 ¹⁸⁹
[‡] miR-17 ¹⁹⁰	*miR-9 ¹⁸⁹	[‡] miR-22 ¹⁹¹	miR-26a ¹⁸⁴
miR-18a ¹⁸⁸	miR-26a ^{168,184,187,192}	miR-93 ¹⁸⁴	miR-29c ^{184,193}
[‡] miR-20a ¹⁹⁰	miR-26b ¹⁹²	[‡] miR-572 ¹⁹¹	miR-30e ¹⁸⁴
*miR-21 ¹⁸⁶	miR-29c ^{184,185}	[‡] miR-638 ¹⁹¹	miR-451 ¹⁹⁴
*miR-24 ¹⁸⁶	[‡] miR-29c ¹⁹⁰		miR-98 ¹⁹²
miR-93 ¹⁸⁴	miR-30e ¹⁸⁴		miR-142-3p ¹⁸⁴
miR-141 ¹⁹⁵	miR-34b ¹⁸⁷		[‡] miR-1234 ¹⁹¹
miR-144 ¹⁹⁶	miR-101 ¹⁹²		
miR-146a ^{197,198}	miR-138 ¹⁹⁹		
miR-155 ²⁰⁰	miR-142-3p ¹⁸⁴		
*miR-155 ^{186,201}	miR-200b ²⁰²		
*miR-214-3p ^{189,203}	miR-216b ²⁰⁴		
miR-214-3p ⁸⁶	miR-218 ²⁰⁵		
miR-663 ²⁰⁶	[‡] miR-223 ¹⁹⁰		
*miR-3135a ¹⁸⁹	*miR-378 ¹⁸⁶		
	miR-451 ¹⁹⁴		

MiRNAs included in this table satisfy each of the following criteria: 1) a statistically significant alteration in their expression was identified in NPC patient specimens 2) Some degree of validation (either in additional samples, using an additional analytical method, or functional validation) was reported. **NPC Patients vs. Healthy Control:** expression significantly increased (“up-regulated”) or decreased (“down-regulated”) in tumours/plasma compared to nasopharyngeal tissues/plasma from healthy controls. **Prognostic Association:** expression higher (“positive”) or lower (“negative”) in tumour/plasma samples of patients with poor prognosis. **Superscripts:** expression measured in *plasma, [‡]serum, all others were discovered using tumour samples.

1.2.5 Human miRNA Biology in NPC

In addition to alterations in expression of miRNAs in NPC, we and others have also demonstrated phenotypic roles for miRNAs in NP tumourigenesis using *in vitro* and *in vivo* models. The first miRNA characterized in the context of NPC was miR-29c, which was the main focus of the first miRNA profiling report by Sengupta *et al.*¹⁸⁵. In this initial study, the authors demonstrated a potential tumour suppressive role for miR-29c through targeting mRNAs encoding extracellular matrix proteins (collagens 3A1, 4A1, 15A1, laminin γ 1)¹⁸⁵. They postulated that suppression of miR-29c will subsequently increase the migration and invasion ability of NPC cells through up-regulation of these components of the extracellular matrix; however, they stopped short of testing this hypothesis in the initial study, and further reports supporting these claims have not been published to date. Subsequent studies by two other groups corroborated the role of miR-29c as a tumour suppressor in NPC; however these reports differed on their causal mechanisms, and putative targets. These included promotion of NPC cell migration and invasion through increased expression of T-cell lymphoma invasion and metastasis 1 (TMP1)²⁰⁷, and increased resistance to radiotherapy and Cisplatin through up-regulation of anti-apoptotic regulators Mcl-1 and Bcl-2¹⁹³. This suggests, much like other miRNAs, that miR-29c can act through a number of pathways to suppress proliferation, survival and motility of NPC cells.

Another moderately well studied miRNA in the context of NPC is miR-9. Not only has miR-9 been identified as a potential circulating marker of advanced NPC¹⁸⁹, several potential targets and functions have also been reported. Recently, two reports on various putative tumour suppressive mechanisms for miR-9 in NPC have been published. Lu *et al.* described that miR-9 hyper-methylation and subsequent under-expression led to up-regulation of its putative target

CXCR4, resulting in increased cell growth, migration and invasion through activation of the MAPK pathway²⁰⁸. In contrast, Gao *et al.*²⁰⁹ postulated a role for miR-9 in modulating the immune response to NPC by targeting several interferon (IFN)-induced genes, multiple members of the major histocompatibility complex (MHC) class I molecule, as well as a number of interleukins and related genes.

Other potentially functionally active miRNAs in NPC include, but are not limited to, miR-26a^{210,211}, miR-98¹⁹², miR-155^{200,212}, miR-200a/b^{202,213,214}, miR-205^{215,216}, and miR-216b²⁰⁴. Proposed targets of these miRNAs represent a wide array of processes and pathways including EMT²¹³, Notch²⁰², PI3K-Akt²¹⁵, and ERK/MAPK²⁰⁴ signaling pathways. Intriguingly, function, pathways, and targets affected by many of the miRNAs involved in NP tumourigenesis overlap. One such example is miR-26a and miR-218, which have both been found to target Enhancer of Zeste homolog 2 (EZH2), leading to a reduction in multiple tumourigenic properties such as migration, invasion and cell survival^{192,211}. This overlapping functional impact of multiple miRNAs adds an increased layer of complexity when attempting to elucidate their biological role; however, this property could also increase our confidence in the biological importance of affected genes and pathways, given that there are multiple mechanisms that malignant cells exploit to activate or inhibit the same gene or pathway.

1.2.6 EBV encoded miRNAs in Nasopharyngeal Carcinoma

The EBV, which is present in the vast majority of NPCs, also encodes a number of its own miRNAs. Twenty-five EBV encoded precursor-miRNAs (pre-miRNAs) processed into 44 mature miRNA sequences have been verified to date (miRBase, Release 20). They emanate from two major regions of the EBV genome: a) the BamHI-A rightward transcripts (BART);

and b) the open reading frame of the *BHRF1* gene. Originally cloned from EBV-infected Burkitt's lymphoma cells²¹⁷, BHRF1-miRNAs do not appear to be expressed in EBV-positive NPC primary tissues^{218,219}. Indeed, the first report of comprehensive EBV-miRNA profiling in primary NPC samples reported substantial expression of all 35 of the EBV-BART-miRNAs interrogated in the majority of samples, whereas none of the 4 EBV-BHRF1-miRNAs were detected. In contrast, NPC derived cell lines, C666-1 and HONE-Akata, additionally expressed all 4 BHRF1-derived miRNAs²¹⁸. Subsequent studies have identified several targets of EBV miRNAs; both host and viral. Viral targets include LMP1; putatively targeted by several miRNAs from the BART region (ebv-miR-BART1, 9, 16, 17)^{218,220,221}, and LMP2A; identified as a target of ebv-miR-BART22²²². Subsequent functional experiments suggest that BART-miRNA modulation of these virally encoded proteins can influence multiple cellular properties including cell proliferations, survival, and evasion of host immune response²²⁰⁻²²². Host targets of various BART-miRNAs include pro-apoptotic effectors p53 up-regulated modulator of apoptosis (PUMA)²²³, Bcl-2 interacting mediator of cell death (Bim)²²⁴, and Translocase Of Outer Mitochondrial Membrane 22 Homolog (TOMM22)²²⁵, as well as several genes thought to influence host immune response, including MHC class I-related chain B (MICB)²²⁶, importin 7 (IPO7)^{225,227}, and Dicer²²⁸. Overall, EBV encoded miRNAs play a complementary role to the viral proteins expressed in NPC; contributing to evasion of the host immune response, and promoting survival and proliferation of NPC cells.

1.2.7 MicroRNA Expression Profiling Platforms

The small size and extensive sequence similarity of miRNAs present as unique challenges when attempting to quantitate miRNAs in a biological solution. The “first-

generation” tools for miRNA expression analysis included northern blotting, quantitative reverse transcription real-time PCR (qRT-PCR), and microarray-based methods. While qRT-PCR and microarray-based techniques are still the most widely used methods for miRNA expression analysis, a second generation of counting-based strategies has also emerged. These include the fluorescent-barcode counting method employed by the nCounter platform from Nanostring, and small-RNA sequencing methods, using any of the major next-generation sequencing platforms. Given its limitation in sensitivity and multiplexing compared to other techniques, the use of northern blot analysis for measurement of miRNA expression has waned. Thus, the following review will focus on the other four methodological categories.

1.2.7.1 Quantitative RT-PCR

Two major differences set miRNAs apart from mRNAs pertaining to quantification by qRT-PCR. First, their lack of poly(A) tails means conventional poly-T-primer reverse transcription (RT) utilized for mRNA, can only be applied to miRNA RT with prior addition of poly(A) adapters²²⁹. Second, conventional PCR primers are generally ~18-22 nt in length to optimize melting and annealing kinetics; thus, given the fact that mature miRNAs are ~22 nt in size themselves, conventional PCR primers are clearly unsuitable for analysis of miRNAs. Two major qRT-PCR chemistries exist, which address both of these issues uniquely.

The Taqman® MicroRNA Assays from Life TechnologiesTM (Foster City, CA) first reported by Chen *et al.* in 2005²³⁰, employ “stem-loop” primers in the RT step to provide sufficient stability of the annealed RNA-primer complex, with complementarity to only 6-9 bases at the 3’ end of the miRNA. A forward primer specific to the miRNA of interest, and a generic reverse primer are then employed in the “Real-Time” step, along with a miRNA-unique TaqMan probe, which provides a further level of specificity. These assays have also been

incorporated into Life Technologies Taqman Low-Density Arrays (TLDA) to allow multiplexing up to 384 primer pairs on a single qPCR plate.

The other major strategy used to improve the binding affinity of primers with short sequences of base-complementarity is the chemical modification of the oligonucleotide backbone. Various such synthetic molecular classes exist; the major categories being peptide- and locked-nucleic acids (PNA and LNAs)²³¹. The latter class is the intellectual property of ExiqonTM, a company that specializes in techniques for the analysis and manipulation of miRNAs. The C3'-endo sugar conformation of these so-called "locked-nucleic acids" acts to reduce the flexibility of the ribose ring, thereby increasing the affinity of LNA containing oligonucleotides to complementary RNA/DNA sequences²³². Exiqon's miRCURY LNATM Universal microRNA PCR System starts with the ligation of a poly-A tail to mature miRNAs to facilitate universal RT using poly-T primers. This is followed by a qPCR step which employs miRNA specific LNA primers to amplify the sequence of interest, and quantifies the PCR product in real-time using the classic double-stranded DNA dye, SYBR® Green (Life Technologies). Exiqon also offers a line of miRNA qPCR panels which enable the user to multiplex >750 miRNA primer sets in a single plate.

1.2.7.2 MiRNA Microarrays

Similar to PCR primers, microarrays rely on base-pairing between complimentary nucleic acid (NuA) sequences, thus raising issues similar to those encountered in PCR pertaining to the small size of miRNAs. As such, similar backbone modification methods to those detailed above, have been exploited to address these concerns for miRNA microarrays as well.

Microarrays employ oligo-nucleotide probes, which are immobilized on a solid, usually glass surface. Hybridization of labeled sample NuA sequences to complementary probes can then be detected and quantified using a microarray scanner. Exiqon once again offers a line of miRNA microarrays, which capitalize on the binding properties of their LNA chemistry. In addition, the majority of biotechnology companies offering microarrays (e.g. Affymetrix, Agilent, Illumina) also offer miRNA options utilizing their respective methodologies originally developed for mRNA expression profiling. These include variations in fabrication (e.g. printing), labeling (e.g. fluorophore), and experimental design (e.g.. one-colour vs. two-colour) (see review by Heller²³³).

1.2.7.3 Digital molecular-barcoding expression analysis

In 2008, Nanostring Technologies launched their first “second-generation” gene expression-profiling platform, nCounter. This platform was subsequently extended in 2010 to include a miRNA expression assay. The nCounter system employs fluorophore-barcodes to label and subsequently “count” specific NuA sequences. The miRNA assay works in much the same way as its mRNA counterpart, with the addition of a ligation step, increasing the length of the miRNAs to improve subsequent hybridization. Much like miRNA microarrays, the nCounter system is inherently multiplexable, with up to ~800 unique target sequences interrogated on a single chip. Unlike most other multiplexed platforms however, the nCounter human miRNA assay v1 also included 80 viral miRNAs. Subsequent versions have since eliminated the viral miRNAs in favour of additional human miRNAs.

1.2.7.4 miRNA-Sequencing

In line with other miRNA profiling technologies, miRNA-Sequencing (miRNA-Seq) borrows much from its mRNA counterpart, RNA-Seq. RNA- and miRNA-Seq both exploit massively parallel high-throughput (HT) DNA sequencing technologies to gather both sequence and abundance information about the transcriptome of samples analyzed. MiRNA-Seq targets miRNA molecules through specific chemical properties (unique 3' and 5' end modifications), as well as PAGE (polyacrylamide gel-electrophoresis) size fractionation and purification²³⁴. Following reverse transcription and PCR amplification, size-selected cDNA molecules are run through the HT DNA sequencing platform of choice (e.g. Illumina or ABI SOLiD sequencers). Unless discovery of novel miRNAs is the objective, reads from the sequencing run are mapped to known miRNA sequences, and counting the number of reads which map to a specific miRNA provides the basis for quantification using this method. One major characteristic that sets miRNA-Seq apart from other miRNA measuring techniques is the ability to detect all small RNA molecules in a single reaction, including not only mature miRNAs, but pre-miRNAs, as well as small nucleolar RNAs.

1.2.7.5 Comparison of technologies

While there is no clear-cut “gold-standard” methodology for miRNA profiling, various experimental design characteristics should be considered when selecting the appropriate miRNA expression profiling technology. Factors such as the number and identity of miRNAs interrogated, quantity and quality of RNA required, platform design flexibility, sensitivity, specificity, robustness to contaminants in analyte solution, number of samples to be analyzed, and budget are all variables that must be considered when selecting the appropriate miRNA profiling platform. While several studies have directly compared various miRNA profiling

platforms, none have included all the available techniques in a single study, and there is no consensus regarding the best method for all scenarios. With these caveats in mind, Table 1-2 summarizes the current knowledge comparing the different platforms as a function of various pertinent factors such as the type and required amount of the starting material, cost, and reported sensitivity and specificity.

Table 1-2. Comparison of different miRNA profiling platforms and technologies

	Multiplex qRT-PCR		Microarray									Digital Expression			
	Stem-loop	LNA		Affymetrix	Ambion	Agilent	Exiqon	Illumina	Invitrogen	LC Sciences	Toray	nCounter	Illumina HTS	SOLID HTS	
Correlation with qRT-PCR[†]	-	-		5	2	3	6	4	7	NA	NA		NA	1	NA
FFPE vs. Frozen Correlation²³⁵	NA	NA		0.91	NA	0.83	NA	0.94	NA	NA	NA		0.89	0.89	NA
ID novel miRNAs?	N	N		N	N	N	N	N	N	N	N		N	Y	Y
miRNA throughput²³⁶	+			++									++	+++	
time requirement²³⁶	1 day			1-2 days									1 day	1 wk+	
RNA quantity required²³⁶	10-700ng			100 - 10µg									200ng	250ng–10µg	
Cost per sample per miRNA²³⁷	\$\$	\$\$		\$									\$	\$\$\$	
Sensitivity²³⁶⁻²³⁸	+++			++									++	+++*	
Specificity^{237,238}	++			+									++	+++	

[†] Six studies^{235,239-243} where direct comparisons were performed between miRNA profiling technologies and single-well qRT-PCR were used to rank each platform based on its concordance with this widely accepted methodology. Only platforms tested in at least 2 studies were included in the ranking. Platforms were percentile ranked within each study, then their average percentile rank across studies was used to determine their final rank.

* Higher depth of sequencing results in higher sensitivity with the trade off of increased cost and RNA requirements
HTS; high-throughput sequencing, NA; not available, Y; yes, N; no. +; low, ++; medium, +++; high

1.2.7.6 miRNA expression data analysis

In order to glean interpretable information from any of the miRNA expression platforms discussed above, the raw data produced by these arrays, cards, and sequencing runs must first be correctly normalized to minimize the effects of systematic experimental variation. Indeed, the normalization methodology used can have a profound impact on the conclusions drawn, reproducibility, and cross-platform agreement²⁴⁴. Data processing is an issue that largely needs to be addressed on a case-by-case basis; certainly amongst platforms, and likely amongst various experimental conditions. However, there is no single agreed-upon normalization strategy for any technology; let alone disease or experimental condition.

Similar to the platforms and technologies themselves, miRNA normalization strategies borrow much from their mRNA counterparts. Users of qPCR-based techniques often employ purportedly “invariant” small RNAs, similar to the so-called “house-keeping genes” often used to normalize mRNA qRT-PCR data, as endogenous controls to account for sample-to-sample systematic variation. However, small RNA endogenous controls, most commonly small-nucleolar RNAs (snoRNAs), are subject to biological variation themselves, and as such can distort the data they are meant to be improving²⁴⁵⁻²⁴⁷. Global miRNA expression approaches, such as arrays, chips and sequencing methods allow the user to take advantage of the comprehensive nature of the data obtained, and normalize individual miRNAs against the entire micronome. Numerous mathematical algorithms originally developed for use with mRNA assays such as loess, quantile, variance stabilizing normalization (VSN), global geometric mean, and invariant selection, have all been successfully applied to miRNA platforms^{244,248-251}. For miRNA sequencing data, the somewhat simplistic reads-per-million (RPM) technique is still the most widely used normalization method, although, there are multiple others

available²⁵². It is unlikely that there will be one normalization strategy identified that is optimal in all circumstances, even for a given platform. As such, the experimental variables and objectives of the study must always be taken into account when selecting the appropriate data processing methods.

1.3 Research Objectives

The majority of NPC patients receive curative RT, with concomitant CRT in cases of locally advanced disease, and these current treatment strategies achieve a modest 5-year survival rate of ~75%²⁵³. Moreover, despite recent improvements in local control for NPC patients, distant metastasis remains a major cause of death in this patient population²⁵⁴, underscoring a need to better treat and identify those patients at risk of distant metastases. MiRNAs are thought to regulate the majority of protein-coding human genes^{255,256}, and it is also apparent that miRNAs play important roles in many, if not all human malignancies²⁵⁷. Hence, this thesis proposes to explore NPC biology and clinical behaviour through the lens of miRNAs. The work presented herein can be broken down into three broad Aims:

1. To identify miRNAs that are dysregulated in NPC.
2. To characterize the biological cause and impact of dysregulated miRNAs in NPC.
3. To determine the clinical relevance of dysregulated miRNAs in NPC.

Chapter 2 presents the identification (Aim 1) and functional characterization (Aim 2) of dysregulated miR-375; whereas, **Chapter 3** demonstrates a validated 4-miRNA signature associated with distant metastasis in NPC patients (Aims 1&3) and preliminary pathway

enrichment analysis indicating a potential role in cell-cycle control for members of this signature (Aim 2). In addition, **Chapter 4** examines copy number alterations in DNA from NPC samples through a comprehensive approach similar to those used to identify aberrantly expressed miRNAs. Notably, several miRNAs were located within frequently altered genomic regions, providing a potential mechanism for miRNA dysregulation in NPC (Aim 2).

In summary, this work provides evidence that miRNAs can provide important insights into both the biology and prognosis of NPC patients. Moreover, the discoveries made herein provide valuable information that could aid in the development of novel targeted therapies and patient stratification methods that will improve outcome for future NPC patients.

Chapter 2

Significance of Dysregulated Metadherin and MicroRNA-375 in Head and Neck Cancer

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2. Significance of Dysregulated Metadherin and MicroRNA-375 in Head and Neck Cancer

2.1 Abstract

2.1.1 Purpose

Despite recent improvements in local control of head and neck cancers (HNCs), distant metastasis remains a major cause of death. Hence, further understanding of HNC biology, and in particular, the genes/pathways driving metastasis is essential in order to improve outcome.

2.1.2 Experimental Design

Quantitative RT-PCR was used to measure the expression of miR-375 and metadherin (MTDH) in HNC patient samples. Targets of miR-375 were confirmed using qRT-PCR, western blot analysis and luciferase assays. Phenotypic effects of miR-375 re-expression and MTDH knock-down were assessed using viability (MTS), clonogenic survival, cell migration/invasion, as well as *in vivo* tumour formation assays. The prognostic significance of miR-375 or MTDH in nasopharyngeal carcinoma (NPC) was determined by comparing low vs. high expression groups.

2.1.3 Results

MiR-375 expression was significantly reduced ($p=0.01$), and conversely, MTDH was significantly increased ($p=0.0001$) in NPC samples. Quantitative RT-PCR, western blots and luciferase assays corroborated MTDH as a target of miR-375. Re-expression of miR-375 and siRNA knock-down of MTDH both decreased cell viability and clonogenic survival, cell migration/invasion, as well as *in vivo* tumour formation. NPC patients whose tumours expressed high levels of MTDH experienced significantly lower survival, and in particular, higher distant relapse rates (5-year distant relapse rates: 26% vs. 5%; $p=0.005$).

2.1.4 Conclusions

Dysregulation of miR-375 and MTDH may represent an important oncogenic pathway driving human HNC progression, particularly distant metastases, which is now emerging as a major cause of death for HNC patients. Hence, targeting this pathway could potentially be a novel therapeutic strategy by which HNC patient outcome could be improved.

2.2 Introduction

Head and neck squamous cell carcinomas (HNSCC) constitute the 5th most common malignancy worldwide²⁵⁸. Patients with locally advanced HNSCC have 5-year overall survival (OS) rates hovering around 30-45%²⁵⁹, underscoring a considerable opportunity for improving outcome. Nasopharyngeal carcinoma (NPC) is another malignancy of the head and neck region; however, NPCs are distinct from other head & neck cancers (HNCs) due to their unique etiological, clinical/biological and epidemiological characteristics. Locally advanced NPC patients have a 5-year overall survival rate of ~70%¹³, also demonstrating a need for improvement. Hence, it is imperative to acquire a deeper understanding of HNC biology to guide the development and evaluation of novel therapies, in order to improve patient outcome.

MicroRNAs (miRNA) are a novel class of gene regulators which are involved in many biological systems, and recognized to play important roles in human cancers²⁶⁰. We have recently completed a miRNA profiling study of locally advanced HNSCC, and reported down-regulation of miR-375 as one of the most frequently detected aberrations²⁶¹. Preliminary functional analysis demonstrated a potential tumour suppressive role for miR-375 in HNSCC²⁶¹. In the current study, we further investigated the role of miR-375 down-regulation in HNC, and determined that metadherin (MTDH) is a target of miR-375 in HNC. MTDH, also known as astrocyte elevated gene 1 (AEG-1) was only recently cloned²⁶²; however, it is rapidly emerging as an important oncogene in many human cancers²⁶³. Furthermore, MTDH over-expression has been observed to significantly correlate with poor prognosis and distant metastases in breast²⁶³, lung⁴⁰ and gastric²⁶⁴ cancers, warranting its further examination in HNC.

2.3 Materials & Methods

2.3.1 Patient information & tissues

With approval from the Institutional Research Ethics Board (REB), 20 diagnostic formalin-fixed paraffin-embedded (FFPE) blocks were collected from HNSCC patients with locally advanced (Stage III or IV) disease, who were previous participants in a Phase III randomized study of hyperfractionated radiotherapy conducted between 1988-1995²⁶⁵. Normal epithelial tissues derived from three FFPE blocks of individuals who underwent a tonsillectomy (University Health Network), plus three additional FFPE blocks derived from normal laryngeal squamous epithelial tissues post-laryngectomy (commercially purchased from Asterand, USA) served as controls. In addition, 94 primary FFPE biopsy samples from NPC patients diagnosed from 1993-2000 were evaluated. Eight FFPE blocks of normal nasopharyngeal epithelial tissues derived from patients who underwent a diagnostic quadroscope served as normal controls. Clinical characteristics of these cancer patients are provided in Table 2-1.

Table 2-1. Clinical characteristics of HNC patients studied.

Clinical Descriptors for the 94 NPC Patients		
Age		
median	46	
range	16-79	
Stage	Frequency	Percent
I	8	8%
II	32	34%
III	28	30%
IV	26	28%
Relapse	Frequency	Percent
No relapse	64	68%
Local relapse only	7	7%
Local + Distant relapse	13	14%
Distant relapse only	10	11%
Treatment	Frequency	Percent
Radiation only	69	73%
Radiation + chemo	25	27%
Clinical Descriptors for the 20 HNSCC Patients		
Age		
median	59	
range	35-75	
Stage	Frequency	Percent
III	8	40%
IV	12	60%
Relapse	Frequency	Percent
No relapse	4	20%
Local relapse	6	30%
Regional relapse	4	20%
Local + regional relapse	6	30%
Treatment	Frequency	Percent
Radiation #1 (D)	9	45%
Radiation #2 (T)	11	55%
(T = twice daily) and (D = standard)		

2.3.2 RNA purification from FFPE samples

To ensure that all tissues analyzed contained >70% tumour cells, a representative section from each patient was stained with H & E, then reviewed by a HNC pathologist (B P-O) to ascertain regions with malignant epithelial cells for macro-dissection. All blocks were processed randomly, with clinical outcome unknown, to avoid experimental bias. Total RNA enriched for small RNA species was isolated using the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Ambion, Inc.), according to the manufacturer's instructions.

2.3.3 Cell lines and reagents

The human hypopharyngeal HNSCC cell line, FaDu, was obtained from American Type Culture Collection (Manassas, VA), and cultured according to specifications. The Epstein-Barr virus (EBV) positive NPC cell line C666-1³¹ was maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Wisent, Inc.), and 100 mg/L penicillin/streptomycin. The normal oral epithelial (NOE) cells (Celprogen, Inc.) served as normal controls. All cells were authenticated at the Centre for Applied Genomics (Hospital for Sick Children, Toronto, Canada) using the AmpF/STR Identifier PCR Amplification Kit (Applied Biosystems), and maintained at 37°C with 5% CO₂.

2.3.4 5-aza-2'-deoxycytidine treatment and quantitative CpG methylation analysis

C666-1 and FaDu cells were seeded overnight in 12-well plates and media containing 3µM 5-aza-2'-deoxycytidine (Sigma Aldrich) was added to cells 2 and 4 days after seeding, respectively. On day 5, cells were harvested and RNA was extracted for qRT-PCR analysis of

miR-375 expression levels. The CpG islands in the genomic region of miR-375 were identified using the EMBL-EBI CpG Island Finder and Plotting Tool (Fig 2-1A) and 3 amplicons spanning these regions (Fig 2-1B) were analyzed for CpG methylation using the EpiTYPER (Sequenom, Inc.) at the Analytical Genetics Technology Centre (AGTC), University Health Network, Toronto.

B

Amplicon Sequences:

```
GACCAAGGGAGTGTGTCAGGGAGTGACATCTCAGGATGTCAGCAGCACCACAGAGATCCGAGGAGTGGATT
TGAGGCTCCATGGTGGCCCTCAGCTTCCCCCGCCAGATTTCCACACCTCAGCCTTGGCTCCAACTCCG
TGAGGGCCTGCATCTTCCCCAGCATGACCAGGAGCCCAGGAACCGGGACAGGAGGCTGGGGAGGGCCT
AGCCCCTGCGGGAGGCCAGACCAACCGTTCACTGAGCAAATGGGAAAACCAAGAGGCCGGGAAATGACT
TGCCCAAGGCCATAGAAGTCGCGGGGACAGAGGCGGACTAGAATCCGGGTTTCCACCTCCAGAAGGGTT
TCTGTTCTGGGCCACGGCCCCCTTCGCCACCCGCTGGAGCCGGCTCAGCCCGTATTACGACGCAGAATG
GAGCCTGGCAGCCCCAGAAGCTGCGGGCAGGGCAAGGCAGGGCGGGTGGGGCCGGATGGGAGCCGGAT
CTCCCCTGGAGCGGCGCACAGCCTCTCCACCCGTACGTTGAGATGGCGGTGGCGGTGGCGGGCGGCA
GGTGCCTGCGTGGCGATCAGGCCGGCCCCCGGGCTGGTGCTGAGAGGCCGCCCCCTGCCTCACGCGAG
CCGAACGAACAAAACGCTCAGGTCCGGTTTGTGCGAGGGGCTCGTCGCGGGGCGGGAGCGGAGGGCC
CGGGGCTTGGCGGTGCGCCTCGGTGATCTCCTGGTCTTCCGCGAGCTCAGTGCTGCACAGCG
CCCCGCTAGCCTCCGCCCTCACCTGAACGCATCTGCGGCTGACACGTGCGCGGCCGCGGCGGCCACCG
CAGGTGCGCACCTGGCCCCGCCCTCCTTTCCAGCCACCCTCCGCCCGGCTTTCCTGGCCCCCTCCGCC
CGGCCAGCCTAGCTTGGCTTCGTCCCCAGCCCCACGCCTTGGAGCTTGTCCCGAGCCCGGGCTCCGT
GTGCTCTTATACTGCCTGTTTCAGTCCCCCGCCCCAGACACACTCACACCTGTCCAGGCGCCCCGTTT
GCTCGGGGCAAATATTGACTCATGGAGGCGGAGCGCTCCCCCTCCCCCGCCGGGCTCTGAGACCGG
AGCTTCTCCGAGGCCTTGGTGGCGGGGGCGGAGGGGAGGGTGTGGGGACCCTG
```

Amplicon #1 (blue): Length: 355 bp

Amplicon #2 (pink): Length: 489 bp

Amplicon #3 (green): Length: 442 bp

Note: Sequence marked in yellow represent regions of overlap between amplicons.

Figure 2-1. Genomic location of miR-375 at chromosome 2q35.

The CpG islands in the genomic region of miR-375 were identified using the EMBL-EBI CpG Island Finder and Plotting tool.

2.3.5 Quantification of miRNA and mRNA

MicroRNA expression was assessed by qRT-PCR analysis using TaqMan[®] microRNA Assays (Applied Biosystems) as previously described²⁶⁶. Quantitative RT-PCR was also utilized to analyze expression changes of four previously published miR-375 targets: MTPN, USP1, PDK1, ADIPOR2, plus five newly identified miR-375 targets: MTDH, GMFB, RANBP3, SPAG9 and ZNF462. Total RNA was isolated from cells using the Total RNA purification Kit (Norgen, Inc.). One µg of total RNA was reverse-transcribed using SuperScript II Reverse Transcriptase (Invitrogen Canada) as specified by the manufacturer. Quantitative RT-PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems) and an ABI PRISM 7900 Sequence Detection System (PerkinElmer Biosystems). Primers for PCR amplifications (Table 2-2) were designed using Primer 3 Input (version 0.4.0). Relative mRNA levels were calculated using the $2^{-\Delta\Delta C_t}$ method²⁶⁷.

Table 2-2. Primer sequences for qRT-PCR of potential miR-375 targets.

Gene Symbol	Forward Primer	Reverse Primer
SPAG9	5'-GCTGACCAGATTAGCAGACTTGA -3'	5'-GGATTCTAGTTGATCACTCCCTGA -3'
MTDH	5'-GATGATGAATGGTCTGGGTAAA -3'	5'- GACCTTTTGATCATCAGGAATTG-3'
USP1	5'-GACAAGGGAAATTGCAAAGA-3'	5'-TGTGTTAAGCAGTCGCCTTG-3'
ZNF462	5'- CTCAAAGCTCATGCTGGTGA-3'	5'- GTGGGAGTGTGAGGAGAGGA-3'
MTPN	5'-GCCAAGGGAGAAGATGTCAA-3'	5'-AGCACCTTTGACAGAAGCA-3'
GMFB	5'- TAAACGCCTGGTGGTACTGG-3'	5'-TCAGGCTTACATCCAACAGGA -3'
RANBP3	5'-GAGAACTGGCAGGGAGGTC -3'	5'-CCACTGCTCCTTCCTCTGA-3'
YAP1	5'- CGTTTTGCCATGAACCAGAG-3'	5'-GCAGCCTCTCCTTCTCCATC -3'
AEBP2	5'- GTCAGCGAGGAGGGGTATTT-3'	5'-CATTGCAGCCACCAACAA -3'
PDK1	5'-ACATCTGTAGGTTTTGGTTTCCA-3'	5'-AAAGACACGTGCAATGATGG-3'
ADIPOR2	5'-TTTGTGTGTAAGGTATGGGAAGG-3'	5'-TGTGTCCAAATGTTGCCTGT-3'

2.3.6 Construction of plasmids and luciferase assays

Wild-type and/or mutant fragments (150-200 bp) from the 3'UTR of MTDH, MTPN and USP1 containing predicted binding sites for miR-375 were individually amplified by AmpliTaq gold DNA polymerase (Applied Biosystems) using the primers listed in Table 2-3. The PCR products were subsequently purified, digested with SPEI and HINDIII, and then cloned downstream of the firefly luciferase gene in the pMIR-REPORT vector (Ambion, Inc.) to produce the following plasmids: pMIR-MTDH, pMIR-MTDH-MUT, pMIR-MTPN, and pMIR-USP1. Subsequently, FaDu and C666-1 cells were seeded onto 24-well plates one day and three days before transfection, respectively. Cells were then transfected with 100 nM of pre-miR-NEG or pre-miR-375 in the morning; 6 hours later, cells were co-transfected with 100 ng of the reporter plasmid of interest, plus 50 ng of pRL-SV40 (Promega BioSciences) containing the *renilla* luciferase gene. Luciferase activity was measured 48 hours post-transfection using the Dual-Glo luciferase assay system according to manufacturer's instructions (Promega BioSciences). Firefly luciferase activity was normalized to that of the *renilla* luciferase.

Table 2-3. Primer sequences used for cloning and screening of pmiR-REPORT luciferase encoding vectors.

Gene	Forward Primer	Reverse Primer
MTDH	GTTGTTACTAGTATGTTGTCATTATATT TGACTGTGGTT	GTTGTTAAGCTT AAGGCCAGTCTTC AGCAAAA
	Reverse screen: AACCATTTTGTTTCGTTTAGCTTTC	
MTDH- mutant	GTTGTTACTAGTATGTTGTCATTATATT TGACTGTGGTT	AAGGCCAGTCTTCAGCAAACTAAA ACCATTCGTCGATTTTAGCTTTCCTA GTCTGAC
	Reverse screen: GTTGTTAAGCTTAAGGCCAGTCTTCA GCAAACTAAAACCATTCGTCGATTTT AGCTTTCCTAGTCTGAC	
USP1	AGTGAAATTTGAATTACTGAAAACCA	AAGCAAACACAAAAGACTGGTG
	Reverse screen: TGGTGTAATCCAAGTGTTTAAATG	
MTPN	GACTAGTGCTTTGTGCATTTTGA	TGTAGCACAATTCAAGTTTATTCCA
	Reverse screen: CAAGTTTATTCCATTTGTTCTTGC	

2.3.7 Viability and clonogenic assays

Viability of pre-miR-375, or siMTDH-transfected FaDu or C666-1 cells was examined using the CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTS), according to the manufacturer's protocol (Promega BioSciences). The cellular effects of these manipulations were further investigated in FaDu cells using clonogenic assays as previously described²⁶⁶. Briefly, cells were re-seeded at low density in 6-well plates in triplicate, and incubated at 37°C under 5% CO₂ at 3 days post-transfection. After 10-12 days, plates were washed, fixed in 50% methanol and stained with 0.1% crystal violet, and then the number of colonies was counted. The fraction of clonogenically viable cells was calculated by comparison of pre-miR-375 or siMTDH transfected cells with pre-miR-NEG or scrambled siRNA (siNEG) transfected cells, respectively.

2.3.8 Cell cycle analysis

Cell cycle analysis of pre-miR-375 transfected C666-1 cells was performed as previously described²⁶⁶. Briefly, cells were harvested and washed with fluorescence-activated cell sorting (FACS) buffer (PBS/0.5% BSA), then re-suspended and fixed with ice-cold 70% ethanol. After washing, cells were re-suspended in FACS buffer and incubated with propidium iodide in the dark before analysis in the BD FACScalibur using the FL-2 channel. The flow cytometry data were analyzed using FlowJo software (Tree Star, Inc.).

2.3.9 Western blot analysis

Total protein extracts were harvested from cell lines and prepared for immunoblotting as previously described²⁶⁸. Membranes were probed with anti-Phospho-Akt (Ser473) (D9E) XP[®]

rabbit mAB (Cell Signaling Technology), anti-Akt (pan) (C67E7) rabbit mAB (Cell Signaling Technology), anti-MTDH polyclonal (clone L-19; 1:1000 dilution; Invitrogen, Inc.), anti- β -actin (8H10D10) mouse mAB (1:20,000 dilution; Cell Signaling Technology), or anti-GAPDH monoclonal antibodies (1:15,000 dilution; Abcam, Inc.), followed by secondary antibodies conjugated to horseradish peroxidase (1:2000 dilution; Abcam, Inc.) or IRDye® fluorescent Secondary Antibodies (1:20,000 dilution; LI-COR Biosciences). GAPDH or β -actin protein levels were used as loading controls. Western blots were quantified with the Adobe Photoshop Pixel Quantification Plug-In (Richard Rosenman Advertising & Design, Toronto, Canada), or the Odyssey Application Software 2.1 (Li-cor Biosciences).

2.3.10 *In vitro* migration and invasion assays

Invasion and migration of HNC cells were assayed using the BD BioCoat™ Matrigel™ Invasion Chambers and Control Inserts (BD Bioscience), respectively. Each well of a 24-well plate contained an insert with an 8 μ m pore size PET (polyethylene terephthalate) membrane. Inserts coated with a thin layer of matrigel basement membrane matrix were used to measure the ability of the cells to invade through the reconstituted basement membrane. For both migration and invasion assays 1×10^5 cells were seeded inside the insert with medium containing 0.5% serum. High serum (20%) medium was then added to the bottom chamber of 24-well plates to serve as a chemo-attractant. After 48 hrs, the membranes were washed, stained, then separated with a sterile scalpel and mounted on a glass slide. The number of migrating or invading cells was then counted under a light microscope.

2.3.11 Tumour formation assay

All animal experiments utilized 6-8 week-old severe combined immunodeficient (SCID) BALB/c female mice in accordance with the guidelines of the Animal Care Committee, Ontario Cancer Institute, University Health Network (Toronto, Canada). Cells were first transfected with siNEG, siMTDH, pre-miR-NEG, or pre-miR-375 72 hrs before they were harvested and their viability was assessed by the trypan blue exclusion method. Subsequently, 2.5×10^5 viable cells were suspended in 100 μ L of growth medium, and injected intramuscularly into the left gastrocnemius muscle of female SCID mice. Tumour growth was monitored by measuring tumour plus leg diameter (TLD) three times a week. Mice were euthanized by CO₂ once TLDs reached 14 mm.

2.3.12 Immunohistochemical (IHC) detection of MTDH expression

Protein expression of MTDH was evaluated in 94 NPC patient samples using immunohistochemistry, which was performed on 5 μ m FFPE sections of each patient's tumour using microwave antigen retrieval, in combination with the Level-2 Ultra Streptavidin system (Signet Laboratories). MTDH expression was detected with the rabbit polyclonal anti-MTDH antibody (1:50 dilution; Invitrogen, Inc.). Expression levels of MTDH were graded according to staining intensity.

2.3.13 Statistical analyses

All experiments were performed at least three independent times, with the data presented as the mean $\pm$ SEM. The statistical differences between treatment groups were determined using a Student's *t* test when comparing two treatment groups, or a one-way

ANOVA followed by Tukey's method when comparing more than two treatment groups. Statistical analyses and graphing were performed using Microsoft excel and Graphpad Prism software (Graphpad Software, Inc.).

To determine the potential prognostic significance of miR-375 or MTDH expression, NPC patients were dichotomized into low ($<$ median) or high ($\geq$ median) expression groups which were compared based on several clinical parameters: overall survival, disease-free survival, local relapse-free survival, nodal relapse-free survival, or distant relapse-free survival. Median follow-up time for this cohort was 3 years. Using the log-rank test, a p-value < 0.05 was considered significant.

2.4 Results

2.4.1 Down-regulation of miR-375 in HNC cell lines and primary NPC patient samples

We have previously reported a high frequency of miR-375 down-regulation in HNSCC samples²⁶¹. To further validate the importance of this miRNA in HNC, expression of miR-375 was measured in an additional seven HNC cell lines, and compared to that of the normal oral epithelial (NOE) cell line (Fig 2-2). Down regulation of miR-375 was noted in five of the seven HNC cell lines analyzed (UTSCC-42a, VU147T, HONE-1, HK-1 and C666-1); the C666-1 cells were observed to have the lowest level of miR-375 expression (~16-fold lower than NOEs). We previously reported that pre-miR-375 transfection of FaDu cells inhibited cell growth, along with a modest increase in the sub-G₀/G₁ population²⁶¹. These same analyses demonstrated that miR-375 transfection in C666-1 cells caused a similarly significant reduction in viability to ~60% by day 3, maintained for at least 6 days (Fig 2-3A). Cell cycle analysis

corroborated that transfection of C666-1 cells with pre-miR-375 resulted in a significant increase in the sub-G₀/G₁ fraction compared to cells transfected with pre-miR-NEG (11.6% *vs.* 1.5%; $p=0.002$); no other phases of the cell cycle appeared to be affected (Fig 2-3B). The expression level of miR-375 was then examined in 92 FFPE NPC compared to 8 normal nasopharyngeal epithelial samples. This analysis demonstrated significantly lower miR-375 expression in NPC *vs.* normal samples (Fig 2-3C; $p=0.01$). Using a cut-off of 2-fold below the mean expression of normal nasopharyngeal epithelial tissues, miR-375 was under-expressed in 69 out of 92 (75%) NPCs; its down-regulation however, was not significantly associated with clinical outcome (Fig 2-4).

Figure 2-2. miR-375 expression in HNC cell lines.

Quantitative RT-PCR analysis of miR-375 expression in 7 HNC cell lines, compared to that of the normal oral epithelial (NOE) cell line. Relative expression was calculated by normalizing to RNU44 and RNU48 expression, in comparison to that of NOEs.

Figure 2-3. Down-regulation of miR-375 in HNC cells

(A) Viability of C666-1 cells as assessed by the MTS assay 3 and 6 days post-pre-miR-375 or pre-miR-NEG transfection. (B) Cell cycle analysis as performed by flow cytometry on C666-1 72 hours post-pre-miR-375 or pre-miR-NEG transfection. (C) MiR-375 expression in 92 primary NPC biopsy samples, compared to 8 normal nasopharyngeal epithelial control tissues as measured by qRT-PCR. (D) Bisulfite sequencing analysis of the CpG sites within a ~1200 bp region around the genomic locus encoding miR-375. Methylation status of three amplicons was analyzed on C666-1 and FaDu cells, treated with either 5-aza-2'-deoxycytidine (FaDu-AZA/C666-1-AZA), or DMSO (FaDu-V/C666-1-V). (E) qRT-PCR analysis of miR-375 expression in FaDu and C666-1 cells after treatment with 5-aza-2'-deoxycytidine relative to vehicle-control (DMSO) treated cells. Each experiment was performed at least three independent times, with the data presented as the mean $\pm$ SEM. *indicates $p < 0.05$

Figure 2-4. Prognostic significance of miR-375 expression in NPC patient samples. Kaplan-Meier plots dichotomized based on miR-375 transcript expression level for NPC patients. Plots show (A) overall; (B) disease-free; (C) distant relapse-free; (D) nodal relapse-free; and (E) local relapse-free survival.

2.4.2 Methylation status of the genomic region containing miR-375

Methylation has been reported as a potential mechanism of miR-375 silencing in hepatocellular carcinoma (HCC)²⁶⁹; hence we examined this possible mechanism in both FaDu and C666-1 cells using both bisulfite sequencing and methylation-status related expression analysis. Bisulfite sequencing conducted on three amplicons spanning the genomic region previously reported to be methylated in HCC²⁶⁹ demonstrated that ~73% and 65% of the CpG sites in this area were indeed methylated in C666-1 and FaDu cells, respectively (Fig 2-3D). Furthermore, treatment of both cell lines with the global de-methylating agent 5-aza-2'-deoxycytidine (5-aza) significantly reduced methylation at all CpG sites (Fig 2-3D), which was associated with the subsequent re-expression of miR-375 (Fig 2-3E), clearly indicating that one mechanism for miR-375 suppression in HNC cells is hyper-methylation of the promoter or coding regions of miR-375.

2.4.3 Identification of mRNA targets of miR-375

To identify the mRNA targets of miR-375, genome wide mRNA expression analysis was performed comparing pre-miR-375 transfected HNC cells with that of pre-miR-NEG transfected cells. Transcripts with >2-fold down-regulation in pre-miR-375 transfected cells were compared to *in silico* predicted targets from 7 publicly available databases (miRanda, miRDB, RNA22, PITA, miR-Walk, RNAHybrid, TargetScan) generating a list of nine candidate target mRNAs (Table 2-4). As confirmation of the validity of our methodology, two of these genes (MTPN, USP1) had been previously reported as targets of miR-375^{171,270}. The effect of miR-375 transfection on gene expression was analyzed for 6 of the 7 identified genes (optimal primers for AEBP2 could not be generated), as well as two previously reported miR-

375 targets (PDK1, ADIPOR2; ^{171,271}). Transfection of FaDu and C666-1 cells with pre-miR-375 led to down-regulation of all potential mRNA targets with the exception of ADIPOR2 in FaDu cells (Fig 2-5). Of note, MTDH was consistently down-regulated to the lowest level in both HNC cell lines; moreover, MTDH was the only target predicted by all seven *in silico* algorithms (Table 2-4).

Table 2-4. Potential miR-375 targets identified using both DNA micro-arrays and *in silico* prediction algorithms.

Gene Symbol	Fold Change	Location	Number of <i>In silico</i> predictions
SPAG9	0.349	17q21.31	1
MTDH	0.373	8q22.1	7
USP1	0.388	1p31.3	2
ZNF462	0.418	9q31.2	4
MTPN	0.421	7q33	3
GMFB	0.444	14q22.2	2
RANBP3	0.46	19p13.3	3
YAP1	0.461	11q13	1
AEBP2	0.465	12p12.3	4

Figure 2-5. Expression of putative targets in HNC cells after miR-375 transfection.

Quantitative RT-PCR analysis of candidate miR-375 mRNA targets for (A) C666-1 cells; and (B) FaDu cells transfected with miR-Neg or miR-375 (20 nM each), assayed 48 hrs or 24 hours post-transfection for C666-1 and FaDu cells, respectively. Relative expression was calculated by normalizing to GAPDH expression, in comparison to cells transfected with pre-miR-NEG.

Down-regulation of MTDH by miR-375 was confirmed at both the transcript and protein level by qRT-PCR and Western blot analysis, respectively (Fig 2-7A). The direct interaction between miR-375 and MTDH was then verified using luciferase assays. Figure 2-6 depicts the *in silico* predicted binding sites between the miR-375 seed region with the MTDH transcript. The predicted binding site located at nucleotides 3518-3539 in the 3'UTR of the MTDH transcript was supported by all seven *in silico* databases and was therefore selected for plasmid construction. In comparison to control cells transfected with pMIR-REPORT, FaDu and C666-1 cells transfected with pMIR-REPORT-MTDH demonstrated a significant reduction in luciferase activity of 18% and 20% respectively, when co-transfected with pre-miR-375 (Fig 2-7B). Upon mutating the miR-375 binding site, this inhibitory effect was completely abrogated, corroborating that the observed decrease in luciferase activity was indeed dependent upon an intact miR-375 binding site. Two previously reported mRNA targets, MTPN and USP1, were also demonstrated to be potential targets of miR-375 in both HNC cells (Fig 2-8).

MTDH mRNA:

Figure 2-6. Schematic depicting the location of predicted miR-375 binding sites on the MTDH transcript.

Circled location was the only site predicted by all seven *in silico* algorithms, and was the site cloned into the luciferase expression plasmid.

Figure 2-7. Identification of mRNA targets of miR-375

(A) MTDH expression at both the mRNA (left) and protein (right) level after pre-miR-375 or pre-miR-NEG transfection in C666-1 and FaDu cells. (B) Relative luciferase activity in C666-1 or FaDu cells after co-transfection with pMIR-REPORT, pMIR-MTDH or pMIR-MTDH-MUT vectors and pre-miR-375 or pre-miR-NEG. (C) MTDH expression at both the mRNA (left) and protein (right) level after siMTDH or siNEG transfection in C666-1 and FaDu cells. (D) Relative viability in C666-1 and FaDu cells transfected with siMTDH compared to that in cells transfected with the negative control sequence. OD, optical density; *indicates $p < 0.05$

Figure 2-8. Interaction of miR-375 with MTPN and USP1 3' UTRs.

Luciferase assay results illustrating the interaction between miR-375 with its predicted binding sites in the 3'UTRs of the MTPN and USP1 transcripts in both C666-1 and FaDu cells.

* indicates $p < 0.05$

2.4.4 Effects of MTDH knock-down on HNC cells

The potential oncogenic role of MTDH was investigated by evaluating the phenotypic consequences of transfecting HNC cells with siRNA directed against MTDH (siMTDH). MTDH knock-down by this siRNA sequence was confirmed at both the transcript and protein level (Fig 2-7C). Similar to the effects of pre-miR-375 transfection, siMTDH transfection significantly reduced cell viability in both C666-1 (>50%), and FaDu (~20%) cells (Fig 2-7D). To ensure that the observed cytotoxicity was not due to off-target effects, the MTS experiments were repeated using a second distinct siRNA sequence targeting MTDH, demonstrating a similar effect (Fig 2-9A). As yet another level of corroboration, a rescue plasmid expressing a MTDH transcript refractory to siRNA was co-transfected with siMTDH, which completely abrogated any siMTDH-mediated cytotoxicity, confirming that this was indeed an MTDH knock-down-specific effect (Fig 2-9B).

Metadherin is frequently reported to promote metastasis in human cancers; hence, the effect of miR-375 and MTDH on HNC cell migration and invasion were evaluated using *in vitro* trans-well migration assays. Compared to their corresponding negative controls, transfection with pre-miR-375 significantly reduced migration of both C666-1 and FaDu cells by 57% and 80%, respectively (Fig 2-10A). Similar inhibition of migration was observed in cells transfected with siMTDH, with 75% reduction in C666-1 cells and 86% in FaDu cells (Fig 2-10A). Moreover, siMTDH and pre-miR-375 transfection also resulted in 86% and 92% reduction in invasion of FaDu cells, respectively (Fig 2-10B). C666-1 cells are unable to penetrate the matrigel coating used in the invasion assay; hence this property could not be assessed in the NPC model.

Figure 2-9. Specificity of siRNA mediated cytotoxic effects

(A) The effect of an additional, distinct siRNA sequence targeting MTDH on viability of FaDu cells as measured by the MTS assay. B) Rescue of C666-1 cells from siMTDH-mediated cytotoxicity by co-transfection with a plasmid encoding a si-resistant MTDH transcript (pMTDH). *indicates $p < 0.05$

Figure 2-10. Migration and invasion of miR-375 or siMTDH in HNC cells

(A) Representative images (left) and bar graphs (right) depicting the migratory ability of C666-1 and FaDu cells after pre-miR-375 or siMTDH transfection compared to corresponding negative controls. (B) Representative images (left) and bar graphs (right) depicting the invasive ability of FaDu cells after pre-miR-375 or siMTDH transfection compared to corresponding negative controls. *indicates $p < 0.05$; **indicates $p < 0.01$; ***indicates $p < 0.001$.

2.4.5 Effects of miR-375 transfection and MTDH knock-down on PI(3)K-Akt signalling

Western blot analyses were performed to investigate the signaling pathways downstream of miR-375 over-expression or MTDH knockdown in FaDu and C666-1 cells. The PI(3)K-Akt pathway has been previously reported to be activated by MTDH over-expression; specifically increased phosphorylation of Akt (p-Akt), although the mechanism by which this induction occurs remains poorly understood²⁷²⁻²⁷⁴. Concordantly, a significant reduction in p-Akt was observed in both C666-1 and FaDu cells after siMTDH knockdown (Fig 2-11A&B), phenocopied by miR-375 transfection in FaDu cells (Fig 2-11B), but not so for C666-1 cells (Fig 2-11A). Total Akt expression remained unchanged under these conditions.

Figure 2-11. Down-stream signaling, and *in vivo* effects of miR-375 and siMTDH in HNC models.

Representative western blot images and relative quantification of p-Akt protein levels in (A) C666-1 and (B) FaDu cells transfected with pre-miR-375 or siMTDH, compared to corresponding negative controls. (C) & (D) Tumour-plus-leg diameter measurements of (C) C666-1 and (D) FaDu cell xenografts transfected with siMTDH, pre-miR-375 or corresponding negative controls. OD, optical density; * indicates $p < 0.05$; ** indicates $p < 0.01$.

2.4.6 MTDH knock-down and miR-375 transfection delayed tumour formation *in-vivo*

To investigate the effects of miR-375 over-expression and MTDH knock-down on head & neck tumour forming ability, FaDu and C666-1 cells transfected with siMTDH, pre-miR-375 or corresponding negative controls were injected intra-muscularly into SCID mice. The results demonstrated that both miR-375 over-expression and MTDH knock-down resulted in a significant delay in tumour formation wherein such transfected tumours attained the humane endpoint (TLD = 14mm) ~10-15 days later than tumours transfected with the corresponding negative controls (Fig 2-11C, 2-11D).

2.4.7 Over-expression of MTDH in primary HNC samples

The relevance of MTDH in HNC was further investigated in primary human HNC samples in the previously evaluated 20 HNSCC²⁶¹, as well as the same 92 NPC samples for which miR-375 expression has already been examined (Fig 2-3C). Significantly higher MTDH transcript levels were detected in both HNSCC ($p=0.048$) and NPC patient samples ($p<0.0001$) compared to corresponding normal controls as measured by qRT-PCR (Fig 2-12A & B). Using a 2-fold cut-off, MTDH mRNA over-expression was detected in 65% and 93% of HNSCC and NPC samples, respectively. Over-expression of MTDH at the protein level was corroborated in 94 NPC patient samples (including the 92 previously investigated cases) using immunohistochemistry (IHC), illustrating that MTDH expression was predominantly localized to the cell membrane or cytoplasm, with little to no immuno-staining observed in the surrounding stroma (Fig 2-12C). The vast majority of NPC samples had cytoplasmic MTDH immuno-expression (91 out of 94 cases, or 97%), with only one sample showing nuclear

staining (Fig 2-12D). Strikingly, when dichotomized based on MTDH transcript expression level, NPC patients with high expression ($\geq$ median) had significantly worse overall (HR=2.34; $p=0.04$), disease-free (HR=2.08; $p=0.04$), and distant relapse-free (HR=6.38; $p=0.005$) survival compared to patients with low MTDH ($<$ median) expression (Fig 2-12E). The association of MTDH expression with two additional factors that are potentially predictive of distant relapse in NPC patients was also explored; namely treatment, and stage. Firstly, MTDH expression was not different as a function of treatment (chemoradiation *vs.* radiation alone; Fig 2-13A). Additionally, when the radiation only group of patients was analyzed, MTDH remained a powerful predictor of distant relapse (Fig 2-13B). A similar trend was observed for the chemoradiation patients, but the difference was not statistically significant ($p=0.17$), likely due to the small sample size (Fig 2-13B). Secondly, MTDH expression was interestingly inversely related to stage, being lower in Stage III/IV *vs.* Stage I/II patients ($p=0.04$; Fig 2-13C). Importantly, however, when patients were further categorized according to both stage (I/II *vs.* III/IV), as well as MTDH expression ($<$ median *vs.* $\geq$ median), this was a highly significant association ($p=0.006$; Fig 2-13D). Specifically, Stage I/II patients with low MTDH expression had 0% distant relapse; conversely, Stage III/IV patients plus increased MTDH expression had the highest risk of distant relapse (5-year rate of 37%), suggesting that using both variables could be quite informative in terms of prognosticating for metastatic disease in NPC. Due to the small sample size and mal-distribution of outcome of the HNSCC patients (the majority of the 20 samples had relapsed disease), survival analysis was not performed on this cohort.

Figure 2-12. MTDH Expression in HNC patient samples.

MTDH expression measured by qRT-PCR in (A) 20 HNSCC and (B) 92 NPC patient biopsy samples, in comparison to 6 normal laryngeal epithelial tissues, and 7 normal nasopharyngeal epithelial tissues, respectively. (C) Representative images of immunohistochemical analysis of MTDH expression in primary NPC biopsy samples. Arrows indicate tumour cells exhibiting (C) cytoplasmic membrane or (D) nuclear expression of MTDH. T, tumor cells; E, endothelial cells. (E) Kaplan-Meier plots of overall survival, disease-free survival, and distant relapse-free survival for NPC patients dichotomized based on high ($\geq$ median) vs. low ($<$ median) MTDH transcript expression level.

Figure 2-13. Association of MTDH expression with treatment modality and tumour stage. (A) MTDH expression in patients treated with chemoradiation (n=22) vs. radiation alone (n=66). (B) Kaplan-Meier plots dichotomized based on MTDH expression level for NPC patients treated with radiation alone (left) or chemoradiation (right). (C) MTDH expression in patients with stage I/II (n=36) vs. stage III/IV (n=53) disease. (D) Kaplan-Meier plot with patients divided into four groups based on stage (I/II or III/IV) and MTDH expression (<median or ≥median).

2.5 Discussion

Our recent study profiling miRNA expression in locally advanced HNSCC reported a high frequency of miR-375 under-expression in these tumours, and suggested its potential tumor suppressor function²⁶¹. The current report demonstrates that miR-375 is also down-regulated in 75% of NPCs and confirms a tumour suppressor function for this miRNA in HNC cell lines. Furthermore, MTDH has been verified as *bona fide* target of miR-375 in HNC. During the preparation of this report, MTDH was also identified independently by another group as a target of miR-375 in HNSCC²⁷⁵. However, to the best of our knowledge, this study is the first to support a role for the dysregulation of miR-375 and MTDH in promoting metastases in HNCs; particularly in NPC.

MiR-375 was first described in the development and function of pancreatic islet cells^{276,277}, and more recently in human embryonic stem cell development²⁷⁸. Mounting data however, suggest that miR-375 might also be playing an equally important role in human cancers. Several groups have recently demonstrated a tumour suppressor role for miR-375^{261,271,279,280}, congruent with our own data in this study. Reduced miR-375 expression has in fact been reported to be associated with worse outcome for patients with esophageal carcinoma^{281,282}; high miR-221 to low miR-375 ratio has also been suggested as a potential diagnostic marker for HNSCC²⁷⁹. Conversely, several groups have also described a potential oncogenic role for miR-375^{283,284}, underscoring that similar to many other miRNAs, miR-375 can function as both a tumour suppressor and an oncogene, probably dependent upon context and cancer type.

Chromosomal loss and promoter hypermethylation are two common mechanisms for inactivation of tumour suppressor genes. Deletion of chromosome 2q35 has indeed been

reported in HNSCC²⁸⁵. In our current work, bisulfite sequencing demonstrated that CpG islands proximal to the miR-375 locus are highly methylated (Fig 2-3D), and exposure to 5'-aza-2-deoxycytidine resulted in a significant re-expression of miR-375 (Fig 2-3E), corroborating that hypermethylation is indeed one important mechanism for miR-375 under-expression in HNC, similar to the previous report in HCC²⁸⁶. Hence, chromosomal loss and hypermethylation-mediated silencing are two likely mechanisms leading to miR-375 down-regulation in HNC.

In terms of MTDH over-expression in HNC, miR-375 down-regulation is clearly one documented mechanism, but the down regulation of other miRNAs could also play a role in this process. As an example, miR-26a was recently reported to be under-expressed in breast cancer, targeting both MTDH and enhancer of zeste homolog 2 (EZH2)²⁸⁶. Our own group has previously reported significant miR-26a down-regulation in primary HNSCC²⁶⁰, as well as NPC¹⁹¹; hence, under-expression of miR-26a might indeed be another mechanism for MTDH over-expression in HNC. A third potential mechanism for MTDH over-expression could be chromosomal amplification; given that 8q22 (genomic location for MTDH) is also frequently amplified in both NPC and HNSCC^{26,287}. Indeed, this mechanism for MTDH over-expression has already been reported for both breast²⁶² and hepatocellular carcinomas²⁸⁸. Taken together, these conglomerate reports illustrate that MTDH over-expression in HNC is likely mediated by a complex interplay between several factors including chromosomal amplification and targeting by multiple miRNAs.

The human *MTDH* gene was first cloned in astrocytes, where it was shown to be up-regulated in response to treatment with TNF- α , gp120, or HIV infection; therefore initially denoted as “Astrocyte elevated gene 1” (AEG-1)²⁶¹. The mouse homologue was cloned shortly thereafter in a murine model for metastatic breast cancer; discovered due to its role in

selectively homing metastatic breast cancer cells to the lung²⁸⁹. Since these seminal observations, MTDH has rapidly emerged as an important mediator in the development and progression for several human cancers²⁶². In fact, MTDH over-expression has already been associated with poor outcome for breast²⁶², liver²⁸⁸, esophageal²⁹⁰, renal cell²⁹¹, colorectal²⁹², and non-small cell lung cancers²⁹³, frequently associated with metastases and chemoresistance. However, the molecular mechanisms by which MTDH mediates metastases and/or chemoresistance remain to be clearly elucidated. Some of the suggested downstream signaling events have included activation of PI3K-Akt²⁷², NF- κ B²⁹⁴, MAPK and Wnt²⁸⁸, up-regulation of MMP-9²⁹⁵ and FOXO3a^{273,290}, suppression of FOXO1²⁷⁴, as well as induction of EMT²⁹⁴.

Herein, we report the over-expression of MTDH in both HNSCC and NPC. Due to small sample size (n=20), the significance of MTDH over-expression observed in the HNSCC samples was marginal (p=0.048), therefore we sought out additional evidence to support these findings. Using a publicly available online (<http://www.ebi.ac.uk/gxa/array/U133A>) data set mined by Lukk *et al.*²⁹⁶, we identified that MTDH was also significantly over-expressed in their HNSCC samples by 2.1-4.2 fold (p<0.08). Furthermore, another group has also recently reported the significant over-expression of MTDH in HNSCC using 20 paired HNSCC/Normal samples²⁷⁵; hence we are confident regarding MTDH over-expression in primary HNSCC. We also report, for the first time, a role for MTDH in promoting a metastatic phenotype in HNC, particularly in NPC. The oncogenic function of MTDH was demonstrated both *in vitro* and *in vivo* wherein MTDH affected cell migration and invasion, potentially mediated in part *via* phosphorylation of Akt. The clinical impact of this observation is extremely important; due to the advent of intensity-modulated radiation therapy (IMRT), the 5-year local control rates for NPC are excellent, at ~90%¹³. However, the 5-year OS for NPC remains modest at ~70% due

to distant metastases¹³. Hence, therapies that effectively reduce metastases would be of extreme importance in improving survival for NPC patients. As MTDH is primarily expressed at the plasma membrane of breast cancer cells²⁸⁹, a DNA vaccine therapy targeting MTDH has been developed, demonstrating some preliminary success in reducing tumour burden and lung metastases in a mouse model of breast cancer²⁹⁷. However, such approaches might not be applicable to HNC, given that MTDH expression appears to be predominantly cytoplasmic in this disease (Fig 2-12C).

In conclusion, a novel pathway of miR-375 down-regulation, leading to MTDH over-expression has been documented in human HNC, which has significant clinical implications as a mechanism by which HNC metastases could develop (Figure 2-14). Further unraveling of the MTDH protein structure and function, as well as the molecular mechanisms by which it activates a metastatic cascade could lead to the development of small molecule inhibitors, which can be examined as a potential therapeutic strategy by which outcome can be improved for future patients with HNC.

Figure 2-14. Working model detailing the role of the miR-375-MTDH axis in NPC.

Chapter 3

The Identification of a microRNA Signature Associated with Risk of Distant Metastasis in Nasopharyngeal Carcinoma

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3. The Identification of a microRNA Signature Associated with Risk of Distant Metastasis in Nasopharyngeal Carcinoma

3.1 Abstract

3.1.1 Background

Despite significant improvement in locoregional control in the contemporary era of nasopharyngeal carcinoma (NPC) treatment, patients still suffer from a significant risk of distant metastasis (DM). Identifying those patients at risk of DM would aid in personalized treatment in the future. MicroRNAs (miRNAs) play many important roles in human cancers; hence, we proceeded to address the primary hypothesis that there is a miRNA expression signature capable of predicting DM for NPC patients.

3.1.2 Methods

The expression of 734 miRNAs was measured in 125 (Training Set) and 121 (Validation Set) clinically annotated NPC diagnostic biopsy samples. A signature associated with risk of DM was generated by fitting a penalized Cox Proportion Hazard regression model to the Training data set, and subsequently tested in the Validation set. Pathway enrichment analysis was then performed on validated targets of the miRNAs comprising the final signature.

3.1.3 Results

A 4-miRNA expression signature associated with risk of developing DM was identified in a Training set (HR 8.25; $p < 0.001$) and validated in an independent Validation set (HR 3.2; $p = 0.01$), and observed to be the strongest independent predictor when multivariate analysis was performed. Pathway enrichment analysis indicated that the targets of these miRNAs appear to be converging on cell-cycle pathways.

3.1.4 Conclusion

This 4-miRNA signature adds to the prognostic value of the current “gold standard” of TNM staging. In-depth interrogation of these 4-miRNAs will provide important biological insights that could facilitate the discovery and development of novel molecularly targeted therapies to improve outcome for future NPC patients.

3.2 Introduction

Nasopharyngeal carcinoma (NPC) has a unique set of etiological, epidemiological and biological characteristics that renders it distinct from other epithelial malignancies of the head and neck region. The primary curative treatment for NPC is radiotherapy (RT) for patients with early stage disease, and concomitant chemoradiotherapy (CRT) for those with locally advanced disease. Advances in RT delivery such as intensity-modulated radiation therapy (IMRT), and increasing use of CRT have led to significantly improved loco-regional control (LRC) for NPC patients, with 5-year LRC rates now well over 90%²⁹⁸. However, distant metastasis (DM) rates have not improved, and late metastatic disease remains the major cause of mortality in this patient population²⁹⁸. Currently, TNM staging is the primary tool utilized clinically to prognosticate outcomes for NPC patients. Other indicators such as tumor volume²⁹⁹, plasma Epstein–Barr Viral (EBV) DNA titre^{128,300}, and levels of expression of various proteins and transcripts^{157,301,302} have been reported to correlate with clinical outcomes; to date however, none has been universally adopted in the clinical management of NPC patients.

MicroRNAs (miRNA) are small non-protein-coding RNA molecules that function to reduce the expression of protein coding genes at the post-transcriptional level. Since their identification in 1993³⁰³, miRNAs have emerged as key regulators of gene expression in nearly all biological processes, including cancer²⁵⁶. Additionally, miRNA expression signatures

associated with prognosis have been identified in numerous malignancies²⁵⁹. Hence, we undertook to identify and validate a miRNA expression signature capable of predicting DM for NPC patients.

3.3 Materials & Methods

3.3.1 Patients and Samples

Approval for this study was obtained from the Institutional Research Ethics Board (REB) at the Princess Margaret Cancer Center (PM). Diagnostic formalin-fixed paraffin-embedded (FFPE) blocks were collected for NPC patients treated at the PM who were diagnosed with NPC between 2000-2009 (Training cohort), and 1993-2000 (Validation cohort) (Table 1). Patients with metastatic disease at the time of diagnosis were excluded. Diagnostic and follow-up data were collected through chart reviews; the clinical characteristics for these two patient cohorts are shown in Table 1. It is important to note that despite the greater utilization of CRT (which reflected clinical practice), and the superior LRC rates in the Training cohort, the 5-year distant metastasis-free survival (DMFS) rates for both cohorts was identical, at 87%.

Normal nasopharyngeal epithelial tissues (n=17) were macro-dissected from FFPE blocks of patients who underwent a quadroscope and were not diagnosed with NPC. These tissues were examined by at least one head and neck cancer (HNC) pathologist (BP-O or IW), and deemed to be free of malignant cells.

Table 3-1. Clinical characteristics of patients in the two independent cohorts studied.

	Training Set (Dx '00-'09; n=125)	Validation Set (Dx '93-'00; n=121)	p-value*
<u>Age (years)</u>			
median	52	48	0.30
range	14-89	16-79	
Frequency (%)			
<u>Gender</u>			
Male	86 (67)	56 (71)	0.52
Female	39 (33)	23 (29)	
<u>T stage</u>			
T1	37 (30)	37 (31)	0.26
T2	20 (16)	30 (25)	
T3	28 (22)	24 (20)	
T4	39 (31)	28 (23)	
Unable to evaluate	1 (<1)	1 (<1)	
<u>N stage</u>			
N0	25 (20)	24 (20)	0.05
N1	33 (26)	50 (41)	
N2	52 (42)	38 (31)	
N3	15 (12)	8 (7)	
Unable to evaluate	none	1 (<1)	
<u>TNM Stage</u>			
I (%)	11(8)	10 (8)	0.07
II (%)	22 (18)	37 (31)	
III (%)	41 (33)	39 (32)	
IV (%)	51 (41)	34 (28)	
Unable to evaluate	none	1 (<1)	
<u>Treatment</u>			
Radiation only	34 (27)	86 (71)	1.43E-11
Radiation + chemo	91 (73)	35 (29)	
5-year Survival %			
<u>Survival</u>			
Overall	83%	73%	0.22
Disease-Free	72%	66%	0.35
Local Relapse-Free	90%	76%	0.002
Nodal Relapse-Free	94%	88%	0.08
Distant Relapse-Free	87%	87%	0.89

*Statistical tests: Wilcoxon rank-sum test (Age), Chi-squared test (Gender, T/N/TNM-stage, Treatment), log-rank test (all survival endpoints).

3.3.2 MiRNA Expression Profiling

A representative section from each block was stained with hematoxylin and eosin (H&E) and reviewed by a HNC pathologist (BP-O) to identify regions containing malignant cells. Samples were then macro-dissected to ensure that >70% of the material analyzed was malignant epithelium. Specimens were de-paraffinized with xylene, and total RNA was extracted using the Recover All Total Nucleic Acid Isolation Kit for FFPE (Ambion, Inc.), according to the manufacturer's instructions. RNA was quantified and purity-assessed using a Nanodrop spectrophotometer (Thermo Scientific). Two hundred nanograms of total RNA from each sample was analyzed according to the manufacturer's instructions using the nCounter[®] Human miRNA Assay from Nanostring (v1.0) to measure 734 unique human and viral miRNAs. Data were normalized using the NanoStringNorm³⁰⁴ (v1.1.13) package in the 'R' statistical computing environment (v2.15.2). Raw counts were background-corrected by subtracting the mean + 2 standard deviations of the negative control probes included in the assay, followed by variance stabilization and normalization (vs³⁰⁵) called through the NanoStringNorm package.

3.3.3 Statistical Analysis

All statistical tests were performed in the 'R' statistical computing environment (v2.15.2), with the exception of pathway enrichment analysis. MiRNA expression in tumour vs. normal samples was compared using Welch's t-tests with the Benjamini & Hochberg false discovery rate (FDR) correction for multiple comparisons³⁰⁶. Prior to all regression analysis, the data were standardized (mean centered and standard deviation scaled), using the R package 'ffmanova'³⁰⁷, to maintain consistency across datasets. In order to identify a signature

associated with risk of DM, an L1 penalized (Lasso³⁰⁸) Cox proportional hazard (CoxPH) regression model was fitted to the Training cohort data using the penalized (v0.9-42) package³⁰⁹ in R. Leave-one-out cross validation was used to determine the L1 tuning parameter that yielded the highest log-likelihood, and a penalized CoxPH regression model was fitted using this optimal value. After fitting, the 4 miRNAs most strongly associated with DM were selected for inclusion in the risk score (RS) equation in order to maintain the number of events per variable (EVP) at ~five³¹⁰. The final equation was generated using the regression coefficients from the CoxPH model. RS was calculated for each patient in the Training cohort, and patients were dichotomized to “high risk” ($RS \geq \text{median}$) or “low risk” ($RS < \text{median}$) groups. The difference in DMFS between the two groups was compared using the log-rank test. RS and risk-groups were determined similarly for the Validation cohort, with the cut-point maintained as the median RS in the Training cohort and DMFS compared using the log-rank test. Multivariate analysis was performed by including the 4-miRNA signature risk-group assignment (“high risk” or “low risk”) for each patient adjusted with clinical variables (T-Stage, N-Stage, gender, age, treatment) in a CoxPH model fitted to the combined Training and Validation datasets (n=242; one patient removed due to unevaluable nodal stage, three patients removed due to unevaluable tumour stage). The two variables found to be significantly associated with DM in this model (the 4-miRNA signature RS, and N-stage) were then combined to create five risk groups: **1.** N0; **2.** N1/2 & Low-RS; **3.** N1/2 & High-RS; **4.** N3 & Low-RS; and **5.** N3 & High-RS. The area-under the curve (AUC) was calculated for the receiver operating characteristic (ROC) curves across multiple time points using the ‘survivalROC’ package (v1.0.3)³¹¹ and the c-statistics for various predictive models were calculated using the ‘survConcordance’ function in the ‘survival’ package (v2.37-4)³¹² for R.

3.3.4 TCGA Data Retrieval and Analysis

Level 3 (Reads-per-million; RPM) miRNA-Seq and Level 1 clinical data were downloaded from the Broad Institute Firehose (stddata run 2013_11_14) repository for data generated by TCGA. At the time of analysis, there were 260 HNSCC samples with miRNA-seq values and sufficient follow-up data to determine disease specific survival status. RPM data were $\log_2$ transformed and standardized (mean centered, standard deviation scaled) before risk scores were calculated using the 4-miRNA equation described above. Patients were dichotomized into “low risk” ($<$ median RS) vs. “high risk” ($\geq$ median RS) groups, and then compared using the log-rank test.

3.3.5 Target Identification and Pathway Analysis

Validated targets of the 4 miRNAs in the signature were downloaded from the miRWalk database which uses a text-mining algorithm to extract validated miRNA-target relationships from the literature³¹³. The union of the 4 lists of targets was inputted into the Database for Annotation, Visualization and Integrated Discovery (DAVID; v6.7)³¹⁴ for pathway enrichment analysis. To ensure that the enrichment observed was not due to bias in the validated target database, a complete list of validated targets for all human miRNAs was downloaded from the miRWalk database, and used as the background for enrichment analysis.

3.4 Results

3.4.1 Differentially Expressed miRNAs

Amongst the 734 human and viral miRNAs included in the nCounter human miRNA assay, 531 and 442 miRNAs were expressed above background levels in at least one sample in

the Training and Validation cohorts, respectively. Tumour *vs.* normal tissue comparisons in the Training (tumour n = 125; normal n = 7), and Validation (tumour n = 121; normal n = 10) data sets yielded 124 and 192 differentially-expressed miRNAs, respectively, at a false discovery rate (FDR) cut-off of 0.05. Amongst these differentially expressed miRNAs, 81 overlapped between the two data sets, including 30 EBV-encoded miRNAs from the BamHI-A rightward transcripts (BART) region, plus several miRNAs that have been previously reported as differentially expressed in NPC or other cancers (Table 3-2)³¹⁵⁻³¹⁷.

Table 3-2. Differential expression analysis statistics for 81 miRNAs found to be significantly altered in both Training and Validation cohorts.

	Training				Validation			
	log ₂ FC	Mean	Raw p	Adjusted p	log ₂ FC	Mean	Raw p	Adjusted p
Human								
miR-1978	7.33	4.70	3.04E-50	1.68E-48	5.15	5.35	1.08E-02	4.54E-02
miR-708	7.08	4.54	3.08E-32	8.52E-31	1.97	8.72	1.03E-03	6.80E-03
miR-301b	4.22	2.76	1.36E-24	3.00E-23	1.62	1.08	2.82E-11	5.03E-10
miR-663b	3.18	2.04	2.95E-19	5.42E-18	1.55	1.10	3.22E-09	4.63E-08
miR-1977	3.04	1.95	3.61E-19	6.39E-18	4.62	4.22	4.50E-03	2.24E-02
miR-130b	2.22	1.44	1.70E-15	2.50E-14	4.74	4.77	6.79E-03	3.19E-02
miR-1253	2.97	1.90	2.19E-13	2.85E-12	2.10	1.80	3.42E-04	2.60E-03
miR-1290	8.90	6.00	2.71E-12	3.32E-11	6.00	4.25	3.03E-11	5.23E-10
miR-421	3.88	2.70	1.44E-10	1.67E-09	3.81	3.92	3.03E-03	1.60E-02
miR-452	1.44	0.92	2.24E-09	2.24E-08	3.02	2.49	5.15E-03	2.49E-02
miR-224	1.24	0.80	2.28E-09	2.24E-08	0.63	0.40	2.16E-04	1.74E-03
miR-566	1.74	1.22	1.89E-08	1.71E-07	1.60	1.02	1.06E-08	1.49E-07
miR-1308	12.02	8.49	3.78E-08	3.34E-07	2.56	10.72	1.77E-07	2.29E-06
miR-495	0.95	0.61	8.15E-08	6.67E-07	1.74	1.56	7.45E-03	3.44E-02
miR-1246	10.85	8.12	4.07E-07	3.16E-06	2.78	9.73	2.36E-04	1.88E-03
miR-369-3p	0.66	0.42	1.34E-06	9.69E-06	0.43	0.27	1.89E-03	1.09E-02
miR-2117	7.05	5.42	2.05E-06	1.43E-05	3.81	3.07	4.62E-05	4.50E-04
miR-885-5p	0.85	0.54	1.36E-05	8.35E-05	1.11	0.90	1.58E-03	9.72E-03
miR-362-3p	0.76	0.49	1.74E-05	1.04E-04	3.22	2.53	1.16E-03	7.53E-03
miR-548f	0.63	0.41	2.02E-05	1.18E-04	0.44	0.28	1.53E-04	1.36E-03
miR-653	0.88	0.57	2.40E-05	1.36E-04	0.50	0.34	2.49E-04	1.95E-03
miR-145	-5.64	6.10	4.80E-05	2.52E-04	-2.64	9.19	1.66E-03	1.01E-02
miR-125b	-2.64	9.25	5.71E-05	2.97E-04	-1.18	11.69	9.94E-03	4.33E-02
miR-455-5p	0.63	0.40	3.12E-04	1.41E-03	4.43	3.49	2.32E-03	1.29E-02
miR-551b	0.54	0.35	4.19E-04	1.82E-03	1.48	0.98	3.40E-07	4.18E-06
miR-324-3p	0.36	0.23	9.84E-04	3.92E-03	2.75	2.18	1.20E-03	7.59E-03
miR-21	3.81	10.46	1.48E-03	5.55E-03	3.77	13.03	4.42E-04	3.31E-03
miR-320a	5.69	5.78	1.50E-03	5.56E-03	5.68	6.30	9.14E-03	4.11E-02
miR-196b	0.42	0.27	1.58E-03	5.82E-03	5.55	3.59	6.35E-32	2.34E-30
miR-199b-5p	-5.64	2.91	1.63E-03	5.91E-03	-2.47	6.39	8.54E-04	5.97E-03
miR-494	8.40	13.18	1.68E-03	6.03E-03	4.65	13.19	7.41E-06	8.15E-05
miR-630	6.77	9.56	1.95E-03	6.94E-03	3.44	8.97	1.03E-02	4.44E-02
miR-1274a	1.61	13.10	2.10E-03	7.21E-03	1.50	13.33	2.22E-03	1.26E-02
miR-874	0.37	0.24	2.20E-03	7.47E-03	0.51	0.35	1.07E-04	9.68E-04
miR-493	4.74	6.13	2.27E-03	7.67E-03	5.14	6.40	4.49E-03	2.24E-02
miR-508-3p	0.60	0.39	2.82E-03	9.24E-03	0.31	0.22	1.05E-02	4.49E-02
miR-762	0.26	0.17	3.68E-03	1.13E-02	0.36	0.22	2.64E-03	1.42E-02
miR-18b	0.29	0.18	3.75E-03	1.14E-02	1.61	1.02	2.25E-09	3.33E-08
miR-572	0.25	0.16	4.00E-03	1.21E-02	0.15	0.11	1.17E-02	4.87E-02
miR-208a	0.26	0.17	5.41E-03	1.59E-02	0.79	0.53	9.20E-07	1.06E-05
miR-25	4.27	8.25	5.62E-03	1.65E-02	2.25	11.10	1.90E-03	1.09E-02

	log ₂ FC	Mean	Raw p	Adjusted p	log ₂ FC	Mean	Raw p	Adjusted p
miR-184	0.33	0.22	7.64E-03	2.15E-02	3.74	2.43	8.56E-16	2.21E-14
miR-96	4.48	5.83	7.70E-03	2.15E-02	2.50	8.79	1.74E-03	1.04E-02
miR-1975	1.95	8.23	9.02E-03	2.43E-02	2.74	10.34	2.87E-03	1.53E-02
miR-215	0.20	0.13	1.12E-02	2.95E-02	0.42	0.28	1.62E-04	1.42E-03
miR-574-3p	-3.47	0.45	1.45E-02	3.69E-02	-3.64	4.01	9.92E-03	4.33E-02
miR-106b	2.79	7.45	1.57E-02	3.92E-02	3.09	9.77	1.98E-04	1.63E-03
miR-450a	0.12	0.08	1.63E-02	4.05E-02	2.42	1.54	2.00E-14	4.69E-13
miR-1979	1.10	7.99	1.84E-02	4.38E-02	2.29	9.62	2.25E-03	1.27E-02
miR-2054	0.15	0.10	1.94E-02	4.56E-02	0.28	0.18	1.20E-03	7.59E-03
miR-181c	0.15	0.10	2.07E-02	4.76E-02	4.10	3.62	5.15E-03	2.49E-02
EBV								
miR-BART7	13.62	8.74	2.83E-68	1.25E-65	14.96	9.86	2.45E-69	1.27E-66
miR-BART9	14.49	9.29	1.10E-66	2.43E-64	2.15	8.65	1.65E-04	1.42E-03
miR-BART3	12.81	8.22	1.49E-63	2.19E-61	12.50	8.25	2.31E-61	1.99E-59
miR-BART22	11.81	7.58	9.79E-60	1.08E-57	15.33	10.10	1.63E-68	4.21E-66
miR-BART6-3p	10.10	6.48	1.63E-54	1.44E-52	12.57	8.30	1.52E-63	1.96E-61
miR-BART17-3p	9.54	6.12	1.74E-52	1.28E-50	13.13	8.65	6.87E-64	1.18E-61
miR-BART19-3p	9.56	6.13	2.16E-52	1.37E-50	12.11	7.96	3.94E-59	2.91E-57
miR-BART1-3p	9.69	6.21	1.30E-49	6.36E-48	12.89	8.49	3.01E-63	3.11E-61
miR-BART4	9.46	6.07	8.89E-49	3.93E-47	11.30	7.46	1.28E-57	8.24E-56
miR-BART18-5p	9.03	5.79	6.03E-46	2.42E-44	11.24	7.40	3.53E-57	2.03E-55
miR-BART21-3p	8.52	5.46	2.30E-45	8.46E-44	11.05	7.27	1.14E-55	5.89E-54
miR-BART11-5p	6.35	4.08	3.77E-33	1.19E-31	10.32	6.78	1.29E-52	6.07E-51
miR-BART17-5p	6.25	4.01	3.04E-32	8.52E-31	10.38	6.87	3.22E-47	1.39E-45
miR-BART15	4.20	2.69	2.57E-22	5.17E-21	7.87	5.18	4.08E-42	1.62E-40
miR-BART14	2.14	1.37	2.86E-12	3.42E-11	3.53	2.32	2.18E-18	6.26E-17
miR-BART18-3p	1.70	1.09	1.50E-09	1.58E-08	3.62	2.37	4.73E-18	1.29E-16
miR-BART6-5p	9.01	6.58	5.79E-08	4.92E-07	4.98	9.41	9.17E-07	1.06E-05
miR-BART19-5p	1.98	1.27	7.03E-08	5.87E-07	4.07	2.61	3.33E-15	8.20E-14
miR-BART5	3.45	9.14	1.88E-07	1.51E-06	5.26	3.46	4.78E-27	1.54E-25
miR-BART1-5p	1.19	0.76	1.13E-06	8.34E-06	4.83	3.20	3.42E-23	1.04E-21
miR-BART8	10.73	8.05	2.22E-06	1.49E-05	14.46	10.09	2.36E-11	4.36E-10
miR-BART2-3p	0.77	0.49	5.83E-06	3.68E-05	1.82	1.22	2.48E-10	3.88E-09
miR-BART10	3.42	9.19	8.72E-05	4.33E-04	6.87	10.40	1.12E-03	7.34E-03
miR-BART13	0.74	0.47	1.49E-04	7.00E-04	2.09	1.35	1.94E-11	3.85E-10
miR-BART12	0.36	0.23	3.10E-03	9.94E-03	5.79	3.84	1.26E-29	4.34E-28
miR-BART20-3p	0.31	0.20	3.29E-03	1.04E-02	0.72	0.46	1.04E-04	9.62E-04
miR-BART11-3p	0.36	0.23	7.83E-03	2.17E-02	0.53	0.34	9.85E-04	6.65E-03
miR-BART21-5p	0.23	0.14	1.55E-02	3.90E-02	2.20	1.42	2.09E-11	4.01E-10
Other Viruses								
hsv1-miR-H8	4.11	2.63	1.19E-23	2.51E-22	1.54	6.75	8.37E-04	5.93E-03
hcmv-miR-UL22A	2.79	1.79	2.30E-14	3.18E-13	0.26	0.21	3.97E-03	2.01E-02

3.4.2 Generation and validation of miRNA signature associated with risk of distant metastasis

Using the Lasso method to fit a penalized CoxPH model to miRNA expression data from the Training cohort yielded 33 variables (miRNAs) with non-zero coefficients (Table 3-3). The 4 miRNAs most strongly associated (greatest absolute coefficient values) with risk of DM were combined with their coefficients within the penalized model to yield the following equation:

$$\text{Risk Score} = \text{miR-154-5p}_{\text{Expression}} * 0.417 + \text{miR-449b-5p}_{\text{Expression}} * 0.280 \\ - \text{miR-140-5p}_{\text{Expression}} * 0.653 - \text{miR-34c-5p}_{\text{Expression}} * 0.311$$

The RS was calculated for each patient in the Training cohort, allowing the patients to be dichotomized into either a “low risk” (<median), or a “high risk” ($\geq$ median) group. A highly significant relationship was observed between the RS and the likelihood of DM whether the RS was treated as a continuous (Wald test; HR=2.76; $p=2.8 \times 10^{-5}$), or a binary (log-rank test: HR=8.25; $p=8.0 \times 10^{-4}$) variable (Fig 3-1A).

When the same miRNA signature equation was applied to the independent Validation cohort of 121 NPC patients diagnosed approximately a decade earlier, a similar relationship was observed (Fig 3-1B). When treated as a continuous predictor, the HR was 1.7 with a Wald test p -value=0.05. When dichotomized by the median from the training cohort, the HR was 3.2, with a log-rank test p -value of 0.014. These results indicate that there might be a greater benefit to utilizing this RS as a binary variable, with an established cut-point, as opposed to a continuous spectrum of risk.

When this 4-miRNA signature was examined further in miRNA-Seq expression data generated by TCGA, there was a significant difference observed in disease-specific survival for patients with a “high risk” score compared to those with a “low risk” score (HR=1.8; $p=0.02$; Fig 3-2), despite the fact that none of these samples were NPCs with the majority derived from the oral cavity or larynx.

Table 3-3. MiRNAs with non-zero coefficients in the penalized regression model fitted to miRNA expression data from the training set.

miRNA	Penalized Coefficient
hsa-miR-140-5p	-0.65265805
hsa-miR-154	0.416841936
hsa-miR-34c-5p	-0.311288747
hsa-miR-449b	0.27970981
hsa-miR-136	0.278252522
hsa-miR-1246	0.252954521
hsa-miR-31	0.246736327
hsa-miR-633	-0.232982777
hsa-miR-375	0.227348203
hsa-miR-646	-0.187297587
hsa-miR-188-5p	-0.184079758
hsa-miR-574-3p	0.167259093
hsa-miR-542-3p	-0.165407907
hsa-miR-1	0.153553988
hsa-miR-561	-0.152880207
hsa-miR-580	-0.147656138
hsa-miR-155	-0.145379233
hsa-miR-34c-3p	0.114592858
hsa-miR-196a	-0.099335183
hsa-miR-296-5p	0.094110203
hsa-miR-107	0.093446126
hsa-miR-663b	0.074681673
hsa-miR-600	0.063613683
hsa-miR-142-5p	-0.060944673
hsa-miR-132	-0.056635448
ebv-miR-BART14	0.048490936
hsa-miR-362-5p	0.043422478
hsa-miR-377	0.04287268
hsa-miR-934	-0.036495034
hsa-miR-144	0.034527264
hsa-miR-188-3p	-0.033462301
hsa-miR-93	0.007480249
hsa-miR-579	-0.003676771

Figure 3-1. Kaplan-Meier curves showing NPC patients dichotomized based on risk score
A) Training cohort; and B) Validation cohort. “High Risk” is defined as a RS $\geq$ the median in the training cohort, and “Low Risk” is defined as a RS $<$ the median in the training cohort. RS; Risk Score, HR; Hazard Ratio, CI; Confidence Interval.

Figure 3-2. Kaplan-Meier curves demonstrating the association Disease Specific Survival the 4-miRNA signature applied to HNSCC miRNA-Seq Data generated by TCGA.

“High Risk” is defined as a $RS \geq$ the median, and “Low Risk” is defined as a $RS <$ the median. RS; Risk Score, HR; Hazard Ratio, CI; Confidence Interval.

3.4.3 Multivariate Analysis

Table 3-4 demonstrates that after controlling for clinical factors such as stage, age, gender, and treatment, the miRNA signature RS remained significantly associated with risk of DM (HR=4.0; $p=7.1 \times 10^{-4}$). Nodal stage was the only other factor significantly associated with DM on both univariate and multivariate analyses (Table 3-2). For this reason, the 4-miRNA signature RS was then combined with N-stage to define five risk groups, based on the visual inspection of the DMFS Kaplan-Meier curves stratified by N-stage (Fig 3-3). Figure 3-4A demonstrates that when patients were stratified into these five distinct groups, the patients in the N1/N2 Group and the N3 Group were further dichotomized by the miRNA RS, thus depicting the ability of this 4-miRNA signature to provide improved risk prediction for DM in these clinically intermediate and high-risk groups. This improved prognostic ability was further illustrated when ROC AUCs were calculated over time and compared amongst clinical factors, the original 4-miRNA RS, and the 5-group N-Stage/miRNA risk-stratification (Fig 3-4B). The area under the ROC curves was consistently greatest in this 5-group stratification (save for year-1, where the 4-miRNA signature alone marginally outperformed the model including N-stage). C-statistics for each CoxPH model were also calculated (Table 3-5), further demonstrating the significantly greater prognostic value for this combined parameter (c-statistic = 0.78), compared to all other models.

Table 3-4. Univariate and multivariate CoxPH analysis of clinical factors and miRNA-signature Risk-Score in the combined dataset from both the Training and Validation cohorts (n=242).

	Univariate		Multivariate	
	HR (95% CI)	p-value	HR (95% CI)	p-value
MiRNA RS (High Risk vs. Low Risk)	4.5 (2.04-9.90)	1.9×10^{-4}	4.0 (1.8-8.9)	7.3×10^{-4}
T stage (T1&2 vs. T3&4)	1.7 (0.86-3.3)	0.13	1.5 (0.70-3.2)	0.30
N stage (N0&1 vs. N2&3)	2.5 (1.2-5.0)	8.0×10^{-3}	2.7 (1.2-5.7)	0.01
Age	1.0 (0.98-1.0)	0.86	1.0 (0.98-1.0)	0.93
Gender (Female vs. Male)	1.31 (0.60-2.9)	0.50	1.3 (0.57-2.9)	0.53
Chemotherapy (- vs. +)	0.97 (0.5-1.9)	0.94	0.46 (0.21-1.0)	0.05

Figure 3-3. Kaplan-Meier curves demonstrating cumulative incidence of distant metastasis based on clinical Nodal-stage in the combined dataset (n=245).

Figure 3-4. Comparison of 4-miRNA signature with clinical variables.

A) DM Kaplan-Meier curve showing patients assigned to risk groups based on the combined N-stage and 4-miRNA signature Risk Score. **B)** ROC AUCs over time demonstrating the ability of various clinical factors and the 4-miRNA signature RS to predict DM in NPC patients. DM; distant metastasis, ROC; receiver operating characteristic, AUC; Area Under the Curve, RS; Risk Score.

Table 3-5. C-statistics for comparison of various CoxPH models' ability to predict DMFS.

Covariates included in the model (levels for each variable)	C-Statistic $\pm$ SE
4-miRNA Signature (High Risk/Low Risk), N-stage (N0&1/N2&3)	0.78 $\pm$ 0.048
5-miRNA Liu et al. Signature (High Risk/Low Risk), N-stage (N0&1/N2&3)	0.74 $\pm$ 0.048
4-miRNA Signature (High Risk/Low Risk)	0.71 $\pm$ 0.050
TNM-stage (I/II/III/IV)	0.67 $\pm$ 0.049
N-stage (N0&1/N2&3)	0.62 $\pm$ 0.043
5-miRNA Liu et al. Signature (High Risk/Low Risk)	0.60 $\pm$ 0.044
T-Stage (T1&2/T3&4)	0.55 $\pm$ 0.043

Figure 3-5 demonstrates the ability of this 4-miRNA signature to predict risk of DM in advanced stage (III & IV) patients who were treated with RT alone (HR=7.13; p=0.003; Fig 3-5A), as well as those who were treated with CRT (HR=3.35; p=0.045; Fig 3-5B). These data suggest that even in NPC patients with locally-advanced disease, this 4-miRNA signature could potentially identify a low-risk group of patients, whose likelihood of developing DM at 5-years was so low (<10%), that adding CT to their RT might have no benefit to their survival (Fig 3-5A). Conversely, there remained a high-risk group, whose outcome could be potentially improved with the administration of combined CRT, since RT alone was associated with a 5-year risk of 45% in developing DM (Fig 3-5A), vs. 20% when treated with CRT (Fig 3-5B).

Figure 3-5. Kaplan-Meier curves showing DM in NPC patients dichotomized based on miRNA risk score in advanced stage patients (Stage III/IV).

Patients treated with **A)** RT alone; and **B)** combined CRT. “High Risk” is defined as a RS $\geq$ the median in the training set, and “Low Risk” is defined as a RS $<$ the median in the training set. RT; radiotherapy, CRT; chemoradiotherapy, RS; Risk Score, HR; Hazard Ratio, CI; Confidence Interval.

3.4.4 Validated miRNA Target Pathway Enrichment Analysis

When the miRWalk database was queried, 292 miR-target relationships were identified for the 4 miRNAs in the prognostic signature. When pathway enrichment analysis was performed, several biological pathways were observed to be significantly enriched (corrected p-value <0.05) within the union of validated targets for all 4 miRNAs (Table 3-6). The “Cell Cycle” KEGG pathway, and the “Cell Cycle: G1/S Check Point” Biocarta pathways were the first and fourth most significantly enriched pathways, respectively. These were also the only two pathways identified to be significantly enriched in the targets for 3 of the 4 miRNAs (miR-449b-5p, miR-34c-5p, miR-140-5p) when individual pathway analysis was also performed, suggesting that the underlying biological processes of cell cycling or cellular proliferation may be mediating the clinical observations.

Table 3-6. Pathways observed to be significantly enriched within validated targets of the four miRNAs in the prognostic signature using DAVID (v6.7).

Database	Term	Count	Fold Enrichment	Raw p-value	Corrected p-value
KEGG_PATHWAY	Cell cycle	22	4.23	3.97E-09	4.44E-07
KEGG_PATHWAY	Melanoma	18	4.38	1.02E-07	5.72E-06
KEGG_PATHWAY	Pathways in cancer	38	2.3	1.69E-07	6.30E-06
BIOCARTA	Cell Cycle: G1/S Check Point	15	5.09	3.76E-08	6.95E-06
KEGG_PATHWAY	Glioma	16	4.39	6.83E-07	1.91E-05
KEGG_PATHWAY	Pancreatic cancer	17	3.91	1.52E-06	3.40E-05
KEGG_PATHWAY	Chronic myeloid leukemia	17	3.71	3.29E-06	6.13E-05
KEGG_PATHWAY	Bladder cancer	13	4.79	4.26E-06	6.82E-05
KEGG_PATHWAY	Colorectal cancer	17	3.53	6.73E-06	9.43E-05
KEGG_PATHWAY	Prostate cancer	18	3.31	8.27E-06	1.03E-04
BIOCARTA	p53 Signaling Pathway	11	5.27	3.92E-06	3.63E-04
KEGG_PATHWAY	p53 signaling pathway	13	3.72	7.61E-05	8.52E-04
BIOCARTA	ATM Signaling Pathway	10	5.09	2.11E-05	1.30E-03
PANTHER_PATHWAY	p53 pathway	16	3.07	5.75E-05	1.70E-03
PANTHER_PATHWAY	p53 pathway feedback loops 2	13	3.92	3.02E-05	1.78E-03
KEGG_PATHWAY	Non-small cell lung cancer	12	3.6	2.34E-04	2.38E-03
KEGG_PATHWAY	Small cell lung cancer	14	3.11	2.62E-04	2.44E-03
BIOCARTA	Cyclins and Cell Cycle Regulation	10	4.52	7.27E-05	3.36E-03
KEGG_PATHWAY	Focal adhesion	19	2.35	5.42E-04	4.66E-03
BIOCARTA	Role of BRCA1, BRCA2 and ATR in Cancer Susceptibility	7	5.18	7.49E-04	2.73E-02
KEGG_PATHWAY	Notch signaling pathway	7	4.3	3.74E-03	2.95E-02
KEGG_PATHWAY	Thyroid cancer	7	4.3	3.74E-03	2.95E-02
BIOCARTA	Telomeres, Telomerase, Cellular Aging, and Immortality	7	4.75	1.35E-03	4.09E-02

3.5 Discussion

Given the continued challenge of DM in NPC patients, the validated prognostic 4-miRNA signature presented herein could prove to be a valuable tool in guiding treatment decisions for patients. To date, there are no clinically utilized prognostic biomarkers in NPC; plasma levels of EBV DNA titres have certainly been associated with clinical outcome, particularly in predicting risk of relapse when the titre remained elevated, or increased post-treatment^{128,300}. However, this has not contributed to treatment decisions, which to date, have relied on TNM staging, with administration of combined CRT for patients with locally-advanced NPC. However, such CRT regimens are also associated with significant acute and long-term morbidity, and even mortality, diluting any potential gains with these aggressive treatments³¹⁸⁻³²⁰. Hence, the utility of this 4-miRNA signature, which has been validated in an independent cohort (Fig 3-1) and was demonstrated to add to the prognostic value of the TNM staging category (Fig 3-3), is extremely promising. Furthermore, the apparent ability of this 4-miRNA signature to identify a low-risk group of patients with locally-advanced NPC, who could be cured with RT alone (Fig 3-5), suggests a potential predictive value of this signature, which to date, has never been previously documented. However, given the inherent risk of selection bias that comes with a retrospective study such as this, in order to confirm the ability of this signature to predict treatment success for patients treated with RT alone, further verification in an additional independent cohort of randomized controlled trial (RCT) participants randomly designated to RT alone *vs.* CRT arms would be needed. This would potentially make it possible to de-escalate treatment for patients with truly a favourable prognosis, thus sparing them the increased toxicity of combined CRT. In contrast, patients at high-risk will have a 60% risk of DM at 5-years, despite CRT; for this group of patients, the

need to identify and evaluate novel therapeutics cannot be over-emphasized. Indeed, identifying such targetable pathways may be achieved by probing the underlying biology of these miRNA signatures.

There exists another validated prognostic 5-miRNA signature set for NPC, generated by a group from Guangdong, which was associated with overall, disease-free, and distant metastasis-free survival¹⁸³. We have evaluated their 5-miRNA signature, compared to either our 4-miRNA signature alone, or each combined with N-stage, and our signatures appeared to demonstrate a more robust performance, based on direct comparisons and a ROC curve analysis (Fig 3-6, and Table 3-6). Specifically, comparing the HR of our 4-miRNA signature to the HR of the 5-miRNA signature for DM (Fig 3-7A vs. B), our current 4-miRNA signature appeared to perform better, with a higher HR and greater statistical significance. Similarly, the greatest AUC in the ROC analysis was observed with our 4-miRNA signature combined with N-stage (Fig 3-7C). Of note, there is a complete lack of overlap between their 5-miRNAs and our 4-miRNAs. This is not surprising, given the differences in platforms (in-house vs. Nanostring nCounter), different population cohorts, and redundancy in biological processes; this phenomenon has been similarly observed with other miRNA signatures^{321,322}, as well as mRNA signatures³²³. Future analysis on additional independent datasets would be required to definitively determine the optimal signature; as well as the potential importance of geographical and population differences.

Figure 3-6. Kaplan-Meier curves showing local-regional relapse (LRR) in NPC patients dichotomized based on miRNA RS in advanced stage patients (Stage III/IV).

Patients treated with A) RT alone and B) combined CRT. “High Risk” is defined as $RS \geq$ the median in the training set, and “Low Risk” is defined as a $RS <$ the median in the training set. RT; radiotherapy, CRT; chemoradiotherapy, HR; Hazard Ratio, CI; Confidence Interval.

Figure 3-7. Comparisons of the current 4-miRNA signature vs. the 5-miRNA signature reported by Liu et al.³⁴

(A&B) Kaplan-Meier curves showing distant relapse in the combined data set including both the training and validation cohorts (n=246). Patients were dichotomized by A) the 4-miRNA signature described in the current study or B) the 5-miRNA signature described by Liu et al.³⁴. “High Risk” is defined as a RS $\geq$ the median in the training set, and “Low Risk” is defined as a RS < the median in the training set. C) ROC AUCs across multiple time points showing the ability of the 4-miRNA signature described herein, the 5-miRNA signature reported by

Liu et al.³¹ and N-stage alone or combined with either signature to predict DM in NPC patients. These data demonstrate that the model including N-stage and the 4-miRNA signature is the most robust prognostic signature. HR; Hazard Ratio, CI; Confidence Interval, N; Nodal, ROC; receiver operating characteristic. AUC; Area under the curve, RS; Risk Score.

Preliminary pathway enrichment analysis suggests a role in cell cycle regulation for the 4 miRNAs in this signature set. MiR-34c-5p, miR-140-5p, miR-154-5p and miR-449b-5p have all been previously reported to target important members of the cell cycle control/G1-S checkpoint pathway³²⁴⁻³²⁷. Given the relationship to DM reported in this study, one could potentially hypothesize that since both chemotherapy and RT primarily target actively dividing cells^{328,329}; the “high-risk” tumours might be rendered more resistant by exhibiting a quiescent cellular phenotype. However, this is merely a postulate, which would require extensive pre-clinical investigations before any conclusions could be drawn regarding the underlying biological basis of these miRNAs in promoting metastasis in NPC.

Despite the numerous publications purporting the identification of prognostic miRNA signatures (reviewed by Nair et al¹⁷⁷); none to date has been utilized in the clinic. We are hopeful that this 4-miRNA signature set will be verified and implemented, based on several strengths of this current NPC study: a) reasonable sample size (both Training and Validation cohorts were in excess of 100 patients); b) uniformity of treatment at a single institution; c) identical DMFS in both cohorts despite the difference in time period and increased use of chemotherapy in the Training cohort; d) a single experimentalist to ensure technical consistency; e) the same type of tissues (both cohorts were FFPE blocks); and finally, f) consistency of the miRNA profiling platform. Indeed, if this 4-miRNA signature can be verified in yet a third independent RCT cohort, this signature could be theoretically readily translatable, given that FFPE processing is broadly utilized around the world.

In conclusion, we have successfully validated a 4-miRNA signature that can prognosticate for DM in patients with NPC. This signature adds to the prognostic value of the current “gold standard” of the TNM staging categories, and there is a suggestion of its potential

predictive role in selecting NPC patients for de-escalating treatment to RT alone, despite locally-advanced disease at presentation. Further interrogation of the underlying biological pathways such as cell cycle and proliferation could render the selection of patients who might be sensitive to HDAC or EGFR inhibitors. Such novel molecular targeted agents have been demonstrated to have promising efficacy in the clinic^{10,330,331}, as well as in pre-clinical models³³²; the utilization of a prognostic miRNA signature would enrich for potentially sensitive patients, thereby improving clinical outcome for future patients with NPC.

Chapter 4

Genomic characterization of NPC

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4. Genomic characterization of NPC

4.1 Introduction

In addition to the pivotal role of EBV infection in NPC tumourigenesis, a number of genetic alterations have also been established as contributors to NPC development and progression. Early genetic analysis of NPC samples focused on gross chromosomal aberrations interrogated through low-resolution genome-wide methods such as PCR²², comparative genomic hybridization (CGH)²⁶, and more recently, the higher resolution array CGH (aCGH)⁴³. These studies have identified several recurrently amplified and deleted regions, which have provided valuable insight into the genetic basis of NPC (reviewed in chapter 1). However, the quality and quantity requirements of the genomic DNA required for such techniques has limited the sample size and clinical follow-up data available for such studies³³³.

Recent advancements in molecular inversion probe (MIP) array technologies have made interrogating a large number of single nucleotide polymorphisms (SNPs) relatively simple and inexpensive. In addition to sequence information, intensity values for SNPs tiled across the genome can also be used to generate genome-wide copy-number data³³⁴. Moreover, specific methodologies have been developed to enable the generation of high-quality copy-number data from relatively small amounts of genomic DNA (<20 ng in some cases) extracted from formalin-fixed paraffin embedded (FFPE) samples^{335,336}. This revived usability of routinely collected FFPE biopsy samples for copy-number analysis immediately broadens the number of available specimens, especially in the case of a disease such as NPC which is very rarely treated with surgery. Given the specific challenges associated with NPC FFPE biopsy specimens, which are often small in size, with significant lymphocytic infiltration, a pilot study was

undertaken to test the feasibility of analyzing these samples using a SNP array specifically designed by Affymetrix for use with DNA extracted from FFPE specimens (OncoScanTM).

4.2 Material and Methods

4.2.1 Patients and Samples

FFPE diagnostic blocks from 15 patients diagnosed with NPC between the years of 2002-2008 and treated at Princess Margaret Cancer Center (PM) were collected with approval from the institutional research ethics board (REB). Clinical and follow-up data were collected through chart review; summarized in Table 4-1 for these 15 NPC samples. In addition, 7 FFPE tonsillar specimens with no evidence of malignancy that were archived during the same time period were utilized as reference controls for down stream copy-number calculations.

Table 4-1. Clinical characteristics of 15 NPC patients analyzed using OncoScan platform

Age (years)	
median	52
range	17-63
Frequency (%)	
Gender	
Male	11 (73)
Female	4 (27)
T stage	
T1	4 (27)
T2	5 (33)
T3	4 (27)
T4	2 (13)
N stage	
N0	2 (13)
N1	2 (13)
N2	8 (53)
N3	3 (20)
TNM Stage	
I (%)	2(13)
II (%)	2 (13)
III (%)	6 (40)
IV (%)	5 (33)
Unable to evaluate	none
Treatment	
Radiation only	4 (27)
Radiation + chemo	11 (73)
5-year %	
Survival	
Overall	80%
Disease-Free	79%
Local Relapse-Free	91%
Nodal Relapse-Free	100%
Distant Relapse-Free	87%

4.2.2 DNA extraction

Samples were macro-dissected to ensure that at least 70% of the material analyzed was malignant epithelium. Genomic DNA was extracted from eight 4- μ m sections using the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Ambion) according to the manufacturers instructions. DNA was quantified using the Quant-iT™ PicoGreen® dsDNA Assay and 20ng of DNA was diluted with water into 25 μ L volume and sent to Affymetrix for processing.

4.2.3 SNP Microarray Analysis

DNA from each sample was analyzed by the Affymetrix Research Service Laboratory (ARSL; Santa Clara, CA, USA) using the OncoScan™ FFPE Express 2.0 Service. The MIP based assay employed includes ~335,000 markers for copy number and allele frequency measurement, and 541 markers for somatic mutations frequently observed in cancer³³⁶. Samples were prepared and analyzed according to manufacture's instructions. Briefly, following overnight probe annealing to template DNA, gaps in hybridized probes are filled by a DNA polymerase reaction in the presence of dNTPs, followed by ligation, removal of un-ligated probes, linearization of circularized probes, PCR amplification, HaeIII digestion, and denaturing. The fragmented, denatured PCR product is then hybridized to OncoScan microarrays overnight, followed by washing, staining, and finally scanning of the arrays. Output .CEL files from the Affymetrix GeneChip® Scanner are then converted to one Nexus (Biodiscovery) compatible .cbzip file for downstream analysis.

4.2.4 Quality control

Several quality control (QC) metrics were used to determine which samples were likely to provide high quality data. First, sample concentration was re-analyzed on site by the ARSL and all 15 NPC samples were found to have >20ng available DNA by PicoGreen. Second, samples are run on 3% agarose gels from both the first PCR reaction and the second PCR reaction/HaeIII digestions to ensure high quality products at the correct size. The array intensity values themselves are then assessed for noise using the Median Absolute Pairwise Difference (MAPD) metric. The MAPD algorithm calculates the $\log_2$ distance between neighbors for all pairs of probes and then the median is taken across all samples. This represents the typical difference between marker pairs with respect to $\log_2$ ratios. The higher the MAPD, the greater the noise between the array of interest and the reference set. Samples with a median MAPD value > 0.6 are excluded. Finally, Gender is inferred from the ratio of signal from Y chromosome markers to signal from X chromosome markers. This provides some degree of sample tracking as well as further evidence of useable data quality.

4.2.5 Copy-Number Calculation

All copy-number analysis was performed using Nexus for Affymetrix (Biodiscovery). In order to calculate copy-number values from SNP chip intensities, Nexus first calculates $\log_2$ ratios for each probe in experimental samples compared to the mean of the same probe in control samples. Ratios are then corrected for GC content and fragment length using the quadratic correction method in Nexus. Regions with copy-number alterations are then identified using a Hidden Markov Model (HMM) based approach called SNP-FASST2 (Fast Adaptive States Segmentation Technique 2) with the following parameter settings: significance threshold = 5×10^{-7} , max contiguous probe spacing = 1000kb, min number of probes per

segment = 3, copy-number gain threshold = 0.3, “high” copy-number gain threshold = 1.2, copy-number loss threshold = -0.3, homozygous copy-number loss threshold = -1.2.

In order to identify regions significantly altered within the set of NPC samples, the global frequency statistic approach of the Significance Testing for Aberrant Copy number (STAC) method³³⁷ was employed with a significance cut off of $p=0.05$ and a frequency cut-off of $\geq 40\%$ (i.e. at least 6/15 samples).

4.2.6 Sequence Variant Identification

Scores were calculated for each somatic mutation assay by taking the $\log_2$ ratio for the mutant vs. the reference allele. Scores above 9 were considered valid somatic mutations as per the manufacturer’s instructions.

4.3 Results

4.3.1 Quality Control

All NPC samples passed each step of QC, however only 2 of 7 reference (normal tonsillar) samples passed all QC steps. As a result, an additional frozen sample was added to the analysis by the ARSL to increase the number of reference samples to 3. The gender was also correctly identified for each sample submitted.

4.3.2 Copy-Number Events

With a STAC p-value cut-off of 0.05 and a recurrence cut-off of 40%, a total of 16 regions were found to be significantly amplified or deleted in this small cohort of 15 NPC samples (Figure 4-1, Table 4-2). These significantly altered regions cover 203 genes, including 59 miRNAs. (Table 4-2)

Figure 4-1. Visual representation of genome-wide frequency of CNVs across 15 NPC samples.

Table 4-2. Genomic regions with significant ($p < 0.05$) CNV events.

	Copy-Number Gain Events				
	Region	Cytoband	Frequency (%)	P-Value	Genes in region
a.	chr2:68,600,507-68,664,870	p13.3	6 (40)	<0.001	PLEK
b.	chr6:26,145,344-26,292,109	p22.2	6 (40)	<0.001	19 members of Histone cluster 1 (HIST1H)
c.	chr10:90,744,235-90,771,558	q23.31	6 (40)	<0.001	ACTA2, FAS-AS1, FAS
d.	chr11:65,220,293-65,440,529	q13.1	6 (40)	<0.001	MIR548AR, MIR4690, MIR4489, MALAT1, SCYL1, LTBP3, LOC254100, SSSCA1, FAM89B, EHBP1L1, KCNK7, MAP3K11, PCNXL3, SIPA1, RELA
e.	chr11:69,241,838-69,466,493	q13.3	6 (40)	<0.001	CCND1
f.	chr18:60,812,527-60,911,023	q21.33	6 (40)	<0.001	BCL2
g.	chr3:172,038,603-172,267,351	q26.31	9 (60)	0.001	FNDC3B, GHSR, TNFSF10
h.	chr12:6,319,912-6,453,021	p13.31	7 (47)	0.004	CD9, PLEKHG6, TNFRSF1A
i.	chr12:6,497,742-6,584,837	p13.31	7 (47)	0.004	LTBR, CD27-AS1, CD27, TAPBPL, VAMP1
j.	chr1:160,375,767-161,044,446	q23.2 - q23.3	8 (53)	0.014	VANGL2, SLAMF6, CD84, SLAMF1, CD48, SLAMF7, LY9, CD244, ITLN1, ITLN2, F11R, TSTD1, USF1, ARHGAP30, PVRL4

Table 4-2. Continued

Copy-Number Loss Events					
	Region	Cytoband	Frequency (%)	P-Value	Genes in region
k.	chr3:54,486,627-54,653,831	p14.3	8 (53)	0.001	CACNA2D3
l.	chr16:86,067,066-87,094,764	q24.1	6 (40)	0.029	LOC146513, LOC732275, FOXF1-AS1, FOXF1, MTHFSD, FLJ30679, FOXC2, FOXL1
m.	chr14:96,137,364-96,360,975	q32.13 - q32.2	7 (47)	0.042	TCL6, TCL1B, TCL1A, LINC00617
n.	chr14:99,749,669-100,328,304	q32.2	7 (47)	0.042	SETD3, CCNK, CCDC85C, HHIPL1, MIR5698, CYP46A1, EML1
o.	chr14:101,101,661-102,179,241	q32.2 - q32.31	7 (47)	0.042	54 miRNAs (including miR-154), 38 small nucleolar RNAs, DIO3, DIO3OS, DLK1, LINC00523, MEG3, MEG8, MEG9, RTL1
p.	chr14:104,532,592-105,597,051	q32.33	7 (47)	0.042	ASPG, MIR203, MIR3545, KIF26A, C14orf180, TMEM179, MIR4710, INF2, ADSSL1, SIVA1, AKT1, ZBTB42, LINC00638, CEP170B, PLD4, AHNAK2, C14orf79, CDCA4, GPR132

4.3.3 Sequence Variation Events

Of the 541 known somatic mutations in cancer assayed, only 2 met the rough somatic mutation score cut-off of 9 in at least one NPC sample. The first, a synonymous variant at residue 561 of fms-related tyrosine kinase 3 (FLT3), was present in 4 of 15 NPC samples. The second, a missense mutation in Ataxia telangiectasia mutated (ATM) resulting in a phenylalanine to leucine amino acid change at residue 858, was present in only one sample.

4.4 Discussion and Conclusions

These data provide us with evidence supporting the feasibility of using the OncoScan (V2) SNP array through Affymetrix's FFPE Express 2.0 Service for copy-number analysis of small FFPE NPC biopsies. However, the application of this technology for identification of sequence variants is less convincing. First of all, only two "somatic mutations" were identified, providing limited evidence either for or against the validity of the OncoScan assay for somatic variant identification. It could be the case that of the 541 mutations included in the assay, only these two were within our 15-sample set, or it could be that the probes were insufficient to detect mutations that were indeed present. Further verification using an alternate technique would be required to fully address this issue.

Of the two sequence variants identified, one was recurrent. This variant was a FLT3 A>G transition and was present in 4 of the 15 NPC samples. However, there are three reasons why this variant is unlikely to be of major interest biologically. First, it is synonymous with the reference allele, and therefore unlikely to have a functional impact on the translated protein. Second, this particular variant corresponds with a known polymorphic locus (dbSNP ID: rs34374211) with a global minor allele frequency (MAF) of 12%, and a 16% frequency in

Asian populations; not far from the frequency observed herein (4/15, 27%). Finally, although this variant is included in the catalogue of somatic mutations in cancer (COSMIC), neither of the two studies reporting this variant confirm it is indeed somatic^{338,339}. Given these facts, it is likely that this variant is a germline polymorphism, and not related to tumourigenesis in these patients. The other detected sequence variant was a phenylalanine to leucine amino acid change at residue 858 in ATM. This variant also corresponds with a polymorphic locus (dbSNP ID: rs1800056), and while the MAF (0.01%) is considerably lower than that of the FLT3 mutation, it is similar to the frequency of detection in the current dataset (1/15, 0.07%). In addition, like the FLT3 mutation, neither of the reports cited in this variant's COSMIC entry verify that it is indeed somatic^{340,341}. Thus, the inclusion of these probes in the OncoScan assay is likely due to shortcomings in the selection process for these purported "somatic mutation assays".

The somatic mutation assay shortcomings notwithstanding; this pilot study did yield some interesting observations from copy-number analysis. In addition to corroborating copy-number changes previously found to be recurrent in the NPC genome, several novel amplified and deleted regions were also identified. Indeed, gains in chromosomes 1q, 3q, and 12p previously discovered by several groups^{26,27,342} were identified in this pilot study. As well as losses in chromosome 3p, 16q^{22,342}, and 14q; which has been previously reported to be lost in up to 85% of NPC patients²². Two of the most significant and most focal recurrently amplified regions identified were located at 11q13.3 and 18q21.33, which encode Cyclin D1 (CCND1) and B-Cell CLL/Lymphoma 2 (BCL-2), respectively. Both CCND1 and BCL-2 are well characterized for their roles in the tumourigenesis of NPC; particularly through promotion of cell growth and survival³³³. While CCND1 is also an established target of chromosomal

amplification in NPC⁴³, the mechanism of BCL-2 overexpression is not well understood in this disease³⁴³. The most likely reason for this is a lack in resolution of copy-number strategies previously employed in the analysis of NPC. The majority of early cytogenetic studies in NPC have used relatively crude methods such as Giemsa- (G) banding¹⁹ qPCR of microsatellite loci, or the higher-resolution aCGH methods. Routine resolution of these techniques ranges from ~3-10 Mb (G-banding) to ~1Mb (aCGH); although modern aCGH platforms have been reported to detection alterations as small as 15kb³⁴⁴. By contrast, modern SNP arrays can reach a resolution as low as 700bp/probe³⁴⁵. Moreover, the particular array utilized for this study provides increased coverage of ~200 known cancer genes to increase resolution in these areas³³⁶. In the case of BCL-2, of the 6 samples with an increase in copy-number detected, 5 harboured amplified regions less than 400kb in length (Figure 4-2). These small events would be impossible to detect using G-band and early CGH techniques, and extremely difficult even on the most dense aCGH chips, which could explain the lack of previous evidence for recurrent amplification of this region in NPC, despite the reproducible detection of over-expressed BCL-2 in NPC^{157,343,346}.

In conclusion, this study supports the use of MIP-based SNP array techniques, and in particular the OncoScan platform from Affymetrix, to examine genome-wide copy-number changes using small FFPE NPC biopsy samples. This pilot study also validated a number of previously identified recurrent copy-number alterations in NPC, and generated interesting hypotheses for future testing.

Figure 4-2. Representative sample showing a small (318kb) amplification at Chr18q21.33. Shaded region indicate segments with a median Log₂ratio above the threshold (0.3) for copy number gain.

Chapter 5

General Discussion, Future Directions, and Conclusions

5. General Discussion, Future Directions, and Conclusions

5.1 Introduction

In current clinical practice, the majority of patients who present with NPC are staged using AJCC recommended criteria, and treated with curative RT, with concomitant chemotherapy (5-FU plus a platinum-based compound) in cases of higher stage disease. Advances in radiation delivery methods (i.e. IMRT), and increased adoption of CRT for treatment of NPC patients have resulted in a significant improvement in long-term local control, with 5-year locoregional relapse-free survival rates in excess of 90%. However, despite these improvements, distant metastasis remains a major cause of death for NPC patients, with up to 17% of advanced-stage NPC patients experiencing a distant relapse event within 5 years²⁵³. As a result, distant metastasis is the major cause of NPC related mortality in the current era, underscoring the need for better identification of patients at risk for metastatic disease, as well as developing innovative treatment strategies. Thus, a major focus of this thesis is to address these challenges.

This chapter will discuss the findings expounded in this thesis, put forth corroborating and enhancing observations, as well as outline future studies that will be required to propel these discoveries into clinical use to the ultimate benefit of future NPC patients.

5.2 General Discussion

5.2.1 The role of microRNA-375 and metadherin in NPC

Chapter 2 described our identification of MTDH as an important target of miR-375 in the context of NPC as well as HNSCC. Our study demonstrated dysregulation of both miR-375 and MTDH in primary NPC and HNSCC specimens, as well as the functional impact of these

alterations in HNC models, including one potential mechanism *via* activation of the PI3K-Akt pathway. We also identified a potential prognostic role for MTDH in NPC wherein over-expression of MTDH mRNA was associated with an increased risk of distant relapse. The validity of our report has been subsequently corroborated to varying degrees in ensuing studies performed by others, which will be reviewed below.

5.2.1.1 Scientific impact and subsequent research

Since the publication of our study “Significance of dysregulated metadherin and microRNA-375 in head and neck cancer” in October 2011³¹⁵, it has been cited in 23 articles, including both reports of original research and scientific reviews. To the best of our knowledge, our study was the first to report a role for MTDH in NPC, and the second, preceded only by ~3 months²⁷⁵, in head and neck cancer (HNC). Intriguingly, simultaneous to our own study, Nohata *et al*²⁷⁵ also identified MTDH as a target of miR-375. Subsequently, this same relationship has been identified in hepatocellular carcinoma³⁴⁷, breast cancer³⁴⁸, and esophageal squamous cell carcinoma³⁴⁹ models. Moreover, since the publication of our study, miR-375 and MTDH have been corroborated in numerous cancer types as either a tumour suppressor, or an oncogene, in 84 and 94 publications, respectively. Notably, several of these publications also reproduced our own findings in that MTDH promoted a malignant phenotype by activating the PI3K-Akt pathway³⁵⁰⁻³⁵⁴. Furthermore, many studies have corroborated the role of high MTDH expression as an unfavorable prognostic marker in multiple malignancies, including oral³⁵⁵, cervical³⁵⁶, ovarian³⁵⁷, hepatocellular³⁵⁸⁻³⁶⁰, renal cell³⁶¹, colon³⁶², breast³⁶³, bladder³⁶⁴ and non-small cell lung cancer³⁶⁵; all strong indicators that MTDH is likely to be an important mediator of human cancer development and progression.

5.2.2 MiRNA Signature of Distant Metastasis in NPC

In Chapter 3, I described a 4-miRNA signature associated with risk of distant metastasis in NPC. This signature was generated in a training set of 125 NPC samples using a penalized CoxPH method for variable selection and coefficient shrinkage. Using the coefficients obtained from the model, the following equation was derived to calculate a risk score for each patient: **RS = miR-449b*0.28 + miR-154*0.417 – miR-140.5p*0.653 – miR-34c.5p*0.311**. When dichotomized to high (RS $\geq$ median) vs. low (RS $\leq$ median) risk groups, those patients in the high risk group had a significantly increased risk of developing distant metastasis compared to the low risk group (HR=8.25; p=8.0x10⁻⁴). This signature was then validated in the second independent set of 121 NPC patients (HR=3.2; p=0.014). Furthermore, a multivariate analysis demonstrated the strong independent prognostic value of this 4-miRNA signature, which was able to add prognostic value to nodal involvement; the best current predictor of distant metastasis.

5.2.2.1 Additional Analysis

Given that Chapters 2 and 3 both reported on the identification of expression signatures associated with distant metastasis in NPC, I was interested to determine if combining the three variables consistently associated with distant metastasis in NPC (MTDH expression, our 4-miRNA signature, and N-stage) in one model could further improve prognostic ability. When 88 samples with both MTDH and 4-miRNA risk-score data were used, a CoxPH model with MTDH, 4-miRNA signature and N-stage as variables generated the following equation:

$$\text{Risk Score} = (4\text{-miRNA risk group}) \times 5.08 + (\text{MTDH risk group}) \times 6.81 + (\text{N-stage}) \times 1.71$$

Where risk groups were defined as 0 for low risk and 1 for high risk, and N-stage was

numerical 0-3 for N0-N3. Not surprisingly, when samples were dichotomized by median Risk Score, patients in the high-risk group had a significantly higher risk of distant metastasis (HR = 12; $p=0.002$; Figure 5-1F). Most intriguingly, this combined model appeared to perform the best across multiple time points compared to each signature alone or individually combined with N-stage (Figure 5-1G,H). Interpretation of these results is relatively limited due to the small sample size, and relatively few metastasis events (13), therefore further studies should be undertaken in independent cohorts of patients to determine the full utility of this combined signature.

Figure 5-1. Comparison of the prognostic ability of MTDH, the 4-miRNA signature and N-stage; combined and individually.

88 samples from the previously described cohort of NPC patients diagnosed between 1993 and 2000 were used to identify the optimal signature of distant metastasis risk in NPC.

5.2.3 Genomic Characterization of NPC Samples

Although brief, our pilot study to characterize the genome of 15 FFPE NPC biopsy samples provided two major insights: 1) that CNA profiling of small FFPE NPC biopsies is technically feasible using a MIP-based technology and 2) that this technique can be used to confirm known and identify novel CNA events in NPC. To the best of our knowledge, there is no published article to date reporting the use of an MIP assay for CNA profiling in NPC samples. Moreover, the results suggest that the superior resolution of this platform compared with methods previously used for CNA analysis in NPC can provide novel insights into the genomic landscape of NPC. In particular, the observation of frequent amplification of BCL-2 is especially intriguing. While BCL-2 is a well established oncogene which is consistently overexpressed in NPC^{157,343,346}, and chromosomal amplification is a mechanism of Bcl-2 overexpression in B-cell lymphomas³⁶⁶, recurrent amplification of this genomic region has yet to be described in NPC. Indeed, it may well be the increased resolution afforded by this modern technique that enabled the detection of these relatively small (~400kb) regions of amplification, where lower resolution methods had failed. Thus, MIP-based arrays are a feasible and informative method to interrogate archival FFPE NPC biopsy specimens.

5.3 Future Directions

5.3.1 The role of microRNA-375 and metadherin in NPC

5.3.1.1 Clinical Implementation

The findings in our 2011 study have two major potential clinical applications: 1) miR-375, MTDH or downstream/upstream effectors are potential targets of novel therapeutics; and 2) MTDH expression could serve as a prognostic/predictive biomarker in NPC. However, in

order to translate these findings into clinically useful tools, additional investigation must be undertaken.

We and others have put forth strong cases supporting miR-375 as a tumour suppressor, and MTDH as an oncogene in HNC. While our report remains the only study to demonstrate a role for MTDH or miR-375 directly in NPC, the applicability in this disease is perhaps the most compelling given the strong correlation between MTDH expression and distant metastasis. Two obvious options present themselves for translation of these findings into therapeutic approaches: replacement of miR-375 or knock-down/inhibition of MTDH in NPC patients. While much headway has been made towards so-called “gene-therapy” for cancer treatment, ectopic replacement of tumour suppressor genes or RNA-interference mediated knock-down of oncogenes are still in the pre-clinical/early clinical stages of evaluation, and continued advancements in delivery methods will be necessary before such techniques can be applicable in the clinical arena³⁶⁷. As such, small molecule inhibitors of MTDH or downstream signaling pathway members might be more viable options. To date, no small molecule inhibitors of MTDH have been reported, and the development of such chemicals might be difficult due to a lack of traditionally “druggable” features such as a kinase domain or a known ligand^{368,369}. Thus, the current state of the art dictates that inhibition of MTDH signaling pathway members might be the only imminently available approach. Our study demonstrated that MTDH knock-down in NPC cells resulted in a significant reduction in phospho-Akt (Figure 2-11A), and indeed, several pre-clinical studies have shown efficacy of several PI3K, mTOR, and dual inhibitors in this disease³⁷⁰⁻³⁷³. However, given that PI3K is upstream of Akt, MTDH overexpression in NPC could circumvent the inhibition of this receptor *via* alternative phosphorylation of Akt. In contrast, mTOR inhibitors primarily inhibit the mTORC1 complex

which is downstream of Akt³⁷⁴, perhaps making this a more viable candidate for targeted therapies in MTDH over-expressing patients. Thus, MTDH expression could potentially be used as a predictive biomarker for future clinical trials employing PI3K-mTOR-Akt pathway inhibitors.

For MTDH expression to be a clinically useful prognostic or predictive biomarker, an analytical method for detection must first be validated. Our study reported the use of qRT-PCR in RNA extracted from FFPE biopsy samples; however, this technique is not routinely applied in pathology departments, hence would be difficult to implement broadly. MTDH expression measured by IHC staining, a much more ubiquitously utilized diagnostic technique, has been found to be prognostic in cervix³⁵⁶, ovarian³⁵⁷, prostate³⁷⁵, oral³⁵⁵ and breast cancers³⁶³. However, our initial MTDH IHC staining results in NPC samples were skewed towards high intensity staining, suggesting saturation in the signal (Figure 2-5C&D). Consequently, IHC staining did not correlate well with the qRT-PCR data, nor were these values associated with metastasis (data not shown). It is possible that particularly subtle MTDH expression variation in NPC excludes IHC as an appropriate analytical technique in this disease, however it is likely that optimization of the staining protocol could remedy this challenge.

5.3.1.2 Biological Investigation

In addition to overcoming the challenges of implementing our discoveries in the clinic, we are also interested in further elucidating the biological functions of miR-375 and MTDH within NPC cells, which still remains largely unknown.

First, our preliminary data suggests that hypermethylation of the miR-375 genomic region plays a role in its suppression, however the mechanism by which this region is

methyated as well as a direct role in transcription of these regions has not been established. There is evidence to suggest that LMP1 is capable of increasing the expression of several DNMTs, as such, over-expression and/or knock-down of LMP1 expression and subsequent miR-375 expression and CpG island methylation analysis would provide further evidence that LMP1 mediated methylation is indeed responsible for the wide-spread under-expression of miR-375 observed in NPC samples.

Second, additional targets of miR-375 may very well also be important to NPC development, and exploration of these in *in vitro* and *in vivo* models, similar to those detailed in Chapter 2, could provide further insight into the biology of NPC. Similarly, while we have established that at least one function of MTDH in NPC cells is the activation PI3K/Akt signaling, MTDH is also known to target a number of additional pro-growth and anti-apoptotic pathways in a variety of cancers. Rescue experiments using constitutively active Akt in conjunction with MTDH knockdown could provide evidence that this is the pathway required for MTDH mediated survival signaling in NPC. In contrast, probing other pathways such as NF- κ B, c-Myc, ERK/MAPK, Wnt/ β -catenin pathways, which are also responsive to MTDH mediated activation³⁷⁶, could determine if more than just the PI3K/Akt pathway is mediating MTDH's survival, growth, migration and invasion promoting effects in NPC.

5.3.2 MiRNA Signature of Distant Metastasis in NPC

5.3.2.1 Clinical Implementation

In order to fully elucidate the clinical utility of the prognostic signatures presented herein, and to determine the optimal signature for prediction of distant metastasis in NPC, examination in an additional cohort of patients would be of immense benefit. Furthermore, the

treatment homogeneity and randomized nature of samples collected prospectively as part of a randomized clinical trial would improve the scientific validity and clinical applicability. Hence, we have established a collaboration with the Pamela Youde Nethersole Eastern Hospital in Hong Kong who have agreed to provide an additional 189 FFPE NPC blocks from participants in an RCT, whereby NPC patients with locally-advanced disease were randomized to CRT vs. RT alone^{14,377}. These samples will be analyzed in a similar manner to that described above, with qRT-PCR used to measure the 4 miRNAs from our signature, the 5 miRNAs from Liu et al.'s recently published signature¹⁸³, as well as MTDH and housekeeping controls. Through statistical analysis, we will then attempt to design an optimized signature associated with distant metastasis. The randomized nature of this study will also allow us to draw important conclusions regarding the ability of these signatures to predict success/failure of each treatment modality (CRT vs. RT alone).

5.3.2.2 Biological Investigation

Preliminary pathway enrichment analysis presented in Chapter 3 indicated that validated targets of the members of our 4-miRNA signature converged on the cell-cycle control pathway. However, extensive functional characterization is still required in order to establish if and how these miRNAs regulate the oncogenic phenotype in NPC. These functional experiments will be conducted in a similar fashion to those described in Chapter 2 regarding the characterization of miR-375. Briefly, we will first establish the phenotypic impact of over or under-expression of these miRNAs in NPC cell lines *in vitro*. Targets of functionally relevant miRNAs will then be identified using a combination of *in silico*, and *in vitro* approaches. Candidate mRNA targets will be confirmed using the luciferase reporter assay, followed by phenotypic (e.g. cell cycle,

migration, invasion), and biochemical (Western blotting, qRT-PCR for mRNA level) analyses *in vitro*, and in mouse xenograft models of NPC. One limitation is a lack of a robust mouse model of metastatic EBV-positive NPC. The only consistently EBV positive cell line, C666-1 does not readily metastasize from the widely used intra-muscular or subcutaneous xenograft sites. A few studies have reported alternative methods such as intravenous or intra-hepatic injections; however these clearly do not accurately recapitulate true NPC metastasis^{206,378}. One group has, however, successfully established an orthotopic model of NPC wherein C666-1 cells are injected directly into the nasopharynx of NOD/SCID/gamma mice, with the subsequent development of distant metastases to the bone, lung, and liver³⁷³. While this is likely to be a technically challenging technique, establishment of this model in our own laboratory would be of great benefit for further investigation into the functional impact of the various miRNAs and their targets of putative importance in metastatic NPC. It will also provide a valuable NPC model for pre-clinical evaluation of novel targeted therapies.

5.3.3 Genomic Characterization of NPC Samples

The work described in Chapter 4 was a pilot study to determine the feasibility of obtaining genome-wide copy-number, and potentially mutation data from FFPE NPC biopsies using a MIP-based approach. Given the positive results of this study, work is currently underway to similarly analyze an additional ~200 NPC samples as part of an integrated DNA, RNA, and protein profiling effort by our group at the Princess Margaret Cancer Centre. The data generated will be used to both provide useful insight into the biology of NPC as well as to generate a prognostic model which integrates the relevant data types to optimize predictive value. In the short term, confirmation of BCL-2 amplification in a larger cohort of NPC

samples would provide further evidence that this novel observation is indeed an important event in NPC development.

5.4 Conclusion

The main focus of the studies outlined within this thesis aimed to provide an understanding of NPC biology and clinical behavior through the lens of miRNAs. Chapter 2 focused primarily on the biological impact of the robust down-regulation of miR-375 observed in NPC. These studies led to the identification of MTDH, an emerging oncogene in human cancers, as a potentially important promoter of NPC progression and metastasis. Armed with these data, future studies may determine strategies to target this gene and its affected pathways, or exploit it as a marker of aggressive disease. In Chapter 3, we identified and validated a miRNA signature associated with distant metastasis in NPC patients. This signature could better enable physicians to tailor treatments based on an individual patient's prognosis; those patients at low risk of distant failure could potentially be spared the toxicity of aggressive treatments, while providing currently employed aggressive approaches or novel targeted therapies to those patients who could benefit from them. Finally, in Chapter 4 we took a somewhat different approach; utilizing copy-number data to identify potentially important genomic aberrations in NPC samples. Overall, these studies bring us closer to the ever-more attainable goal of truly personalized treatment for NPC patients.

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