Investigating the Role of miRNA-34 family in human thyroid cancer

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Dedication

“A Journey of a Thousand Miles begins with a Single Step.”

- Confucius

For my Mother, whose faith in me led to that first step; for my Sister, who held my hand every step to the end; and for my Dad, just because he’s my biggest fan.

Abstract

Thyroid cancer is the most rampant endocrine malignancy accounting for >80% of endocrine malignancy and 1.8% of all recently distinguished cancer reports. The most important risk factors identified so far, are alcohol consumption, tobacco, radiation exposure, iodine deficiency. There are also additional factors which can cause thyroid cancer but are not still understood.

Histological and clinical differentiation evidence have shown two categories of carcinoma, papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) that comprise up to 94% total thyroid cancer cases (nearly 80% PTCs and 12-14% are FTCs). Nevertheless, different alteration of papillary thyroid carcinoma and follicular thyroid carcinoma can create two rare subgroups, which are called poorly differentiated thyroid carcinoma (PDTCs) and anaplastic thyroid carcinoma (ATC).

PTCs have an indolent behaviour and may recur and disseminate to distant sites such as the lung, as a primarily metastasis site. Life expectancy of patients who have papillary thyroid cancer is age depended and has increased to the eighth position of females' cancer and shows an increase of 4% annually in the world.

Due to the extensive vascular supply of thyroid gland, the large range of lesions and the spectrum of aggressiveness, have made thyroid cancer a suitable pattern for a study on cancer mechanism and angiogenesis.

Angiogenesis is an essential process for tumour growth, metastasis, and tumours, which have lost growth regulatory function and therefore proliferate aberrantly. Controlling of tumour-associated angiogenesis is a tactic for inhibition of cancer progression. Angiogenesis is primarily activated when growing tumour creates a little oxygen microenvironment.

The cancer cell undergoes an angiogenic switch directly leading to the secretion of angiogenic factors such as Vascular endothelial growth factor (VEGF), also indirectly, activation of proliferating genes such as B-cell lymphoma 2 (*Bcl-2*) and Notch homolog 1 (*Notch1*).

Since the discovery of microRNAs (miRNAs) in *Caenorhabditis elegans*, the evidence is emerging that alteration in miRNAs expression may play a key role in cancer development and progression. miRNAs can be described as small non-coding RNAs which are responsible for regulating expression of multiple target proteins. In mammalian, mature miRNAs have been recognised to function at the post-transcriptional level through interaction with the 3' untranslated region (3’ UTR) of the specific target messenger RNA (mRNA). Their primary function is to suppress translation or occasionally induce their degradation in the major cellular pathways such as cell proliferation and differentiation. miRNAs can act as a tumour suppressor or oncogene in a context-depend manner. Therefore, better understanding the molecular mechanisms by which miRNAs play an important function in derailed cellular signalling in the thyroid cancer cell might be helpful to develop better therapeutic strategies for thyroid cancer treatment.

In mammalians, the miR-34 microRNAs precursor family were computationally discovered and later verified experimentally. The two distinct precursors are processed into three mature miRNAs: a and b/c. The mature miR-34 family are a part of the p53 tumour suppressor network; therefore, it is hypothesised that miR-34 family dysregulation is involved in the development of some cancers. This family is transcribed from two different sets of genes located on chromosome 1 and 11. Studies have shown a preference in tissue with lower expression of miR-34a in brain and miR-34b and miR-34c in the lungs. Their promoter region has a p53 binding site. Therefore, they are induced by p53 and thus involved in cell proliferation, survival, apoptosis, migration, invasion

and angiogenesis. Many controlling genes are regulated through the actions of this family. For instance, ectopic expression of this family increases factors involved in cell cycle regulation and DNA damage response (DDR) and the suppression of cell cycle promoting genes.

Recent studies have highlighted the role of miR-34b as a tumour suppressor in a different type of tumours including non-small cell lung cancer (NSCLC), small cell lung cancer cell SCLC, prostate cancer, lung cancer, colorectal cancer also thyroid cancer. However, very little is known about the role and expression state of miR-34b in thyroid cancer.

The overall aim of this study was to determine the functional role of miR-34b in thyroid cancer progression.

In our first study, it pointed out for the first time that overall expression of miR-34b-5p is much lower than miR-34b-3p miR-34a and miR-34c in all thyroid carcinoma cell lines. Therefore, miR-34b was chosen for further investigation in this study. Interestingly, the expression levels of miR-34b were also significantly downregulated in thyroid carcinoma tissue samples and associated with T-stages of thyroid carcinomas ($p=0.042$). High protein expression of VEGF-A, Bcl-2 and Notch1 in thyroid carcinoma cells were noted in cells with low expression of miR-34b when further compared to miR-34b transfected carcinoma cell lines ($P<0.05$). Therefore, from this study, it was clear that there is a link between miR-34b expression and VEGF-A, Notch1 and Bcl-2 expression in thyroid carcinoma.

In the second investigation, I transiently transfected thyroid carcinoma cell lines with miR-34b to investigate its effect on dominant genes involved in angiogenesis cell cycle regulation including VEGF-A, Notch1 and Bcl-2, respectively. miR-34b was overexpressed in thyroid cancer cell lines. We found through immunofluorescent and western blot assay that mir-34b overexpression leads to a significant downregulation of

VEGF-A, Notch1 and Bcl-2 expression ($P<0.05$). miR-34b transfection induced significant accumulation of cells in G0-G1 of the cell cycle by blocking of their entry into the S transitional phase as well as increasing the total apoptosis. ELISA confirmed a $\simeq$ 50% decreased expression of VEGF in all cultured media of the thyroid carcinoma cell lines after transfection with miR-34b. So, it is reasonable to assume that miR-34b can regulate proliferation, migration and angiogenesis *in vivo* as well.

Therefore, our third and final investigation was to use a hydration-of-freeze-dried-matrix (HFDM) formulated liposomes (PEGlyated-miR-34b), as a more efficient transfection approach, for systemic delivery of this microRNA to the thyroid cancer *in vitro* and *in vivo*. We confirmed the effect of miR-34b on VEGF-A downregulation, as a regulator of proliferation and angiogenesis *in vitro* and *in vivo*, using PEGlyated-miR-34b.

miR-34b expression was low and significantly ($P<0.05$) overexpressed following transfection with PEGlyated-miR-34b in thyroid cancer cell lines. Using Western blot and ELISA assay we found that protein level of VEGF-A remarkably reduced in thyroid cancer cell line after transfection of cells with PEGlyated-miR-34b. Furthermore, miR-34b overexpression significantly ($P<0.05$) reduced proliferation, wound healing potential, cell cycle progression and increased apoptosis in thyroid cancer cell lines. *In vivo* xenotransplantation mouse model also showed a functional role for miR-34b in thyroid cancer cell biology in response to its overexpression. Xenotransplantation model further indicated that smaller and low-vascularized tumours were formed upon intravenous (IV) liposomal administration.

Taken together, miR-34b play a pivotal role as a tumour suppressor via modulation of angiogenesis in thyroid carcinoma and suggest a miR-34b/proliferation axis with potential therapeutic implications. This finding also provides a mechanistic insight into the miR-34b regulation of thyroid cancer proliferation, and angiogenesis

and delivery of miR-34b using this cationic liposome may provide a useful therapeutic delivery strategy for thyroid cancer treatment.

Statement of originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

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Hamidreza Maroof

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Abbreviations

ATC	anaplastic thyroid carcinoma
(AJCC/UICC TNM)	The American Joint cancer committee / Union Internationale Contre le Cancer Tumour Node Metastasis
Ang1	angiopoietin-1
Ang2	angiopoietin-2
APAF-1	apoptotic protease-activating factor-1
Apoptosis	programmed cell death
AR	androgen receptor
AD	Alzheimer's disease
ANOVA	Analysis of Variance
aFGF	fibroblast growth factor a
Bcl-2	B-cell lymphoma 2
bFGF	basic fibroblast growth factor
B-CLL	chronic lymphocytic leukaemia
BMCs	bone marrow-derived mononuclear cells
BT-ICs	breast tumour-initiating cells
BM-MSCs	bone marrow mesenchymal stem cells
BTG4	B-cell translocation gene 4
CAI	carboxyamidotriazole
CLL	chronic lymphocytic leukaemia
Ch1.Ac	chlorendic acid
C/EBP α-p42	CCAAT enhancer binding protein alpha
c-Met	hepatocyte growth factor receptor
Cdk4	cyclin-dependent kinase 4
CDF	difluorinated curcumin
CRC	colorectal cancer
CLL	chronic lymphocytic leukaemia
CCSCs	colon cancer stem cells

Cdk4	cyclin-dependent kinase 4
CDK6	cyclin-dependent kinase 6
CO2	Carbon Dioxide
CCK-8	Cell Counting Kit-8
DDR	DNA damage response
Dll4	Dlta-like4
DDP	chemotherapy drug cisplatin
DMSZ	German Collection of Microorganisms and Cell Cultures
DMEM	Dulbecco's Modified Eagles Medium
ddH2O	double distilled water
DEPC	Diethyl pyrocarbonate
Dll4	Dlta-like4
ES	embryonic stem
ECM	extracellular matrix
eNOS	endothelial nitric oxide synthesis
EFNA3	Ephrin-A3
EOC	epithelial ovarian cancer
ERK1/2	extracellular single-regulated kinase 1 and 2
EMT	epithelial-mesenchymal-transition
E2F3	E2F transcriptional factor 3 protein
EGFR	epithelial growth factor receptor
ECACC	European Collection of Cell Cultures
ELISA	Enzyme-linked immunosorbent
ECM	extracellular matrix
EHS	Engel broth-holm-swarm
ERK1/2	extracellular single-regulated kinases 1 and 2
ER2	epitope antigen retrieval 2
FTCs	follicular thyroid carcinoma
FGF	Fibroblast growth factor
FGF2	Fibroblast growth factor 2

Flk-1	Kinase insert domain protein receptor
FBS	Fetal Bovine serum
FITC	fluoresceine isothiocyanate
FACS	Fluorescence-activated cell sorting
FFPE	Formalin-fixed paraffin-embedded
G-CSF	granulocyte colony stimulating factor and granulocyte
GM-CSF	macrophage colony stimulating factor
GBM	glioblastoma multiform
HGFR	hepatocyte growth factor receptor
HNSCC	head and neck squamous cell carcinoma
HPMCs	primary-cultured mesothelial cells
HCC	hepatocellular carcinoma cells
HMPCs	human primary-cultured mesothelial cells
HMGA2	high mobility group AT-hook 2
HEK293T	human embryonic kidney cell line
HDAC1	histone deacetylase 1
HUVEC	human umbilical cord vein endothelial cells
hpf	high-power field
HFDM	Hydration of Freeze-dried Matrix
HRP	horseradish peroxidase
IV	intravenous
IL-8	Interloukin-8
IGF-1	insulin-like growth factor-1
IFA	Indirect immunofluorescence assay
IHC	Immunohistochemical
LYVE-1	lymphatic vascular endothelial hyaluronan receptor-1
miRNA	messenger RNA
MMP	metalloproteinase
MMP-2	matrix metalloproteinase-2
MMP-9	matrix metalloproteinase-9

MTC	medullary thyroid cancer cells
MAPK	mitogen activated protein kinase
miRNPs	miRNA-protein complex
MEK1	mitogen-activated protein kinase 1
MP HCL	Methapyrilene HCL
MON	monuron
MEFs	mouse embryo fibroblasts
MET	mesenchymal-epithelial-transition
MNU	1-methyl-1-nitrosouresa
MDM2	murine double minute z-protein
Notch1	Notch homolog 1
NSCLC	non-small cell lung cancer
NF-κB	nuclear factor κB
NPTX1	Neuronal pentraxin-1
NFkB	nuclear factor kappa beta
NPC	nasopharyngeal carcinoma
OD	optical density
PTCs	papillary thyroid carcinoma
PDTCs	poorly differentiated thyroid carcinoma
pericytes	mural cell
PDGF	Platelet-derived growth factor
PDGFRA	Platelet-derived growth factor receptor
PLGF	placental growth factor
PIGF	placenta growth factors
PDGFRA	platelet-derived growth factor receptor A
PAI-1	plasminogen activator inhibitor-1
p53	protein 53
PDGFRβ	platelet derived growth factor receptor beta
PDAC	pancreatic ductal adenocarcinoma
PSA	prostate specific antigen

par-4	prostate apoptosis response protein 4
PMSF	phenylmethanesulfonyl fluoride solution
PI	propidium iodide
PEG	polyethylene glycol
PAI-1	plasminogen activator inhibitor-1
qPCR	Quantitative Real Time PCR
qRT-PCR	quantitative polymerase chain reaction
RTKs	receptor tyrosine kinase
ROS	proto-oncogene tyrosine-protein kinase
RASFs	rheumatoid arthritis synovial fibroblasts
RPMI	Rosewell Park Memorial Institute medium
RES	reticuloendothelial
SNP309	single nucleotide polymorphism 309
THs	Thyroid hormones
TGF-β	transforming growth factor β
TGFβ-1	transforming growth factor β-1
TIMPs	metalloproteinase tissue inhibitors
TSP-1	thrombospondin-1
TSP-2	thrombospondin-2
TRAIL	TNF-related apoptosis inducing ligand
TBST	Tris buffered saline-Tween
SCLC	small cell lung cancer
VEGF	Vascular endothelial growth factor
VHL	Von Hipple-lindau
VM	vasculogenic mimicry
YY1	yin yang 1

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Included in this thesis papers in chapters 2, 4 and 5 for which I am the sole first author. Appreciated acknowledgments of those who contributed to the research however did not qualify as authors are included in each paper.

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3. **Maroof H,** Farhadul Islam**,** Armin Ariana, Vinod Gopalan, Alfred K. Lam. The roles of microRNA-34b-5p in angiogenesis of thyroid carcinoma. Endocrine, 2017. doi: 10.1007/s12020-017-1393-3. (**IF**: 3.87, **Citation**: 1).

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International

6. **Maroof H**., Vinod Gopalan, LanFeng Dong, Alfred KY Lam. The miR-34b-5p is down-regulated in thyroid cancer cells and a potential to eradicating thyroid cancer via a systemic delivery of a PEGlyated miRNA-loaded lipid particles

system. International Student Research Forum (ISRF), Omaha, Nebraska, USA, June 4, 2017 **(Oral Presentation)**.

7. **Maroof H**., Ali Salajegheh., Alfred King-yin Lam., miR-34b: A regulator of VEGF-A, Notch1 and Bcl-2 in thyroid carcinoma. AACR Annual Meeting, New Orleans, Louisiana, USA, April 21, 2016.

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Australian

8. **Maroof H**., Vinod Gopalan, LanFeng Dong, Alfred KY Lam. Tumour Suppressor Functions of miR-34b and its potential to eradicating thyroid cancer via a systemic delivery of a PEGlyated miRNA-loaded lipid particles system. International conference on Bio-Nano innovation, University of Queensland, Brisbane, Australia, September 24, 2017. (Manuscript accepted for oral presentation).
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Publication Tangential to the Thesis

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2. Rahman MA, Salajegheh A, Vosgha H, **Maroof H**, Smith RA, Lam AK. Inhibition of BRAF kinase alone in BRAF V600E mutated undifferentiated thyroid carcinoma results no growth arrest. AACR Annual Meeting, New Orleans, Louisiana, USA, April 16, 2016.

Chapter 1: Aims and Hypothesis

1.1 Aims

This study will investigate the role of miRNA-34b in human cancer angiogenesis, growth and development, which will provide a better understanding of these pathways and their potential target for cancer treatment.

In this research, the expression level of miRNA-34b in different cancer cells will be investigated against normal immortalised cell line. This investigation will then use a variety of molecular methods that will help us understand their relationships, interaction and effect in angiogenesis, carcinogenesis and cancer development. Methods that will be recruited in this research are qPCR-based procedures for identification and measurement of the expression of those genes. Then, Western blot and Immunostaining will be used to evaluate products of these genes and their outcome in the progression of malignant cells. Eventually, transient gene manipulation will be utilised to evaluate the effect of genetic alteration in hindering the pathway of growth, development and angiogenesis *in vitro.* The outcome of each alteration will be assessed via recruitment of different relevant assays. Eventually, the liposomal vehicle will be recruited to deliver the same manipulation effect to the implanted cancer cells *in vivo* for confirmation of the beneficial approach. This will improve our understanding of the role of miRNA-34b expression levels and its relevance with thyroid cancer occurrence.

To improve the understanding of tumour angiogenesis in cancer, the following fundamental aims behind this research are established:

Hypothesis 1: *miR-34b* is downregulated and influences expression of major angiogenic regulator genes in thyroid cancer biology.

Aim 1: Investigate the expression of miR-34b in different thyroid cancer tissue and cell lines compared with their normal tissue and normal immortalized cells. Investigate the protein expression of major vascularisation regulatory genes (VEGF-A, Notch1 and Bcl-2) using different experimentations including qPCR, Immunofluorescent imaging and western blotting. The research question associated with evaluation the expression of these genes is:

Is there any correlation between expression level of miR-34b, VEGF-A, Notch1 and Bcl-2?

Hypothesis 2: *miR-34b* expression moderate cancer proliferation and survival through angiogenic events in thyroid cancer *in vitro.*

Aim 2: Transient introduction of the exogenous miR-34b and evaluation of VEGF-A, Notch1 and Bcl-2 expression after this manipulation, and its subsequence effect on angiogenesis, proliferation and apoptosis by assaying its influence such cellular pathways: Angiogenesis, Proliferation and Apoptosis. Research questions associated with this objective are:

a: Does miR-34b overexpression regulates the expression of VEGF-A, Notch1 and Bcl-2 in different type of thyroid cancer cell lines?

b: If so, does miR-34b have significant impact on angiogenesis, proliferation and apoptosis pathways in thyroid cancer cell lines?

Hypothesis 3: Angiogenic modulatory effect of PEGlyated-miR-34b influences thyroid cancer tumour growth *in vivo.*

Aim 3: Determine whether the introduction of the miR-34b (PEGlyated-miR-34b) *in vitro* and *in vivo* could inhibit angiogenesis in cancer tissue and surrounding vasculature.

Confirmation of the effectiveness of these *in vitro* experimental approaches in the *in vivo* modules by the introduction of cancer cell lumps into the animal and investigation of cancer angiogenesis, proliferation and angiogenesis through the permitted and suggested time intervals. Histopathology method will be recruited to make the final confirmation to these finding. Specific research questions are:

a: Does the PEGlyated PEGlyated-miR-34b modulate proliferation, angiogenesis *in vitro*?

b: If so, can PEGlyated-miR-34b impact on tumour growth *in vivo*?

1.2 Significance of study

Cancer is the second leading cause of death in the world. The causes of cancer are diverse, complex, and only partially understood. It is reasonable to think approaches to cancer therapies are shifting to safer remedies of genomics and proteomic because of conventional therapy such as chemotherapy and radiotherapy are comprised of different side effects and insufficiently effective. Various evidence shows that multiple- factors (oncogenes and/or environmental) are required for having successful cancer therapy.

Angiogenesis is necessary for the survival of all mammalian cells because their survival depends on sufficient oxygen and nutrients as well as disposing wastes. It is also an essential process for tumour growth and metastasis. Angiogenesis is controlled by a balance between pro-and anti-angiogenic factors. There are various signalling pathways and promoter genes, which contribute to this switching mechanism. Despite mounting evidence, well-controlled promoters and inhibitors of angiogenesis could be an emerging axis to cancer treatment. Recently, one of the fastest growing cancers in the United States is thyroid cancer. Papillary thyroid carcinoma has an invasive behaviour and may spread to another organ such as lung as a primarily metastasis site. Life expectancy of patients

who has papillary thyroid cancer is age depended and have increased to the eighth position of cancer females and shows an increase of 4% annually in the world.

Recent evidence suggests that a defined class of non-coding 21–25 nucleotides RNA plays critical roles in the regulation of gene expression, especially function at the post-transcriptional level through interaction with the 3' untranslated region (3’ UTR) of target mRNA. Various arrays have demonstrated that miRNAs play a regulatory role in several human cancers as downregulation or overexpression and alteration of expression, which can attribute to cancer development and variation of tumour types. In recent years, studies on miRNA expression pattern have indicated the correlation between the expression level of miRNA and cancer characteristics.

The potential of miRNAs as a therapeutic component and as an oncogenes expression regulator, have achieved great attention because miRNAs play a distinct role in the modulating of expression myriad genes such as Bcl2, Notch1 and VEGF-A by its dysregulation. To date, there is much evidence that has shown the pivotal roles of mir-34b in thyroid cancer development as a regulator. miR-34b is now considered by many researchers as one critical part of the p53 tumour suppressor network of protein. Also, accumulating evidence suggests that miR-34b is involved in the angiogenesis process, modulating new vessel formation through their upregulation or downregulation.

miR-34b, well known as a tumour suppressor, is involved in the inhibition of the angiogenesis process by tight regulation of the VEGF-A/Notch1 feedback loop and promotes apoptosis with downregulation of Bcl-2.

Our research focused on regulating of VEG-A/Notch1 feedback loop and Bcl-2 via manipulation of miR-34b as a tumour suppressor in thyroid carcinoma. We used a new defined approach (PEGlyated-miR-34b) in suppressing proliferation and angiogenesis pathway by targeting VEGF-A in thyroid carcinoma.

1.3 Ethic clearance

Ethical aspects of this study were reviewed and approved by the Animal Research Ethics Committees of Griffith University (GU Ref No: MED/01/17/AEC). The clearance email can be seen in Appendix 1.

Chapter 2: Literature Review

2.1 Introduction to thyroid cancer

Cancer is the second leading cause of death in the world. The causes of cancer are diverse, complex, mutation accumulation and only partially understood. This evidence provides a compelling rationale to the implication of gene regulation in cancer occurrence. Recently proteomic and genomic analysis have changed our insight on cancer and revealed that many genes are involved in the cancer process [1, 2]. Each tumour mass can contain approximately hundred tumour cells with distinguished gene function [3]. Thyroid cancer is the most common endocrine malignancy in the world. Thyroid cancer occurs approximately ~120,000 new cases per year worldwide. Thyroid cancer spreading and its recurrence after surgery is mainly via lymph node metastases and happens in mostly of thyroid cancer patient. Thus, management of patients with thyroid cancer requires a better understanding of the underlying molecular mechanisms of lymphovascular spread of the tumour [1]. It is reasonable to think approaches to cancer therapies are shifting to safer remedies of genomics and proteomics because conventional therapy such as chemotherapy and radiotherapy are comprised of different side effects and insufficiently effective. In recent years, targeted therapies, which have especially focused on known oncogenes, are associated and replaced by conventional therapies [4-6].

2.1.1 Thyroid gland

There are fourth pharyngeal glands from the tongue to the base of the neck that to be called thyroid gland during embryonic life. The thyroid gland has two lobes, which are connected by a bridge of the tissue. Also, thyroid glands have been considered as the highest blood flow rates per gram of the tissue as compared with other organs in the body[2, 3].

Thyroid gland produces two types of hormones (THs) consisting of (T3) 3, 5, 3'-L-triiodothyronine and T4 L-thyroxine. Thyroid hormones regulate the metabolism level in tissues. T3 and T4 induce and regulate oxygen expenditure level of cells. In addition, thyroid hormones control metabolic carbohydrate rate and lipid adjustment. Thyroid hormones have direct contacts to central nervous system, heart, skeletal muscle, intestine, sensory organs, and the skeleton [4].

2.1.2 Thyroid cancer

Thyroid cancer is the most rampant endocrine malignancy accounting for >80 % of endocrine malignancy and 1.8% of all recently separate cancer reports [5, 6].
Some factors involve an attribute to create thyroid cancer such as benign thyroid tumour that has a remarkable ability to transform. Some environmental factors include radiation exposure, alcohol, smoking, iodine deficiency and there are plenty of factors which can case thyroid cancer but are not still understood [7].

Due to the extensive vascular supply of thyroid gland, the broad range of lesions and the spectrum of aggressiveness so far have made the thyroid cancer a suitable pattern for a study on cancer mechanism and angiogenesis [8].

Histological and clinical differentiation evidence have shown two categories of carcinoma, papillary thyroid carcinoma(PTCs) and follicular thyroid carcinoma(FTCs) that comprise up to 94% total thyroid cancer cases, (almost 80% are papillary thyroid carcinoma, and 12-14% are follicular thyroid carcinoma). Nevertheless, differential alteration of papillary thyroid carcinoma and follicular thyroid carcinoma can create two rare subdivides that is called poorly differentiated thyroid carcinoma (PDTCs) and may throughout switched to lose differentiation and convert to anaplastic thyroid carcinoma(ATC) [9, 10].

Papillary thyroid carcinoma has an invasive behaviour and may spread to another organ such as lung as a primarily metastasis site. Life expectancy of patients who has papillary thyroid cancer is age depended and have increased to the eighth position of cancer females and shows an increase of 4% annually in the world [11].The mortality of PTC has a direct relation with the aggressive clinicopathological feature.

In this term, there are some biomarkers that play important roles in the aggressiveness of PTC clinicopathological features and might be able to discriminate tumour organs. It seems so important to identify the major biomarkers that participate in papillary thyroid carcinoma as prognostic or predictive features or predisposing biomarkers [5].

2.1.3 Clinical features of Papillary Thyroid cancer

Papillary thyroid carcinoma is most prevalent tumours of thyroid glands. Papillary thyroid cancer is introduced as good prognosis and low rate of relapse, surgery is considered as the initial treatment of thyroid cancer, an extension of thyroidectomy is still controversial Thyroid cancer is consisted of different types of pathological and clinical feature in neoplasms [12, 13].

The incidence of thyroid cancer in a woman with the median age being about 46 years old, is 2.6 to 6.1 times more frequent than in men (ratio approximately is 3 to 1) [14].
The gender disparity of thyroid cancer is well recognized in incidence, aggressiveness and prognosis but the cause of the disparity is poorly understood. *MET* (mesenchymal-epithelial transition) SNPs (single nucleotide polymorphism) a significant association with gender disparity and more frequent in women [15].
Also, reproductive factors such as estrogen receptors appear to be involved in development of thyroid cancer in women[16]. However, there is no established molecular factors that elucidate gender differences in thyroid cancer. Therefore, high-throughput

genomic and proteomic approaches to the study of thyroid cancer gender disparity could be helpful for better understanding the molecular mechanism of gender differences in thyroid cancer [17].

Papillary thyroid cancer has a typical histologic feature and is comprised to specific tendency with regional lymph nodes metastases and multifocal involvement of the thyroid glands. Early diagnosis of thyroid cancer is feasible and enhances the patient's positive prognosis [18].

2.1.4 Pathological features of Papillary Thyroid cancer

Papillary thyroid carcinomas have papillae asymmetric appearance and papillary ordinance that of them are covered with tumour cell inside that bear typical nuclear alteration with overlapping cell nuclei (ground-glass nuclei with longitudinal grooves) and determined by exposure of papillary structure. Papillary carcinomas are distinguished form follicular and anaplastic and medullary thyroid carcinomas based on the differential alterations. Papillary thyroid carcinoma is well differentiated [14, 18, 19].

2.1.5 Prognostic features

Recently, one of the fastest growing cancers in the United States is thyroid cancer. Although, papillary thyroid carcinoma frequently is associated with good prognosis [20], All types of papillary thyroid carcinomas have histologically been analysed during the 20 years from 1985 to 2005 [21].

In 2012, approximately 230,000 new cases of thyroid cancer were diagnosed among women and 70,000 among men. International comparisons are complex due to differences in diagnosis, ascertainment of the disease and demographic factors such as geographical location, ethnicity, age and sex in worldwide. This is seen an over tenfold difference in incidence across different parts of the world in women, high incidence areas (over 10/100,000 women) including selected countries of South and North America, Italy in

Europe, Japan and the Pacific Islands; the absolute variation is considerable, but relatively smaller in men [22, 23].

In addition, 80% -85% of patient are categorised as being at the low risk level of recurrence. Patients with ages less or equal to 16 have a high risk of recurrence [24, 25]. Prognosis of papillary thyroid carcinoma is excellent, nonetheless, it has been shown that 40% of 53 statistical predictions develop metastasis in loco-regional lymph node, and 28% cases develop metastasis in the central neck. Surgery is the treatment of choice for inhibition of recurrences because some lesions are not responsive to metabolic radiotherapy [12]. The 80 to 95 percent of thyroid carcinomas patients have an overall survival rate around 10 years with middle age adults [26, 27].

More than 22% of patients have one of the 3 types of a tumour: local and regional and distance metastasis or a mixture of them are reported in rare patients. There are many parameters involved incorrect prognosis of recurrence disease:1) histological subtype 2) extent of a tumour 3) patient's age [28-30].

Collectively, histological subtypes of papillary thyroid carcinoma (columnar cell, tall cell variant, diffuse sclerosing carcinoma, poorly differentiated subtype, Hürthle-cell carcinomas) are regarded as the highest risk cohort, whereas, the risk of death might be frequently down. Having appropriate treatment depends on the above-mentioned indicate imprtance of thyroid cancer in future studies.

Angiogenesis and beyond- a comprehensive review

This section includes a co-authored prepared paper. The bibliographic details of co-authored paper, including all authors, is:

Hamidreza Maroof, Vinod Gopalan, LanFeng Dong, Alfred King-Yin Lam.

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Other authors contribution to the submitted paper: Critical review and revisions were conducted by King-Yin Lam.

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Paper 01: **Angiogenesis and beyond- a comprehensive review**

Hamidreza Maroof**,** Vinod Gopalan, LanFeng Dong, Alfred King-Yin Lam

(Manuscript is under preparation)

2.2.1 Abstract

Angiogenesis is defined by development and growth of new blood vessels from the pre-existing vessel. It has become a major type of research as it plays a critical role in normal physiology, cancer development and numerous diseases. Different mechanisms are involved in the growth of vessel plexus, including sprouting angiogenesis, intussusceptive angiogenesis, vascular mimicry, blood vessel co-option and lymphangiogenesis, and the latter three, have only been observed in tumour growth and metastasis. Despite this variety of angiogenic mechanisms, most of the current investigation is focused on the interaction between pro- and anti-angiogenic molecules which are tightly regulated by "on-off switch signalling" between angiogenic factors, extracellular matrix components and cells. Here, we reviewed different angiogenesis mechanisms by which vascular tree are remodelled by the growth of new capillaries from pre-existing vessels in both normal and pathological condition. Furthermore, the tactics used by normal cell in physiological condition to provide their blood supply is discussed about those recruited by cancer cells. Understanding of blood vessel growth in both normal and pathological condition, offer effective therapeutic strategies for cancer.

2.2.2 Introduction to Angiogenesis

Angiogenesis is necessary for the survival of all mammalian cells, because their survival depends on sufficient oxygen and nutrients and as well as disposing wastes. Multicellular organisms need to have more vascular to grow beyond of size, the process of vasculogenesis and angiogenesis will create new vascular [31]. Many growth factors

are associated with angiogenesis such as enzymes and extracellular matrix factors and several cell type [32] eventually leads to the sprouting of new papillary from pre-existing vasculature [33]. Neovascularization is an important process for making a channel for tumour invasion and metastasis and also providing appropriate nutrient and oxygen [34]. Along with this connection, there isn't always a direct correlation between size or capacity of tumour cells to induce angiogenesis and their invasiveness [35]. An excessive or insufficient vascular growth is also directly associated with multiple non-neoplastic disorders. Also, the hallmark of the inflammatory disease is prolonged and excessive angiogenesis. Whereas the hallmark of cancer is sustained angiogenesis. Angiogenesis is controlled by a balance between pro-and anti-angiogenic factors. In various diseases and especially cancer, these factors are often deregulated [31].

It is widely accepted that ischemia can control tumour growth and probably metastasis because, without an important blood supply, the tumour may not be able to invade to another organ and cannot grow beyond a critical size. Subsequently, we cannot deliver an effective dosage of anti-cancer drug around a tumour. Therefore, blocking angiogenesis could be a potent tool for tumour inhibition [31, 36]. The hypothesis later explained that the key role of genetic factors is "angiogenic switching". The concept of "switch off" is that the anti-angiogenic factors balance all pro-angiogenic molecule and "switch on" is unbalanced and make a profit for angiogenesis [36, 37].

There are various signalling pathway and promoter gene, which contributes to this switching. Despite mounting evidence, well-controlled promoters and inhibitors of angiogenesis could be an emerging axis to cancer treatment [34]. There are many human tumours that have long dormancy by balancing between cell proliferation and apoptosis. Furthermore, the spread of the tumours are linked with sprouting development and intussusception from pre-existing vessels Perhaps, the most important group of genomics

factor having a regulatory role are the genes contributing to angiogenesis regulation. Since all tumours use angiogenesis for growth, the deregulated gene expression significantly benefits tumour progression. Recently, most studies have focused on tumour angiogenesis in an animal model, and preclinical and clinical trials have not shown high efficacy in patients [38, 39]. There are many angiogenic inhibitory drugs, which have a broader side effect such as Thalidomide. However, acting as an inhibitor of blood vessel growth, Thalidomide causes limb defect in the developing foetus. Thus, new studies focused on angiogenesis inhibition are necessary and using the responsible regulatory pathway may demonstrate a valuable tool against the process of tumour angiogenesis [40]. This review summarizes the different mechanisms of angiogenesis, the factors (genes) that are involved in the neovascularisation in both normal and pathological conditions.

2.2.3 Normal Angiogenesis

Angiogenesis process typically creates a vast network of arteries and veins in the human body at specific times. For instance, it is necessary for development and growth in the developing fetus in a mother's womb. It also occurs in physiological repair process such as wound healing, damages resulting ischemia and or cardiac failure and ovarian [41, 42] cycle as well as, in women a few days before each month in the lining of the uterus during the menstrual cycle. During this process, initially, a primary network of vascular endothelial cells called vasculogenesis is created. Vasculogenesis creates an endothelial cell lattice and later play significant role as a scaffold for angiogenesis. This primary network remodels into the new capillaries or blood vessel by angiogenesis process that will complete the child's blood circulatory system [43-45].

2.2.4 Mechanisms of physiological angiogenesis

Several divers' process that occurs in the microvasculature conducts the maturation and remodelling of the newly formed microvessel. Sprouting of new the vessel firstly needs to remove mural cell (pericytes) from branching vessel. The result of pericytes removal from endothelium is destabilisation of the vessel by angiopoietin-2 (regulatory factor of normal angiogenesis and antagonises angiopoietin-1 signalling). Consequently, transfer to a plastic, and proliferative phenotype from the stable and growth-arrested state of endothelial cells occurs. VEGF (vascular endothelial growth factor) increases vessel permeability, which can cause spillage of proteases and matrix components from the bloodstream. Endothelial cell activates and produces degradative enzymes called metalloproteinase (MMPs). These enzymes penetrate, and break down extracellular matrix and then endothelial cells proliferate and migrate into surrounding tissues via the remodelled matrix, and then begin to divide and gradually organise in hollow tube form. Mesenchymal cell proliferates and migrate into the new blood vessel as well as differentiate into the mature mural cell. Stabilisation of new blood vessel is depended on the resonance of cell-cell contacts and endothelial cell immobility and minutiae of the new matrix [46-48].

2.2.5 Regulatory factors of normal angiogenesis

Normal angiogenesis is influenced by positive and negative factors, which are active in cell-cell and cell-matrix interactions and hemodynamic effects and soluble polypeptides. Signalling of normal angiogenesis is controlled by an soluble growth factor, molecules bounding membrane and biomechanical forces. Soluble growth factors are the main mediators of angiogenesis (Table 1) [49-62].

Table 1: List of soluble growth factors, which have a significant role in angiogenesis.

Soluble growth factors	VEGF (vascular endothelial growth factor)
	Ang1 (angiopoietin-1)
	Ang2 (angiopoietin-2)
	aFGF, bFGF (fibroblast growth factor a and b)
	PDGF (Platelet-derived growth factor)
	TGF-β (transforming growth factor β)
	TNF α (tumour necrosis factor α)
	EGF, TGF-α, G-CSF, GM-CSF (granulocyte colony stimulating factor and granulocyte/macrophage colony stimulating factor)
	Angiogenin
	Angiotropin
	Tissue factor
	Factor V
	Prostaglandin
	Nicotinamide
	Monobutyrin

In addition to the described soluble growth factors that are positive at an interval from their synthesis site, membrane-bound proteins play a prominent role in angiogenesis via the mediatory function in blood vessel assembly and have a useful role in close distance cell-cell and cell-matrix interactions. There are seven endothelial membrane proteins playing important part in cellular communication including: αv β3-Integrin and

αvβ5-Integrin and α5β1-Integrin (as receptors for extracellular matrix protein and membrane-bound polypeptides comprising of two heterodimeric subunit), EV-cadherin (effective on homotypic interaction between cells), Eph-4B/Ephrin-B2 (eph is a receptor and belong to receptor tyrosine kinase family, ephrin is ligand for eph), Ephrin A1 and A2 [63-68].

The third compelling factor on normal angiogenesis is biomechanical forces that associate to decorate blood vessel in normal angiogenesis processing with the collaboration of blood flow rates and shear stress. The rates of blood flow are directly correlated with the proliferation of vascular endothelial cells. The role of fluid shear stress is depended on blood flow pattern. Expression of endothelial cell stress fiber is increased by laminar blood flow whereas endothelial cells stimulated to divide by turbulent blood flow [69-71]. Along with this theme, VEGF, as a well-characterized angiogenic factor, will describe below.

2.2.6 The biology of VEGF (vascular endothelial growth factor)

VEGF can be assumed as most well characterised angiogenic factor whereby many physiological and pathological processes of angiogenesis during embryogenesis, reproductive function, skeletal growth, tumour neovascularisation and intraocular neovascular disorders are regulated via the modulatory role of VEGF. There is two receptor tyrosine kinase (RTKs) called VEGFR-1 and VEGFR-2. These receptors are the mediators for biological effects of VEGF. The signalling trait of these receptors is completely different. VEGF, also known VEGF-A is located in one family gene region with placental growth factor (PLGF), VEGF-B, VEGF-C and VEGF-D. Investigation of expression of VEGF in parapoxvirus Orf virus has also proved the existence of these homologs [72-74].

2.2.7 VEGF isoforms

The human VEGF gene is comprised of eight exons that are separated by seven introns. Four different isoforms generated by alternative splicing of this exon include VEGF121, VEGF165, VEGF189, VEGF206 and are respectively composed of 121,165,189 and 206 amino acids. Among these isoforms, 165 isoform has the predominant expression. (30.31 of i.c-d). VEGF183 and VEGF 145 are two less frequent VEGF which play a role in splicing of human *VEGF* genes [75].

The property of native VEGF is a homodimeric glycoprotein of 45 kDa that binds heparin [76]. The VEGF mitogenic activity is associated with the activity of heparin-binding domain [77]Among the VEGF isoforms, the optimal bioavailability feature and biology potency has been demonstrated by VEGF 165 [75].

2.2.8 Regulation of VEGF gene expression

The *VEGF* gene expression is regulated by oxygen tension, growth factors and oncogenes. Broad spectrum of gene expressions are influenced by oxygen tension. Pathological and physiological condition and oxygen tension are accompanied to induce VEGF mRNA expression [78] Hypoxic responses could be modulated by the key modulatory function of hypoxia-inducible factor (HIF)-1 [79]. Recently evidence has been discovered that Von Hipple-Lindau (VHL) as tumour suppressor gene modulates HIF-1 function [80].

Investigation in certain VHL diseases such as sporadic clear-cell renal carcinoma and retina and cerebellum capillary hemangioblastomas has been reported the inactivation of *VHL* gene in these patients [81]. VEGF expression and hypoxia-inducible gene are negatively affected by the function of VHL protein [82]. Other studies reported in the deficient condition of VHL in renal cell carcinoma cell line, HIF-1 has been induced [83].

A previous study has shown that VHL targets HIF-1 subunits as a component of ubiquitin-ligase convened [84]. Interestingly, the HIF-1 brings up its demand for association with VHL through hydroxylation at a proline residue by stimulation of oxygen [84, 85]. The upregulation of VEGF mRNA expression is associated with significant growth factors such as platelet-derived growth factor, epithelial growth factor, FGF, Insulin-like growth factor-1, TGF-α, TGF-β, keratinocyte growth factor. Some of these factors via paracrine and endocrine release are related to local hypoxia which in turn regulate VEGF production in the tumour microenvironment [72, 73].

The crucial role of VEGF expression level in inflammatory disorders and angiogenesis has been proven. In particular, cell type such as synovial fibroblasts, the expression of VEGF is influenced by IL-1α and IL-6 as inflammatory cytokines [73]. VEGF expression gene can be induced by transforming event, and in this connection, upregulation of VEGF is influenced by the rate of oncogenic mutation or mutant Ras protein [86, 87].

2.2.9 The VEGF receptors

The expression level of VEGF is depended on the target tissue. For an instant, in a human tumour which angiogenesis is demanded, the level of VEGF is high and likewise in corpus luteum and developing the child, and conversely, in multiple animal tissues, low level of VEGF has been reported [88]. Many cells primarily stromal and mesenchymal cells, secrete VEGF in human tissues [76].

The tyrosine kinase receptors of VEGF are located on the cell surface of vascular endothelial cells and have recently been shown to bone marrow cells surface and vascular smooth muscle cells and blood cells such as monocytes and macrophages, are produced by monocytes in the tissue specimen. These tyrosine kinases are classified in three major cohorts, Flt-1(VEGF-1), KDR/Flk-1(VEGFR-2) and Flt-4 (VEGFR-3) (174 of 1.c-a and

6 of 1.c-d). 7-Ig or *flt* gene region is responsible for the ability to produce VEGF receptors. Both of receptors are determined by having seven immunoglobulin-like domains in the extracellular domains [89, 90]. The expression of VEGFR-1 in skin wound healing and embryonic mice models and adult endothelium is higher than two other ones. Also, the affinity of VEGFR-1 to VEGF is greater than VEGFR-2 and VEGFR-3 [91, 92].

The vascular smooth muscle cells and monocytes also appear to express a high level of VEGFR-1 on their surfaces [93, 94]. Some investigations have shown the correlation between VEGFR-1 mediatory function and particular activity of cells such as proliferation and migration [95]. VEGFR-2 can be considered as second primary receptor tyrosine kinase. The target cells of VEGFR-2 are endothelial cells and hematopoietic precursors. Interestingly, the proliferation of endothelial cells is influenced by the modulatory function of VEGFR-2 in the foetus [96]. The higher expression level of VEGFR-3 in lymphangiogenesis as compared to VEGFR-1 and VEGFR-2, showed that VEGFR-3 is mostly involvement with lymphangiogenesis. [97].

Along with this term, VEGF-3 does not play receptor role for VEGF, however, is a member of tyrosine kinase family that can bind to VEGFC and D[74]. Furthermore, some coreceptors play a binding role for VEGF in parallel to tyrosine kinase family. Indeed, they are neural receptors that are called neuropilin. Neuroplin-1 and neuroplin-2 have significant tendency to interact with VEGF 165 athwart of VEGF 121. In this connection, both neuropline-1and 2 enhance their binding to VEGFR-2 in the endothelial cells, and consequently, the chemotactic abilities of neuropline-1 and 2 matures [98].

Recently studies have been shown that neuropline-1 is associated with angiogenesis process in mice models [99]. Various effect on vascular endothelial cells has been demonstrated by VEGF such as permeability, development of metabolic, circulatory systems from luminal to abluminal and vice versa in epithelial cells of

capillaries [100]. Hypothesised later was the effect of VEGF on the loss of function of adherents junctions between endothelial cells, which thereby increase the permeability of endothelial cells [101, 102].

Spillage of plasma protein due to vessel permeability changes the extracellular matrix formation for migration of stromal cells and endothelial cells [103]. Accordingly, VEGF can be useful in angiogenesis process via the balance of proteolysis necessary for extracellular matrix remodelling. There are many publications, which have elucidated the critical role of VEGF in proliferation and migration of endothelial cells in animal models [51, 104-106]. Particularly notable, the proliferation of lens and corneal endothelial cells, smooth muscle and adrenal cortex cells are stimulated by other factors except VEGF. It is considered that the apoptosis process can be inhibited by the modulatory function of VEGF [107].

Furthermore, sprouting of new blood vessels from pre-existing ones in healing, successful bone marrow grafting and eye disease such as cornea has been observed to accompany with VEGF level [104]. In one study on mice with two miss mutations for VEGF and its receptor Flk-1, has reported the primary role of Flt-1 specifically is in the remodelling of vascular in angiogenesis process [108, 109]. The environment destitute of oxygen is called hypoxia condition, whereby stimulate the expression of VEGF. Hypoxia-inducible factor (HIF) can be influenced on transcription of VEGF gene and constancy of its mRNA via interaction with a *cis* element of VEGF premotor [110].

The entire neovascularization process could be depending on VEGF doses. Besides this issue, low oxygen condition can stimulate angiogenesis process. VEGF showed considerable amino acid homology with other members of growth factors family. For an instant, the homology between VEGF and platelet-derived growth factor (PDGF) is approximately 24% of total amino acid homology, which is existed [111]. Nonetheless,

the receptor of VEGF and PDGF has been discovered to localise in different locations [112], and the VEGF receptor can be a target by molecules which are related with VEGF such as placental growth factor (PIGF) that can bind VEGF-1 and (NP-1)neuropilin-1[113]. In placenta as the maintaining organ of the fetus, the PIGF predominantly leads the angiogenesis process in the embryonic period [114].

The second growth factor that plays a tremendous role in embryonic angiogenesis and adult muscle tissue is VEGF-B (vascular endothelial factor-type B). In versus, the preliminary clinical trial has reported the lower expression of VEGF-B in brain, lung and kidney tissue [115]. The activation of VEGF-B has major correspondence with VEGF-A and VEGFR-1 and neuropilin-1 play binding site role for VEGF–B[116]. However, the proliferation of endothelial cells is influenced by VEGF-B, but the angiogenesis process is performed in the absence of VEGF-B [115].

The VEGF–C and VEGF-D have lesser homology than VEGF-B to VEGF-A VEGFR-2 and VEGFR-3 are often activated by binding to VEGF-C and VEGF-D and eventually results in the elevation of mitogenicity in endothelial cells (3 and 136 of 1.c-a) [117, 118] and both have influenced the angiogenesis process in vivo and in-vitro. Notably, lymphatic vessel expansion and conservation of lymphatic endothelium after differentiation in the adult in angiogenesis can be affected by VEGF-C and VEGFR-3 as the preferred receptor [97, 119]. High expression of VEGF-D has been observed in lung and embryonic phase [120].

VEGF-E: this type of VEGF belongs to *orf* gene region of parapoxvirus and has been assumed as a viral protein, and consequently, can be involved in diseases prevalence of animals such as goats and sheep [121].VEGF-E imposes the stimulatory function of VEGF in angiogenesis process via an effect on VEGFR-2 [122]. The VEGF in mammalian cells may tolerate a genetic drift that is taxed by *orf* virus. Subsequently,

VEGF-E will create. [121]. Also, many inflammatory disorders can be induced by VEGF such as brain oedema. In one study, the upregulation of VEGF and its receptor in rat brain has been observed [123, 124].

2.2.10 Intussusceptive angiogenesis

Different mechanisms are involved in the growth of vessel plexus, including sprouting angiogenesis, intussusceptive angiogenesis (splitting or non-sprouting angiogenesis), vascular mimicry, blood vessel co-option lymphangiogenesis and latter three, have only been observed in tumour growth and metastasis. In addition to sprouting angiogenesis, intussusceptive angiogenesis has main roles in physiologically growing tissue. Intussusceptive angiogenesis is widely involved in remodelling and pruning of exceeding branches of blood vessels in angiogenesis process, whereas sprouting process is assumed as a single mechanism that is purely involved in vascular growth [125-128]. Intussusceptive angiogenesis is categorized in three forms by morphological characteristics.

1-Intussusceptive microvascular growth: during this process, new pillars are expanded from pre-existing vascular networks by rapid expansion.

2-intussusceptive arborization: this process occurs in central arterioles and dense capillary network; this mechanism is involved in creating a new hierarchical vascular tree from the previously hierarchical capillary network by remodelling of a series of pillars.

3-intussusceptive branching remodelling: can be described as changing and branching and pruning of a blood vessel to optimise blood supply for tissue and also contribute in the creation of bifurcation point in tissue pillar close. With the relocation of bifurcation point, the vessel is transformed to two blood vessels [129-132].

The process of intussusception is fast and low metabolic as compared with sprouting process, and because there is no blind ending capillary segment, the

physiological condition has no conflict with the process of intussusceptive angiogenesis. During this mechanism, endothelial cells increase in size and flatten without any proliferation. Intussusceptive angiogenesis is predominate consists of vascular growth and remodelling process that occurs next to initial vascularisation of sprouting angiogenesis [133, 134]. Investigations on intussusceptive angiogenesis are mainly focused on animal disease models such as murine models that are involved with inflammatory disease and liver cirrhosis [135-137].Intussusceptive angiogenesis also contributes in angiogenesis of some cancers including non-Hodgkin's lymphoma, mammary tumours, and diverse types of gliomas, colon carcinoma and renal hepatocellular. In tumours smaller than 8mm in diameter, sprouting angiogenesis has been predominantly observed while intussusceptive angiogenesis has been commonly seen in larger tumours [138-141].

Intussusceptive angiogenesis process enables the vascular network to keep growing. Therefore, the primary treatments are focused on inhibition of sprouting angiogenesis [142]. The regulation of intussusceptive angiogenesis is induced by many factors including hemodynamic factors such as shear stress, cyclic stretch, [134] and molecular factors such as VEGF and VEGF isoforms as most likely candidate and angiopoietin as angiogenic growth factor and fibroblast growth factor 2 (FGF2) and PDGFB, VE-cadherin, ephrin-B, and monocyte chemotactic protein 1 [143, 144].

2.2.11 Vascular co-option

This type of angiogenesis has recently been observed in developing tumours and during initial tumour growth. Vascular co-option is described in well-vascularized tissues such as brain and lung [145-147]. Tumours can grow beyond size by parasitizing on pre-existing vasculature without stimulating an angiogenic response while there is a robust angiogenic response in tumour periphery which unfortunately help to remain of tumour

cell in later stage [125]. These tumours associated with host blood vessels that will embed in tumours and eventually, the initial co-opted vessel. Furthermore, an identical mechanism to vascular co-option has been observed in healing wound tissue. Anti-angiogenic therapies can be effective in inhibition of vessel growth and block angiogenesis but in brain tissue, increasing of co-option of host vessel was described [125, 148].

2.2.12 Vascular mimicry

Adequate blood supply plays a critical role in the improvement of solid tumour growth. Several mechanisms have been identified in tumour vascularization and are involved with the endothelial cell, except one process that called vasculogenic mimicry (VM), Vasculogenic mimicry can contribute to the formation of vessel-like structure in a highly aggressive tumour cell by high plasticity advantage of aggressive tumour cells [149].

Indeed, they do not arise from the angiogenic event and increasing the growth of pre-existing vessel, they are specifically not blood vessels, and their function is plasma and red blood cell transportation. Subsequently, vasculogenic mimicry is divided into two distinctive types; Tubular and patterned matrix type vasculogenic mimicry. Tubular type has a morphological similarity with endothelial cell-lined blood vessels. Patterned matrix type vasculogenic mimicry that have no morphological and topological similarity with the blood vessel. It has been detected in many types of cancer including inflammatory and ductal breast carcinoma, ovarian and prostatic carcinoma, cutaneous and mucous membrane melanomas, uveal, soft tissue sarcomas such as synovial sarcoma rhabdomyosarcoma, and osteosarcoma. Additionally, multiple studies have shown a strong correlation of vasculogenic mimicry in tumours with poor prognosis [149-152].

2.2.13 Lymph angiogenesis

As part of the vascular circulatory system, the lymphatic system is composed of capillaries, collecting vessels and ducts, which drains extravasated fluid, collects lymphocytes and returns it to circulation. Unlike the blood vessels, lymphatic vessels are one-way and open-ended. Studies have shown the role of the lymphatic system in tumour progression, as one of the first signs of cancer is metastasis to the regional lymph nodes and in some cancers the primary mode for spread including breast cancer. There limited information available on the lymphatic system markers and molecular regulation of its development. However, with isolation and culture of lymphatic endothelial cells has made the identification of several specific markers possible [153-155]. Vascular endothelial growth factor receptor-3 (VEGFR- 3) and later on lymphatic vascular endothelial hyaluronan receptor-1 (LYVE-1) was the first identified lymphatic marker that was [97, 156]. Other specific markers identified include podoplanin and transcription factor Prox1 [157, 158].

Under normal conditions, lymphatic endothelial cells are quiescent like blood endothelial cells and possibly the formation of new lymphatic vessels has a similar mechanism as angiogenesis. However, there are no experimental data for a lymphangiogenic switch.

There is evidence that a tumour and inflammatory cells produce lymphangiogenic factors/receptors. VEGF-C and VEGF-D were identified as specific ligands for VEFGR-3. With VEGF-C having a necessary role, as VEGF-C null embryos are deficient in lymphatic vasculature development. Studies have shown that VEGF-C is a growth factor for lymphatic endothelial cells in vitro [159-162] and both VEGF-C and –D were able to increase lymphangiogenesis and metastasis in a mouse tumour model. Interestingly, using

a blocking VEGF-D antibody was able to reduce a tumour spread through lymphatic vessels [163, 164]. Studies have shown that mediators of angiogenesis also induce lymphangiogenesis. However irregular, the interaction of VEGF-A/VEGFR-2 stimulates the development of lymph vessels [165]. It was shown that in a dose-dependent manner, bFGF was able to stimulate both angiogenesis and lymphangiogenesis or only lymphangiogenesis in mouse cornea [166, 167]. Additionally, this study compared the ability of lymphangiogenesis in the PDGF family and observed that PDGF-BB was the most effective. Interestingly tumours that have a higher rate of lymphatic spread express higher levels of PDGFs [168]. Another angiogenic mediator with a role in lymphangiogenesis is angiopoietin-2, which is deficient mice lymphatic capillaries show irregular, and hypoplastic structure [169]. In which the genetic-transfer of angiopoietin-1 was able to correct the abnormality only in lymphangiogenesis. Morisada *et al*, 2005, demonstrated the role of angiopoietin-1 in lymphangiogenesis. It was demonstrated that angiopoietin-1 stimulated the growth and lymphangiogenesis in lymphatic endothelial cells [170]. Other mediators that have lymphangiogenesis stimulating properties include NRP-2, hepatocyte growth factor and insulin-like growth factor-1 and -2 [171-173].

With the available data on specific markers and the lymphangiogenesis development, the role of lymphangiogenesis in tumour progression becomes more important. However, the contradictory results render a complete conclusion impossible. In contrast to THE previous belief that pre-existing lymphatic vessels are used for metastasis, recent studies in animal models and human cancers have shown the presence of peritumoural and intratumoural lymphatic vessels. It is noteworthy that intratumoural lymphatic are uncommon and there are debates on their role in metastasis [174-176].

Other studies have observed that lymphatic indices have prognostic values such as lymph vessel density, lymphangiogenic growth factors [177], or the presence of tumour

cells within lymph vessels or lymph nodes [178, 179]. Therefore, inhibition of lymphangiogenesis has become an attractive avenue for treatment of cancer metastasis. Using a blocking VEGF-D antibody, Staker and co-worker were able to reduce lymphatic metastasis. Another study observed that using a VEGFR-3 fusion protein (VEGF-C/D trap) tumour metastasis and lymphangiogenesis was repressed [164, 180]. It is notable that in addition to the VEGF-C, VEGF-D/VEGFR-3 system, there are significant mediators involved in the regulation of lymphatic vessels that makes the treatment more complicated. In addition to numerous growth factors, an efficient treatment should specifically target a tumour lymphatic marker. Promising results have been reported by Zhang *et al.* and they have shown that lymphatic vessels show diversity relative to organ and stage-specific development of a tumour. Therefore, targeting may become feasible [181] Anti-lymphatic treatment may be a long way from clinical trials; however, this anti-metastatic therapy may be promising in cancer patients by lowering the incidence of lymphatic spread.

2.2.14 Angiogenesis in Disease

Angiogenesis is involved in creating the vessels ranging from fetal vessel growth and their development in the embryo to creation of the largest network in the whole of the body. This process has been arranged in several advanced mechanisms that are associated with a balance between angiogenic factors and endothelial cells and components of extracellular matrix by the paramount role of on and off signals switching. It is plausible to assume that dysfunction of angiogenesis may lead to excessive angiogenesis (adipose tissue, skin, eye, lung, intestines, reproductive system and bone) and insufficient angiogenesis (nervous system, blood vessel, gastrointestinal, lung, kidney, bone, reproductive system, skin).

Cancers commonly are involved an abnormal or excessive angiogenesis with the loss of function in one tumour suppressors and oncogene activation. The occurrence of infectious disease accompanies angiogenic gene expression by pathogen components and transformation of ECs activity also DiGeorge syndrome is characterised by the low expression of VEGF and neuropilin-1 [182-184] Autoimmune disorders may be influenced by mast cell and other leukocytes. It is noteworthy that obesity has a correlation with angiogenesis process, and fatty diet can induce angiogenesis, and subsequently, angiogenic inhibitors can be useful in weight loss [185]. Excessive and abnormal angiogenesis has been reported in some skin disease such as warts, allergic dermatitis, scar keloids, pyogenic granulomas, blistering disease, psoriasis, and associated with Kaposi sarcoma in AIDS patients [183].

Loss of Ang-2 [169, 186] or VEGF164 [187] can be involved in the occurrence of the persistent hyperplastic vitreous syndrome [188-190] as well as inflammatory bowel, periodontal disease, ascites, periodontal adhesion endometriosis, ovarian cysts, ovarian hyperstimulation. Also, uterine bleeding can be assumed as an abnormality and excessive angiogenesis in intestine organ [191].

Conversely, insufficient or vessel regression can also be pathological. For instance, Alzheimer disease is created by vasoconstriction and microvascular degeneration and cerebral angiopathy due to EC toxicity by amyloid β mechanism [192]. Mutation of Notch-3 is associated with stroke due to arteriopathy [193]. The explanation of impaired parallel vessel contributes to atherosclerosis characterisation [194]. Impaired vasodilation or angiogenesis create microvessel rarefaction which has been observed in hypertension disease [195]. Recently evidence revealed that diabetes might be dependent on mechanisms of impaired parallel growth and insufficient angiogenesis in ischemic

limbs and correlate with high neovascularization secondary in the retinal cell to pericytes dropout [196]. Also, hair loss can be due to inhibition of angiogenesis [197].

In the pre-eclampsia disease, it has been observed that soluble Flt-1 induces deprivation of VEGF in the incidence of thrombosis and hypertension [198]. Insufficient HIF-2α and VEGF production can hamper the maturation of lung in neonatal respiratory distress [199]. Meanwhile, TP-1 production disorder contributes to loss of age-related vessel function in nephropathy [200]. The decrease of VEGF expression in angiogenesis process is associated with the impaired bone formation in osteoporosis disease [201]. Also, angiogenesis inhibitors may associate with a hamper of bone fracture healing [202].

Initially, angiogenesis process was considered to involve in limited diseases such as cancer and skin and bone disease. In recent years, reports have implicated excessive or abnormal and insufficient angiogenesis process in many other disorders. Instead, resistance to angiogenic inhibitors is observed. Multiple molecular signalling is likely to be involved in angiogenic diseases thus treatment of cancer as well as other disorders requires a complex challenge between chemotherapeutic drugs and several molecular signals. Antiangiogenic therapy has an active role in inhibition of all blood vessels thus angiogenic-based therapy may be a unique approach to cancer inhibition because it does not target cancer cell. Despite mounting evidence above, effective treatments potentially include chemotherapy drug and angiogenic-based therapy combination [203, 204].

2.2.15 Angiogenesis in cancers

Angiogenesis is an essential process for tumour growth, metastasis, and tumours, which have lost growth regulatory function and therefore proliferate aberrantly. Controlling of tumour-associated angiogenesis is a tactic for inhibition of cancer progression. Many types of tumour cells are similar to a normal cell. However, there are several features, which separates them. Both need to have an adequate supply of oxygen

and nutrients and removing the wastes of metabolic process. These requirements are performed by a vascular supply for the mammalian cell. Physiological vasculogenesis and angiogenesis are relayed on by normal cell and tissue to provide their metabolic demands with vasculature providing.

Tumour expansion is depended on angiogenesis as a critical component. There are several means for establishing the blood vessel supply to the tumour cell. Tumours can induce neovascularization by one process, which is very similar to normal angiogenesis, evoking of a new blood vessel from pre-existing capillaries. In this regard, tumours supply their needs to adequate vascularization with growing around an existing vessel [205].

Circulating endothelial precursors, angioblast-like cell has been observed to contribute to a tumour -derived blood cell; however, these cells are derived from bone marrow cells and enter adult circulation [39]. Tube formation induced by tumours are abnormal structure, though these tubes play a critical role in metabolites delivery [206].

Endothelial cell and tumour cells may contribute to the building of tumour vessel walls [207]. These are pathological features of the tumour vessel that induce tumour cell growth. Tumour vessel morphology is comprised of vessels with thin wall and unbalanced diameter and tortuosity, in absent of pericytes. There are many gaps or fenestrae between endothelial cells in vessel walls that tumour cells can localise into these gaps. With growing tumour cells in the inner region, hypoxic condition increases and angiogenic mediators such as VEGF and FGF and interleukin (IL)-8 are upregulated. Herein, the expression of Ang-2 induced in the pre-existing vessel by tumour growth as far as endothelial cell apoptosis regress and tumour vessels ramify from pre-existing blood vessels [208].

2.2.16 Regulatory factors of Tumour angiogenesis

Many molecules are involved in tumour neovascularization. The action of these molecules has been reported in normal angiogenesis. Most important factors that are discussed here include VEGF (vascular endothelial growth factor), FGF (fibroblast growth factor), Bcl-2 (B-cell lymphoma 2), Notch1 (Notch homolog 1), microRNAs, Heparanase, Ang-2 (angiopoietin-2), IL-8(interleukin), and MMP-2 (matrix metalloproteinase-2).

2.2.17 Vascular endothelial growth factor (VEGF) in tumour expansion

Similar to normal angiogenesis, tumour angiogenesis tends to rely on VEGF. Angiogenesis both in normal and defective conditions require VEGF. In fact, many Tumour cell lines express VEGF *in* vitro at both protein [209] and transcription level including lung, breast, gastrointestinal tract, kidney, bladder, ovary, and endometrial carcinomas, intracranial tumours, glioblastomas, and capillary hemangioblastomas [210]. The high expression of VEGF in metastatic human colon carcinomas and Flk-1(the relative receptor) in associated endothelial cells are reported to be directly correlated with the degree of angiogenesis [211]. The increase in VEGF expression is known to be highly associated with the increase of intratumoural microvessel density, indicating a poor prognosis in breast cancer patients [212]. Furthermore, tumours may also prompt their surrounding tissue to secrete VEGF in addition to their productions. Fibroblasts hosting a VEGF promoter-driven GFP vector showed robust expression for weeks when surrounded by both implanted and spontaneous tumours. Consequently, high levels of both VEGF production and expression of its receptor in a wide variety of a tumour and tumour-associated cells and robust in tumour-associated blood vessels, respectively, propose VEGF as a key player in tumour angiogenesis/vascularization [213].

Inhibition studies strongly promote the causative role of VEGF in tumour

angiogenesis. It has been demonstrated that introducing nude mice harbouring sarcoma and glioblastoma cell tumours with anti-VEGF antibody through intraperitoneal administration, significantly decreases tumour vessel density and declines tumour cell growth [214]. Again, anti-VEGF showed almost complete inhibition of tumour neovascularization during the intravital examination of blood vessels induced in mice administered by tumour spheroids [215]. In general, evidence point to the critical role of VEGF and the effect of inhibition on activity VEGF in vivo suppresses' tumour angiogenesis and growth. The comprised ability of embryonic stem (ES) with inactivated VEGF gene to form teratocarcinoma in nude mice in comparison to control ES cells expresses the direct role of VEGF in stimulating angiogenesis *in vivo* [216].

The VEGF $^{-/-}$ ES cells showed lower numbers of blood vessels with less branching compared to those observed in control group, not only VEGF itself but also inhibiting VEGF receptor signalling can suppress tumour progression in vivo. A dramatic reduction in growth is noted in a variety of tumours, including; mammary, ovarian, lung carcinomas [96] and also C6 glioblastomas [217]. In nude mice when one of the primary negative VEGF receptor, Kinase insert domain protein receptor (Flk-1), is expressed, a reduction in blood vessel formation is observed in the manipulated/transduced neoplasms during histological examination. Although far away from clinical administration, inhibition of VEGF or its receptor not only exerts a significant reduction in tumour angiogenesis and growth but is also in vigorous to host animal, which may prove amenable for bedside properties. The possible mechanism underlying VEGF-mediated tumour angiogenesis relies on oxygen tension. It is well known that solid tumours often contain a central necrotic region resulting from an inadequate, poor oxygen delivery system [218].

Interestingly, hypoxia acts as selection criteria for areas were resistant cells to the damaging effects of hypoxia, or oxygenation-inducing cells may reside. In fact, hypoxia

can select apoptosis-defective cells lacking p53, which is partly accountable for the common observation of mutated p53 in most human cancers [219]. In the case for oxygenation induction, the ability of tumour environment to stimulate VEGF secretion and vascularization, has been observed in cancer cells. Using In situ hybridization, the mRNA of VEGF has been identified in hypoxic areas of glioblastoma neighbouring the necrotic regions, and bundles of vessels adjacent to VEGF-producing cells [220]. The induction of VEGF mRNA by tumour cells in hypoxic conditions in vitro suggests the same in vivo [110]. In all, the prominent role of VEGF in tumour vascularization is observed by these results. The mechanism involved blood vessel growth by VEGF is similar between a tumour and normal vascularization, comprised of a metabolic demand during low oxygen tension stimulates VEGF-mediated neovascularization [221].

The prominent aspect of tumours in the clinic is their ability to metastasize to new locations. In regard to that, VEGF enhances vascular permeability in addition to angiogenesis induction [102, 222, 223]. VEGF can stimulate the formation of leaky blood vessels with fragmented membranes, which are simply penetrable by neoplastic cells to initiate dispersion of a tumour. This fact demonstrates the multiple roles of VEGF in tumour expansion. However, other proteins complement VEGF in its actions for inducing blood vessel growth in tumours [224].

2.2.18 Fibroblast growth factor (FGF)

In the early1970s, Folkman et al. identified the first tumour-derived factor that stimulated endothelial cell proliferation and induced neovascularization, better known as Fibroblast growth factor (FGF). The difficulty in purification of this factor due to the lack of suitable bioassays, and angiogenic activity it was named as “tumour angiogenesis factor” [225].

With the emergence of heparin-affinity chromatography, FGF was identified as the first tumour-derived angiogenic factor [226-228]. The mechanism underlying FGF tumour vascularization was determined using a soluble form of the bFGF receptor. Applying the soluble form of bFGF receptor to suppress tumour growth and decline tumour vessel density in mice with pancreatic cell tumours induced by SV40 T antigen expression promoted by rat insulin promoter established its role in tumour angiogenesis [229]. Furthermore, co-culture of biopsied pancreatic-cell tumours with HUVECs in a 3D collagen matrix demonstrates HUVECs proliferation and migration towards tumour cells. Although induced to secrete FGF receptor, HUVECs discreetly show an angiogenic response [229]. Thus, these results indicate that FGF secretion by neoplastic cells promotes vascularization which stimulates tumour survival and growth *in vivo*. Considering that soluble FGF receptor inhibits tumour growth with a delay compared to soluble VEGF receptor after tumour induction, it is believed that VEGF acts to initiate tumour angiogenesis whereas FGF is essential for its maintenance [229].

It has been shown that bFGF and VEGF collaborate in inducing angiogenesis. In a collagen gel, this cooperation increases proliferation and cord-like structures of bovine capillary endothelial cells [230]. In vivo, in a rabbit model of hind limb ischemia, they were able to augment collateral vessel development [231].

There is also the possibility of induction of VEGF by FGF. Normal mouse mammary cells expressing Exogenous FGF4 show tumourigenic properties in nude mice and increase angiogenic properties of HUVEC cells in collagen and the angiogenic properties is related to induction of VEGF mRNA and translation by FGF4 [232]. The same observation was seen in vascular smooth muscle pulsed by bFGF [233]. bFGF also increases the expression of VEGF receptor in microvascular endothelial cells. Therefore,

these evidences suggest that FGF is involved in tumourigenesis in various pathways, which include synergy with VEGF [116].

2.2.19 Notch and tumour angiogenesis

Notch signalling has been identified to function in the modulation of cell fate determination during development and adult life [170, 171]. Notch family members (Notch1, Notch2, Notch3, Notch4) are single-pass transmembrane receptors that interact with one of five canonical Notch ligands in mammals including Jagged1, Jagged2, and Delta-like Dll1, Dll3, and Dll4. At the cell surface, Notch has been identified as intracellular and extracellular heterodimeric receptor can bind together by noncovalent bonds [172].

Interestingly, Notch has been observed to play a critical role both haematological malignancy, solid tumour and in tumour angiogenesis as either oncogene or a tumour suppressor in context-dependent manner [173]. Dysregulation of Notch ligand Dll4 has been noted in both tumour vasculature and tumour cells [174].

In addition, several lines of genetic studies have clarified that the Notch1 reduction results in hypersprouting phenotype and disruption of vasculogenesis. Thisd eventually leads to decreasing tumour growth due to poorly perfused, non-functional tumour vasculature, suggesting that loss of Notch1 as a potential tumour suppressor leads to widespread vasculature tumours and subsequent lethality via massive haemorrhaging [175-179].

Notch1 is also directly or indirectly implicated in the regulation of genes involved in angiogenesis such as VEGF-A (vascular endothelial growth factor) and vice versa [180]. It has been noted that in response to VEGF, Dlta-like4 (Dll4) in the tip cell activates Notch1 in the stalk cell which, in turn, leads to a reduction of the VEGFR-2 expression in the stalk cells, as an identical receptor for VEGF-A and C [180]. In the mouse Lewis

lung carcinoma tumour model, VEGF inhibition correlates with a decrease in Notch1 expression [181]. In the context of the role of Notch1 in cancer, Notch1 has been implicated in a wide variety of cellular process including the maintenance of stem cells, specification of cell fate, differentiation, proliferation and apoptosis [182].

The first line of evidence that indicates the Notch1 is not exclusively oncogenic and can also act as a tumour suppressive is adapted to studies on mouse skin by inducing Waf1 and repressing Wnt and Shh signalling [183, 184].

A growing body of literature is demonstrating that the reducing of the Notch1 signalling in a different type of thyroid cancers. For instance, Notch1 in downregulated in neuroendocrine tumours such as carcinoid and medullary thyroid cancers and forced expression of Notch1 significantly suppress neuroendocrine tumour growth in vitro [185].

Furthermore, Valproic acid, histone deacetylase inhibitor long used for the treatment of epilepsy, is demonstrating a positive effect of the Notch1 overexpression in medullary thyroid cancer cells (MTC). Valproic acid inhibits MTC cell growth, and induces apoptosis by inhibition of neuroendocrine tumour markers, ASCL1 and chromogranin A, and suppressed calcitonin expression [186].

The first documentation of the role of Notch1 as a tumour suppressor in follicular thyroid cancer and papillary thyroid cancer cells shows that Notch1 activation in thyroid cancer cell lines leads to suppression of proliferation by cell cycle arrest [187].

Taken together, Notch1 studies further exemplify that Notch1 signalling from tumour cell may be able to active activate endothelial cells and trigger tumour angiogenesis mainly through cross-talk between VEGF and Notch signalling pathway in vitro and in vivo.

2.2.20 Crosstalk between VEGF and Bcl-2 in tumour angiogenesis process

The regulation of tumour progression has been attributed to the balance of proliferation and apoptosis in tumour cell population. Beside the role of VEGF in tumour angiogenesis, VEGF has been observed to be a survival factor for endothelial cells and tumour cell, preventing apoptosis by inducing Bcl-2 expression [188].

In agreement with this hypothesis, Pidgeon et al demonstrated that VEGF upregulated Bcl-2 expression and anti-VEGF antibodies reduced Bcl-2 expression. These alterations in Bcl-2 expression were reflected by the levels of tumour angiogenesis progression in both murine (4T1) and human (MDA-MB-231) metastatic mammary carcinoma cell lines [188-190].

2.2.21 Angiogenic potential of tumour cells overexpressing Bcl-2

Several lines of evidence demonstrated the anti-apoptotic properties of Bcl-2 in tumourigenicity, invasion, and metastasis of different type tumour and an enhancement of Bcl-2 expression substantially increase the VEGF expression [191-193]. Since discovered that several cancer cellular pathways such as survival, proliferation, invasion and migration are angiogenesis-dependent process, researchers has evaluated whether the Bcl-2 overexpression can be related to the enhancement of or expand in the angiogenesis phenotype and moreover can interplay with another factor(s) in the regulation of VEGF expression levels. Notably, the involvement of other pro-angiogenic factors Bcl-2 mediated –angiogenesis was completely ruled out.in fact, they found an exact expression level similarity of hypoxia-induced basic fibroblast growth factor (bFGF) and transforming growth factor β-1 (TGFβ-1) in breast carcinoma in control, and Bcl-2 transfects [193-195].

This observation suggests that the effects of Bcl-2 on VEGF expression level can be relatively specific. Many studies were also performed to explain the mechanistic details

of the regulatory role of Bcl-2 in the acquisition of the angiogenic phenotype as detailed below.

Overexpression of Bcl-2 which inhibits mitochondrial metabolism and HIF-1α hydroxylase (as HIF-1α expression regulator) is inactive in hypoxia condition. Also, Bcl-2 expression can directly or indirectly modify post-translational hydroxylation of HIF-1α and also can modulate the expression of sequence-specific RNA binding proteins responsible for VEGF mRNA stabilization [193, 196].

Therefore, Bcl-2 can turn on an angiogenesis switch in response to hypoxia by providing a necessary signal to increase and maintain the angiogenic growth factor production state [192, 197, 198]. These results are in agreement with other report demonstrating that effect of Bcl-2 on VEGF can be modulated by HIF-1α in prostate carcinoma cells under hypoxic conditions [193].

Another possibility for Bcl-2 is stimulation of angiogenesis by P53 downregulation, which was ruled out by a study on breast cancer, which clarified no alteration in p53 protein expression level after Bcl-2 transfection as compared to control breast cancer cell line [193]. Furthermore, it has been indicated that overexpression of Bcl-2 can also promote VEGF expression through activation of transcription factors other than HIF-1. For example, in the MCF7 ADR cell line study, it was illuminated that bcl-2 increases nuclear factor κB (NF-κB) transcriptional activity and blocking of NF-κB resulted in inhibtion of VEGF expression *in vitro* and *in vivo*. Therefore, it is possible to speculate that Bcl-2 expression can regulate the expression level of VEGF through NF-κB alteration [199, 200]. Collectively, these results indicate a new function of Bcl-2 as an oncogene in the regulation of VEGF expression in cancer progression, which can control the tumour progression by inhibition of apoptosis and induction of angiogenesis.

Interacting roles of Bcl-2 and VEGF in Tumour Cells Survival Bcl-2 has been implicated in the faction of VEGF as a cell-specific survival factor in endothelial cell and tumour cell by inducing Bcl-2 and inhibiting tumour cell apoptosis. The bulk of published studies in neuroblastoma, mammary adenocarcinoma and leukemic cell lines demonstrated that VEGF treatments indeed increase cell survival mainly through Bcl-2 upregulation and inhibition of VEGF with anti-VEGF antibodies reduced Bcl-2 expression and induced apoptosis directly in tumour cells and endothelial cell [188, 201, 202]. The molecular mechanism by which VEGF promotes the survival is initiated with induction of heat shock protein 90 (Hsp90) and Bcl-2 expression by VEGF which is activated in VEGF receptor cell. Upon VEGF stimulation by VEGF receptor-2 (KDR), the Hsp90 binds Bcl-2 and apoptotic protease-activating factor-1 (APAF-1) and prevent the degradation of Bcl-2 and APAF-1, resulting in inhibition of apoptosis and increasing of cell survival and consequently activation of the mitogen-activated protein kinase (MAPK) pathway [201].

Ultimately, these studies showed that, besides the role of VEGF in vascular permeability, vascular development and angiogenesis could act either as a survival factor for tumour cells by inducing Bcl-2 expression level and inhibition of apoptosis.

2.2.22 Role of Bcl-2 in Endothelial Cells

In adult and embryonic blood vessels, VEGF also acts as survival factor and in vivo study showed down-regulation of VEGF and destruction of immature microvessels in hormone deficient murine model, which reflects a tumour endothelial requirement to constant survival signal from a tumour to remain viable, and disruption of this signal showed destruction of a tumour capillary bed. This evidence suggests that angiogenic activity of VEGF may be in results of collaboration with Bcl-2 and the enhancing the survival ability of endothelial cells [189, 203, 204].

To investigate how overexpression of Bcl-2 in an endothelial cell can modulate angiogenesis and affects tumour growth, the study conducted in squamous carcinoma showed high intra-tumoural vascular density and an acceleration in tumour growth progress in a tumour microvascular endothelial cells in response to overexpression of Bcl-2 in vivo [205].

In contrast with this data, recent data demonstrated that apoptosis not only occurs during the angiogenesis but also it is a vital to process for correct in vivo remodelling of endothelial cells and inhibition of apoptosis impairs vascular-like structure in vitro and reduces angiogenesis process in in vivo [206]. Ultimately, an overwhelming amount of published paper, clearly indicates that the overexpression of Bcl-2 is not only increased cell survival, improving the ability of these cells to remain viable and functional despite the constraints imposed by the tumour microenvironment.

Furthermore, overexpression of Bcl-2 increased the angiogenic activity of tumour cells but, this effect of Bcl-2 on VEGF remains controversial, seems to be the independent anti-apoptotic activity of Bcl-2, and considered as a new function for this oncogene.

2.2.23 miRNA as angiogenesis modulator

In recent years, many researchers, have shown that many human diseases such as retinopathy, cardiovascular disease, especially various cancers are implicated with the derailed expression level of mRNA. Additionally, miRNA effectively can regulate many cellular processes such as the proliferation of endothelial cells, metabolism, growth and development, apoptosis, homeostasis and angiogenesis by influencing multiple posttranscriptional factors in the gene network. The two enzymes, Drosha and Dicer, are involved in generating miRNA that occurs in consecutive and gradual processes from pre-mature RNA. It is possible to assume that Dicer inhibition can influence an endothelial cell alteration and consequently in angiogenesis process [234]. The

proliferation of T-cells [235, 236], skeletal muscle [237], neurons [238], germ cells [239-241] and heterochromatin formation [242] has been considered to involve with Dicer function. Dicer plays an elegant role in embryonic angiogenesis via regulating the expression level of critical angiogenesis regulators such as VEGF and its receptors including VEGFR-1 and VEGFR-2 and also effect on Tie-1 as its preferred receptor for angiopoietin-2 [243, 244].

Recently evidence has described different levels of Dicer protein in several tissues stimulate angiogenesis in different level [245, 246]. As expected, loss of Dicer in endothelial cells can be considered as an applicable tool for angiogenesis inhibition in the tumour, wound healing and limb ischemia [247, 248].

Correlation between angiogenesis and miRNA was initially described in endothelial cells by miRNA microarrays tools [242]. In general, more than 200 miRNA including both overexpression and attenuate have been identified to be involved in endothelial cell function and as well as in vascular functions [249]. For instant, miR-126 is over expressed, especially in brain and heart as high vascular density organs. Investigation on miR-126 have shown that endothelial cell proliferation, migration and capillary tube stabilization have a delicate correlation with regulatory function of miR-126 [250, 251].

In another example, Kuhnert and colleagues have reported the correlation between VEGF- inhibition angiogenesis and downgulation of miR-126 [250]. The angiogenesis action of VEGF induces the overexpression of miR-2a, miR-21, miR-17-5p, miR-18a, miR-155 and miR-191 in human tumours. It is considerable that these miRNAs has been proven to play regulatory role in angiogenesis, survival, and tumour growth [252-255]. In this connection, growing evidence exist that inflammatory angiogenesis has been

stimulated by function of miR-296, miR-222, miR-221, miR-126, miR-210, miR-126, miR-21, miR-17-5p [256].

In the presence of IL-3 and bFGF in inflammatory microenvironment, vascular cell biology has been observed to be regulate by miR-222 function [257]. Ets-1 and Ets-2 as transcription factors that are involved in angiogenesis and vasculogenesis process in endothelial cells, are assumed as inducible factor for several miRNA expressions such as miR-126 [258]. The blood flow induces angiogenesis sprouting in aortic arch by *klf2a* as "mechano-sensitive zink finger transcription factor", which in turn, modulate VEGF activation and miR-126 expression [259]. It was noteworthy that miRNAs are associated to efficiently regulate the tumour progression and can be used in drug target therapy in breast cancer. Alteration in specific miRNA gene expression has a direct correlation with the occurrence of angiogenesis, tumour growth, drug resistance, metastasis in endothelial cells [260].

The optimization of ectopic angiogenesis and immune response against tumour growth has recently been indicated by the function of angiomiRs (the miRNAs that are involved in angiogenesis process) [261]. Some of these angiomiRs efficiently stimulate angiogenesis with inhibition of negative angiogenesis regulators and implicated to motivate angiogenesis by the promotion of positive angiogenesis regulators [260]. Recent studies showed that miR-34 family antiangiogenic-miRs (the miRNAs that inhibit angiogenesis process) which are a part of p53 tumour inhibitory pathway play a critical role in angiogenesis process [262, 263].

2.2.24 Heparanase

Heparanase can be assumed as a significant inducer in tumour angiogenesis process. It seems that bFGF can have a mediatory function in this process. The effect of Heparanase in angiogenesis process is asymmetrical, direct and indirect. In one study, it

has been observed that Heparanase directly stimulated vascular sprouting and endothelial cell invasion.

Also, it was shown that Heparanase indirectly encourages angiogenesis in the extracellular matrix. This action occurred with the release of bFGF bounded with heparan sulphate from their sites [264].

Investigation on protein and Heparanase gene level in colon carcinoma cell and other metastases cell lines, as well as colon carcinoma metastases to lung, lymph nodes, and liver, revealed highest expression level of Heparanase mRNA and protein in compared to adjacent normal colon epithelial cell. Along with this theme, transfection of Heparanase in healthy colon tissue induced neovascularization in much higher level. Despite the mounting evidence that Heparanase may play an elegant role in tumour angiogenesis, it is not sufficient for inducing angiogenesis, and many studies explain the profound effect of VEGF and FGF as neovascularization mediator in the tumour microenvironment [264].

2.2.25 Ang2 (Angiopoietin-2)

The main action of Ang-1 and Ang-2 are attributed to normal angiogenesis. Remodelling of new capillary tubes are performed by Ang-1 signalling in the accompanying Tie2 receptor interplay between Mural cells, and the endothelial cell is managed to establish new capillary tube by Ang-1 function [265-267]. On the other hand, Ang-2 induces blood vessel regression and mostly is effective on endothelial cells. The role of Ang-2 is VEGF depended. In other words, Ang-2 actions are depended on the absence or presence of VEGF. Ang-2 induces blood vessel regression in the absence of VEGF and remodels the formation of endothelial cells with higher plasticity in the presence of VEGF production. Surviving of Tumour cells is initially depended on pre-

existing vessels and angiogenesis process is not initially induced as long as there are pre-existing vessels [268].

Ang-2 mRNA is highly expressed in new tumour blood cells. It has been assumed that Ang-2 plays ambivalent tasks on tumour angiogenesis [269]. In the first steps of tumour vessel growth, tumour cells induce Ang-2 expression, where destabilization and regression in vessels occur without any changes in VEGF expression. In parallel to tumour growth, tumour demands for metabolic production increase. Tumour cells induce neovascularization by VEGF production and vessel plasticity in the newly formed vessel by increasing level of Ang-2 mRNA expression. Herein Ang2 mRNA can be considered as an indicator of neovascularization in a tumour endothelial cells due to its expression has merely been observed in a tumour endothelial cell [269].

2.2.26 Interleukin-8(IL-8) and matrix metalloproteinase-2 (MMP-2)

Interleukin-8 is considered as a chemotactic and inflammatory cytokine, which is produced, by macrophage and other cells such as epithelial cell and airway smooth muscle cell. Recently evidence has shown that IL-8 play an important role in angiogenesis modulatory function in chronic inflammatory disease as well as it triggers angiogenesis in the rat cornea [124, 270, 271]. Upregulation of IL-8 mRNA in non-small lung tissue, melanoma cell line and other neoplastic tissue compared to healthy cells in vivo introduced an angiogenic function for IL-8 [272, 273]. These finding suggested the relevance of tumour neovascularization rate and expression of IL-8 [274].

Along with this term, endogenous IL-8 that has been produced by transfected gastric carcinoma cells effectively increased the ability of neovascularization in transfected cells as compared to control-transfected cells. Interestingly, the proliferation of HUVEC is induced by transfection of IL-8 into the endothelial cells in tumour angiogenesis process [275]. In an elegant study (Luca *et al.*, 1997), a correlation between

the expression of IL-8-transfected melanoma cell line and activity of MMP-2 was observed which is a compelling factor in the remodelling of extracellular matrix and degradation of the basement membrane. They demonstrated an increase in MMP-2mRNA expression in parallel with the increase in IL-8 expression. Therefore, expression of IL-8 stimulates the production of MMP-2 that provided a suitable situation for invasion and migration in tumour neovascularization process [276].

In another word, extracellular matrix degradation and melanoma cell adhesion are directly influenced by the expression of MMP-2. Consequently, it is plausible to assume that inhibition of MMP-2 expression can play a critical role in inhibition of tumour neovascularization [277, 278]. However, in the IL-8-transfected cells, alongside the increase in MMP-2 expression, the expression rate of VEGF and bFGF were unexpectedly intact This result indicates that IL-8 autonomously stimulates angiogenesis from upregulation of VEGF and bFGF. Consequently, the alteration of VEGF and bFGF expression does not affect IL-8-induced angiogenesis. VEGF is a significant factor in neovascularization of tumour cell, and other factors that are involved in normal angiogenesis are associated with VEGF in blood vessel formation [274, 275].

2.2.27 Tumour angiogenesis inhibitors

Many factors physiologically and pathologically are involved in stimulation of angiogenesis. Among these factors, many substances induce blood vessel growth inhibition. There are more than 40 endogenous inhibitors of angiogenesis that can be classified into 4 cohorts including; metalloproteinase tissue inhibitors (TIMPs), interleukins, interferons, and proteolytic fragments. Many of these inhibitors are used in cancer treatment as clinical therapeutic agents because angiogenesis plays critical role in supplying nutrients and oxygen necessary for tumour growth, as mentioned above.

Respectively, the members of the each of four inhibitor classes will be described below [120].

2.2.28 Interferons

Interferons are glycoprotein components which are secreted from blood vessels and comprise three members including INF-α, INF-β, INF-γ, which were initially classified by their antiviral feature [279]. One of the first components displaying its endogenous angiogenesis inhibitor effect was INF-α, which was useful in the inhibition of endothelial cell chemotaxis in vivo [123]. It has been shown that the extract of tumour cells is associated with "motility of endothelial cells across of gold-plated coverslips" and in hypothesis later, this function was inhibited by the activity of defined doses of INF–α [123].

As mentioned before, Interferons can be useful in the inhibition of angiogenesis in vivo; this is an explanation for suppression effect of INF-α in the chick vascularization in embryo area vascular [280]. The protein level of individual organs such as prostate carcinoma cell, bladder, breast, colon and kidneys, as well as the expression of bFGF mRNA have been downregulated by INF-α and INF-β [281]. The effect of INF-α expression (in vivo) on the migration of endothelial cell has recently been reported [282].

2.2.29 Interleukins

Interleukins secreted from leukocytes function in a diverse spectrum of activities including activation and proliferation of lymphocytes as well as stimulation of IgE production in B cells [283, 284]. Some interleukins can be effective in blood vessel growth, for instance, IL-8 has been reported to upregulate angiogenesis because of its Glu-Leu-Arg amino acid sequence at the NH2 terminus, and in the absence of this pattern, interleukins inhibit angiogenesis such as IL-4 [285]. The IL-4 effectively inhibits tumour angiogenesis via proliferation inhibitory function and stimulation of autoimmune systems

[286, 287]. IL-4 can be considered as a highly potential component in the suppression of neovascularization. Through this connection, IL-4 has been indicated to inhibit the neovascularization and blocking of microvascular endothelial cell migration related to bFGF activation in rat cornea models and in vivo conditions [288].

2.2.30 Tissue Inhibitors of Metalloproteinase

TIMPs are considered as significant components in the angiogenesis field and have a critical regulatory function with effects on growth and migration of endothelial cells. Many factors are involved in the angiogenic response. Among these factors, extracellular matrix (ECM) particularly is a vital component [289]. Formation of new blood vessels is associated with remodelling of ECM as pre-requisite. Furthermore, basement membrane with the provision of collagen type IV, fibronectin and laminin, could create an appropriate scaffold for adherence and migration of endothelial cells [290]. Meanwhile, certain kind of proteases including tissue inhibitors metalloproteinase (TIMPs) and their matrix inhibitors (MMPs) are associated with the destruction of essential remodelling for angiogenesis that whereby can be effective in the progression of angiogenesis in the pathogenic pattern [290-292]. For example, type IV collagen, fibronectin, laminin is broken down by MMP-2 (gelatinase A) and MMP-9(gelatinase B) [293]. Also, b FGF as a microenvironmental factor contributes to upregulation of MMP-2 and MMP-9 gelatinase expression. Along with this theme, recently it has been observed that expression level of MMP-2 is increased by VEGF [294].

Interestingly, many results published explain the paramount role of TIMPs in the regulation of MMP2 as well as inhibition of endothelial cell proliferation, migration, and invasion. Therefore, TIMPs could be an attractive component for designing of anti-angiogenic drugs [295].

2.2.31 Proteolytic fragments

Proteolytic fragments are parts of proteins separated from larger proteins, especially components of extracellular matrix. This is a natural event in proteins remodelling process. For an instant, MMP-2, plasminogen enzymes, fibronectin and collagen are derived from the extracellular matrix and are involved in reversal remodelling of the extracellular matrix.

Angiostatin and Endostatin are assumed as active tumour inhibitors that are recognized as tumour-derived inhibitors. Particularly notable is that primary tumours produce angiostatin which can inhibit distance metastases growth through inhibition of endothelial cell proliferation and consequently neovascularization. Angiostatin is a fragment protein of plasminogen enzyme [296, 297].

Meanwhile, Endostatin shares high similarity with angiostatin because both can inhibit angiogenesis with effects on endothelial cell proliferation. For an instant, Endostatin has been identified as a metastasis inhibition factor in primary Lewis a lung tumour, likewise, Endostatin has been characterized as a component divided by collagen XVIII type and hemangioendothelioma cells produced them. Accordingly, Endostatin mainly can be assumed as a practical part in inhibition of angiogenesis in future anti-angiogenic.

2.2.32 Other angiogenesis inhibitors

Many other molecules and synthetic product are involved inhibition tumour angiogenesis process, but their effects on tumour growth and distant metastases a tumour are not precisely characterized. In one fascinating study on the screening of genes involved in inhibition and promotion of tumourigenesis, TSP-1 (thrombospondin-1) was discovered [298].

In one study, activation of TSP-1 showed a significant inhibition in neovascularization rate, in vitro and in vivo condition [299]. TSP-2 (thrombospondin-2) is another molecule associated with neovascularization inhibition as well as tumour growth and has great homology with TPS-1 although with different expression level in vitro and in vivo [95, 300].

BB94 or Batimastat is a class of synthetic protease inhibitor, investigation on BB94 has shown significant influence in anti-tumour growth and antiangiogenesis activity and prevention of distant and local metastases in vivo and in vitro [301-306]. Interestingly, subsequent work has indicated much less toxicity in compared to natural metalloprotease inhibitors in early clinical trials [301].

The cell-cell connection has been required as a necessary step in angiogenesis process. Adhesive molecules cover Angiogenic endothelium surface. The primary adhesive molecules are Integrins. Using anti-integrins antibodies has been useful in angiogenesis inhibition in experimental tumours. In an elegant study, remodelling peptides containing the residue of three amino acids including Arginine, Glycine, and Aspartic (RGD) in bacteriophage has been investigated. These peptides have shown distinct tendency to interact with integrin, which consequently resulted in inhibition of tumour blood vessel growth [307, 308].

Also, certain pharmacologic agents have angiogenesis inhibitory function such as AGM1470 and TNP470 that are synthetic analogues of fungal fumagillin and play a critical role in inhibition of angiogenesis in vivo and consequently inhibition of endothelial cell proliferation [309].

In another study, it has been reported that thalidomide drug has an angiogenic inhibitory function and besides this term, thalidomide is considered as a teratogen and

can contribute to congenital limb defect in developing a foetus. Furthermore, thalidomide is considered as a treatment component for several eye diseases including macular degeneration , blindness of prematurity and diabetic retinopathy [40]. Kohn and colleagues have focused on the CAI (carboxy amido triazole) as an inhibitor of calcium channel. Effect of this inhibitory function is displayed on barricading of tumour cell migration and tumour cell proliferation. They also indicate to defer metastases by the antiangiogenic effect of CAI in animal models [310].

2.2.33 Conclusion

Angiogenesis is a sophisticated multistep process that relies on many different factors in several cell types. Various mechanisms are involved in the growth of vessel plexus, including sprouting angiogenesis, intussusceptive angiogenesis (splitting or non-sprouting angiogenesis), vascular mimicry, blood vessel co-option, lymphangiogenesis. Latter three, has only been observed in tumour growth and metastasis. Verity of growth factors and cytokines are expressed by cells, immune mediators, fibroblasts and pericytes to interact with the extracellular matrix, which results in proliferation, migration of endothelial cells as well as tube formation and vessel stabilization.

In normal physiological condition, angiogenesis is a highly demanded process for remodelling of the primary vasculature plexus and new blood vessel formation. This complicated process not only depends on chemical mediators such as genes and cytokines but biomechanical forces such as blood flow as well as share stress also play critical role in angiogenesis process. These two factors ensure that only those new vessels which are supporting a physiological function can become a part of the vascular network.

In a pathological condition such as cancer, same mechanisms as normal angiogenesis is used to promote disease, and tumour cells employ same angiogenic factors to recruit new blood vessel supply. This similarity is an advantage for tumour cell

because tumours rely on a same efficient mechanism to promote survival and evasion of the host immune system.

The discovery of endogenous pro- and antiangiogenic factors reveals that tumour survival is indeed dependent on angiogenesis and a balance between these stimulators and inhibitory factors. Because tumour survival is directly involved with blood supply, antiangiogenic therapy of cancer can represent a highly efficient approach for eradicating tumours. Notably, several agents targeting individual factors that regulate angiogenesis process as well as endogenous angiogenesis inhibitor verified an effectiveness of these agents and inhibitors in destroying established tumours. It is extensively demonstrated that tumour angiogenesis is not only dependent on endothelial cell proliferation and it is more complicated than initially thought. Indeed, the process is tightly regulated by cross-talk between several additional factors and mechanisms in both cancer and epithelial cells. Better knowledge of tumour angiogenesis process and particular role of molecular players involved in tumour development is thus urgent and may revolutionize our current understanding of cancer angiogenesis.

Role of microRNA-34 family in cancer with particular reference to cancer angiogenesis

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Paper 02: Role of microRNA-34 family in cancer with particular reference to cancer angiogenesis

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2.3.1 microRNAs

Since the discovery of miRNA in C. Elegans [311, 312], many researchers have focused their attention on elucidating the aspects of miRNA biology and function. Classed as the new generation of epigenetic gene regulators [313-315], miRNAs are 20-25 nucleotides non-coding RNAs which is estimated that about 30% of gene expression is regulated using miRNA [316]. Their main goal is repression of gene expression. After transcription by RNA polymerase II, the pri-miRNA is processed with Drosha (RNase III) and subsequently in the cytoplasm with Dicer to yield a double strand RNA. This form is then cleaved into a single strand RNA as a mature miRNA, which is then incorporated into miRNA-protein complex (miRNPs).

The miRNA in the miRNP complex identifies the seed sequence in the 3' untranslated region of the target mRNA and then either suppresses the translation or degrades the mRNA. Both processes result in downregulated expression of the protein [166, 317, 318]. Additionally, it has been shown that they can directly bind proteins [319, 320]. Therefore, on a hypothetical assumption and considering the account of mRNA genes, their varied expression patterns and consequently the vast potential of miRNA targets, suggest that miRNAs are likely to be involved in an extended spectrum of cellular processes. More than 60% of human protein-coding genes are conserved targets of miRNA [321]. The functional roles of miRNAs have been reported in many biological

events including developmental timing [322, 323], signal transduction [324] and tissue differentiation [318, 325, 326]. Thus, miRNAs play a variety of functions in the biology of a human being.

It has been shown that an alteration in miRNA expression is related to various disease states and pathologic conditions [327]. Even their processing machinery has also been involved in hereditary conditions such as fragile X mental retardation and Digeorge syndrome [328]. miRNA dysregulation is also associated with the initiation and development of cancer[329].

2.3.2 miR-34 family

The miR-34 microRNA precursor family were computationally discovered and later verified experimentally. The two distinct precursors are processed into three mature miRNAs: a and b/c. The mature miR-34a is a part of the p53 tumour suppressor network [330, 331]; therefore, it is hypothesized that miR-34 dysregulation is involved in the development of some cancers [329]. This family is transcribed from two different sets of genes located on chromosome 1 and 11. Studies have shown a preference in tissue expression with higher expression of miR-34a in brain and miR-34b and -c in lungs [332]. The presence of miR-34 products has also been confirmed in embryonic stem cells [333]. Their promoter region has p53 binding site, therefore, they are induced by p53 and thus involved in cell proliferation, survival, apoptosis [334], migration, invasion[335] and angiogenesis [336, 337].

Many_controlling genes are regulated through the actions of this family. For example, ectopic expression of this family results in an increase in factors involved in cell cycle regulation and DNA damage response (DDR) and suppression of cell cycle promoting genes [338]. Each member of this family can induce similar gene expression and repression [339]. Given their similar structure, such pattern was assumable. However,

it seems that each member has an extra affinity to a specific mRNA, which is the result of perfectly complementary sequences, for example, miR34b/c has a higher tendency to suppress c-myc.

miR-34a and miR-34b/c are responsible for cell-cycle arrest in the G1 phase. Also, miR-34b/c inhibited proliferation and colony formation in soft agar. Interestingly, the introduction of miR-34a and miR-34b/c into primary human diploid fibroblasts induced cellular senescence. Microarray analyses after the ectopic introduction of different members of the miR-34 family into various cell lines revealed hundreds of putative, downregulated miR-34targets. Cyclins D1 and E2, cyclin-dependent kinases 4 and 6, mitogen-activated protein kinase 1 (MEK1), R-Ras (RRAS), platelet-derived growth factor receptor A (PDGFRA), and hepatocyte growth factor receptor (HGFR) are among the direct targets that have been experimentally validated [340].

As a member of the p53 pathway, additionally, miR34 regulates the genes involved in apoptosis [336, 341]. Survivin and BCL2 are anti-apoptotic proteins regulated by miR-34a. On the other hand, miR-34 targets the regulatory molecules of p53 which include SIRT1 (silent mating type information regulation 2 homolog1) and YY1 (yin yang 1). SIRT1 is a NAD+ dependent class III histone deacetylase that protects cells against oxidative and genotoxic stress [342]. This downregulation creates a positive feedback loop for p53, enhancing its half-life and function. As p53 increases miR-34a transcription, increased amounts of p53 eventually leads to higher levels of miR-34a [334, 341].

2.3.3 Cancer and miR-34

Many miRNAs are deregulated in cancers via various mechanisms[343]. Genomic abnormalities such as deletion [343], amplification [344-346], and translocation [347] are common in tumourigenesis. miR-15a and -16-1 are an example which is clustered at chromosome 13q14, a frequently deleted region in B cell chronic lymphocytic leukaemia (CLL) and other cancers [348].

Epigenetic factors are heritable transcriptional silencing which can also influence miRNA expression. CpG islands hypermethylation and histone modification in promoter regions result in silencing of tumour-suppressor genes. Microarray analyses have indicated some miRNAs that are repressed by CpG hypermethylation in cancers relative to normal tissue [349]. For instance, miR-9-1 in breast cancer and miR-34a in haematological malignancies are among the hypermethylated [350]. Transcriptional and post-transcriptional regulations can also affect the expression of miRNAs. pri-miRNAs are induced by transcription factors, many of which are oncogenes or tumour suppressors. Many miRNA–transcription factor relationships have been discovered in cancers, for example, P53, c-Myc, and E2F [351].

miRNA processing and stability are also important factors that determine miRNA expression level. Studies have shown that even the expression levels of miRNA processing machinery, Dicer or Drosha , are altered in some cancers, likely due to the copy number gain [352-355].Known to regulate cell cycle, apoptosis, and differentiation, miR-34 is one of the best-characterized tumour suppressor miRNAs to date. It is lost or expressed at reduced levels in numerous cancer types. As stated above, miR-34 functions downstream of p53 by regulating genes to induce cell cycle arrest, cellular senescence and apoptosis and re-introduction of miR-34 mimics growth inhibition in vitro and in vivo [356]. Although p53 has direct activating effects, studies have shown that miR34b is

hypermethylated and therefore silenced in many types of cancer including colorectal[357], gastric [356], mesothelioma [358], breast [359], ovary [360, 361], renal, urothelial [362], pancreas [336], prostate [363], lung [341, 364, 365] and melanoma [360, 364] regardless of presence of wild-type p53 [366]. In this regard, treatment with demethylating agents was able to activate its expression and inhibit malignant growth in vitro [367-369]. Thus, genetic and epigenetic mechanisms contribute to a loss of miR-34 expression. The common conventional therapies with their side effects and chemoresistance tendencies are giving way to more selective non-toxic treatments, which target a defined oncogene able to act in histologically different cancer type [370, 371]. As modulators of gene expression and controllers of many cellular pathways, miRNAs play an important role in the regulation of tumour suppression. Some of important miRNA are let-7, miR-34 and miR-200 [372].

miRNA replacement treatments have resulted in anti-proliferative, pro-apoptotic, and death in cancer cell [373]. miR34 is a well-known tumour suppressor, and as stated above, extensive aberrant expression profile has been observed in many cancers which reintroduction of miR-34a inhibits cancer cell growth and shows its important role in tumourigenesis. Additionally, studies have shown that an important ability of miR-34 is inhibition of cancer stem cells. CD44 or CD133 positive prostate and breast cancer cells express lower levels of miR-34a and ectopic expression of miR34 hampers sphere formation in soft agar and tumourigenicity *in vivo* [374-377]. This impact can be attributed to the inhibitory effects miR34 has on pluripotency genes NANOG, SOX2, and MYCN [376, 378].

Other pathways regulated by miR34 include Wnt signalling [335, 379], AKT pathway [380] and notch [363] which regulate growth, EMT and metastasis. Given that more than 50% of all human cancers show defects in the p53 pathway, miR-34

replacement therapy is likely to become a powerful therapeutic approach. The ability of miR-34 to influence several pathways may be synergistically beneficial when combined with conventional treatments. As experiments have shown, miR-34a alleviates chemo-resistance in various cancer cell models [363]. This attenuation has been partly attributed to the modulatory role of miR-34 on HuR, Bcl2, Sirt1 expression and MAGE-A and p53 expression [381, 382]. As cell models have shown the efficacy of miR-34 treatment, there are few animal studies, which have shown that vector-based delivery of miR-34 has therapeutic potential [365, 383-387].

However, the ultimate therapeutic benefits of miR34 in vivo depend primarily on the delivery system. As promising the animals are, development of a safe clinically relevant system needs further enhancement to achieve the standards of clinical trial drugs. In this regard, miRNA therapeutics initiated a screening process on various delivery systems with the aim of finding the most suitable system. The criteria included were (a) efficacy in mouse models of cancer, (b)miRNA biodistribution, and (c) first safety. Among the tested, the NOV340 technology SMARTICLES (Marina Biotech, Bothell, WA; MiRna Therapeutics Inc., 2011) showed the best blend of efficacy, in vivo targeting, and toxicity.

One important aspect of cancer treatment is disease management and follow-ups. In this regard, miRNAs have been the focus of many studies in cancer prognosis and diagnosis (For detailed summary please refer to reference number) [388]. Studies have shown that miRNAs are secreted as exosomes and can be used as early biomarkers in body fluids for disease diagnosis, prognosis, and response to treatment. As one of the tumourigenesis-related miRNAs, miR-34 has been studied very extensively. miR-34a has been linked to prostate [389], breast [390], and Colorectal [391] cancer metastasis suggesting that it could be a potential biomarker. Additionally, NSCLC patients

undergone resecting surgery having a longer survival, show up-regulated miR-34a expression [392].

In a study by Koufaris *et al*., it has been shown that HCC cells exposed to DNA damage or oxidative stress blocked abnormal cell proliferation when treated with miR-34a [393]. This suggests that miR-34a can be utilized in the detection of HCC. Furthermore, it has been reported that decreased expression of miR-34a is linked with pathogenesis, outcome [393] and poor overall survival [394]. Studies have demonstrated the direct correlation of miR-34 deregulation with epigenetic and genetic mechanisms in cancers of lung, skin [10-13], pancreas, ovary [11, 14, 15], breast , urinary bladder, kidney [11, 15] , colon [16] , prostate [17], liver [18, 19], brain [20, 21], cervix [22, 23], oesophagus [24] and lymphoid system [25-27]. Anti-oncogenic functions of miR-34 have also been noted in cancers of pancreas [11], lung [10], brain [28, 29], liver [18], skin [30, 31], prostate [11], bone [32, 33] as well as in leukaemia and lymphoma [27, 34]. In these cancers, miR-34 family members contribute to carcinogenesis through different mechanisms described below.

2.3.4 Angiogenesis and miR-34

Due to their high metabolic rate, cancer cells are dependent on the extra amount of blood supply. Angiogenesis is one of the hallmarks of cancer. Angiogenesis and vasculogenesis are normal physiological processes utilized in situations which higher levels of nutrients are needed, for example in wound healing and developing embryo [395]. However, the growing tumour cells take advantage of this process.

Several processes are involved in the formation of the new microvasculature. A detachment of pericytes, extracellular matrix degradation and reformation by stromal cells and guided migration and proliferation of endothelial cells by molecular mediators, sequentially govern the formation of new blood vessels [396]. After adequate

proliferation, a monolayer of endothelial cells forms a tube-like structure. Pericytes in the microvasculature, recruit to the abluminal side of the endothelium. Blood flow is then established in the newly formed vessel[397]. Angiogenesis is a highly regulated process, which continues to the extent of need. In the regulatory side, there are positive and negative factors affecting this process; soluble polypeptides, cell-cell and cell-matrix interactions, and hemodynamic forces [398]. As an example, some important factors are named here.

One of the most important and renown mediator of angiogenesis is Vascular Endothelial Growth Factor (VEGF), a highly conserved glycoprotein with high levels of expression in reproductive organs and in mostly of tumours [399]. Initially isolated from tumour cell conditioned medium [400], VEGF has various effects on endothelial cells. Increased endothelial cell proliferation[401], migration [402] and permeability [403], production of plasminogen activators (uPA and tPA), plasminogen activator inhibitor-1 (PAI-1) [404], interstitial collagenase [405], and inhibition of endothelial cell apoptosis [406]are the observed effects in vitro. In vivo studies have shown that VEGF is responsible for capillary sprouts and formation of vascular tube structure[407].

Other angiogenic factors include Angiopoietins and their respective Tie receptors, which are responsible for the integrity and survival of endothelial cells [408]. FGFs promote endothelial proliferation and migration and have major role in wound healing angiogenesis [409]. PDGF is also a necessary factor for pericytes that maintain the stability of capillary wall [410].

Likewise, there are many studies showing the different impacts of miRNAs in the biology of endothelial cells. miRNAs have emerged as an important factor regulating cellular function and responses. The importance of miRNAs in endothelial

cell function was demonstrated by the silencing of the Dicer enzyme, which resulted in a reduction of the mature miRNA profile. Increased activation of the eNOS pathway [411], reduced endothelial proliferation, migration and cord formation was the consequence of dicer knock down [412]. The above results show that miRNAs are important in the physiological function of ECs. As a network, miRNAs regulate the process of angiogenesis in endothelial cells, balancing the pro and anti-angiogenic responses. 27 highly expressed miRNAs have been identified to play a role in endothelial biology, 15 of which were predicted to regulate the expression of receptors for angiogenic factors. For example, the expression of VEGFR2, endothelial nitric oxide synthesis (eNOS) [413] and IL8 [414] is shown to be regulated via miRNAs. Other exemplary pro-angiogenic miRNAs include miR130a, miR210, miR424, let-7 family, miR27b and the miR17-92 cluster. On the other hand, miR-221 and -222 are among the anti-angiogenic miRNAs. The names and function of involved angio-miRs are summarized in table 2.

Table 2: List of miRNAs, which have a role in endothelial physiology and tumour angiogenesis.

Name	Target	Function	Ref.
miR-126	*SPRED1, PIK3R2, VCAM1*	maintaining vascular integrity, endothelial cell proliferation, migration, tube formation and sprouting	[251]
miR-221 and miR-222	*p27, c-kit* mRNA	regulates cell cycle progression, decreased cell migration and downregulation of endothelial nitric oxide synthase expression	[415]
miR-17–92 cluster	*HIF-1 alpha, E2F1, ITGA5*	Endothelial cell sprouting, tube formation, pro-angiogenesis phenotype and reduce p53	[416]
miR-130a	*GAX, HOXA5*	proliferation, migration, and tube formation	(137, 138)
miR-21 and miR-31	*HIF-1alpha* expression, *PTEN* suppression	inducing matrix metalloproteinase expression	[417]
miR-320	*VEGFR2, IGF-1, IGF-1R*	anti-angiogenesis	[418, 419]
let-7 family and miR-27b	*Tsp-1*	sprout formation	[420]
miR-155	*Angiotensin II type I receptor*	anti-angiogenesis	[421]
miR-210	*Ephrin-A3(EFNA3), Neuronal pentraxin-1 (NPTX1)*	pro-angiogenesis, tubulogenesis and VEGF induced migration of endothelial cell growth	[422]
miR-296	*Hepatocyte growth factor-regulated tyrosine kinase substrate*, PDGFR*β*	elevated in a tumour endothelial cells, tubule length and branching of endothelial cells	[423, 424]
miR-378	*SUFU, FUS-1b*	promotes tumourigenesis and angiogenesis in vivo	[425]
miR-20a and miR-20b miR-15 and miR16	*VEGF, BCL2*	induces apoptosis block cell cycle progression	[426]
miR-34a	*SIRT1, Survivin,E2F3, CDK4*	endothelial senescence	[427]
miR-34b	*CREB*	restoration of cell cycle abnormality reduce anchorage-independent growth	[428]
miR-217	*SIRT1, FOXO3A*	endothelial senescence	[429]
miR-424	*CHK1*	migration and proliferation of endothelial cells	[430, 431]
miR-200c	*ZEB1*	senescence in response to proto-oncogene tyrosine-protein kinase (ROS) and increase p53 level	[432]
miR-9	*E-cadherin*	increased migration and angiogenesis	[433]

There are numerous publications reviewing the many factors and their respective functions involved in the process of angiogenesis; however, it is out of the scope of this mini-review. Regarding tumour angiogenesis understanding the pathology of this process is necessary. As mentioned before, the factors that contribute to physiological angiogenesis also play a role in tumour angiogenesis. However, in a tumour microenvironment, there are other additional factors. A growing tumour, demands the extra amount of oxygen and unlike physiological conditions, induces its blood vessels via sprouting of existing capillaries or recruitment of circulating endothelial progenitor cells [434].

Tumours can produce the above-mentioned angiogenic factors in copious amounts. It has been shown that a relatively high amount of VEGF and its receptor is expressed on tumour cells and the respective endothelial and stromal cells [435]. To demonstrate the critical role of VEGF, administration of anti-VEGF or anti-Flk-1 (VEGF receptor) antibodies in vivo was able to decreases tumour vessel density and inhibit tumour growth [436]. These evidences show that inhibition of VEGF activity in vivo results in reduced tumour angiogenesis and tumour growth.

As a cellular view, tumour-induced vessels have an abnormal structure. High amounts of VEGF along with Ang2 expression, induces a rather “leaky vessels” structure with increased permeability, incomplete cellular junction and a lack of basement membrane [437]. The vascular bed is sufficient to provide the tumour cells with adequate nutrient supply and the opportunity to enter the circulation and form distant metastases. In the tumour microenvironment, local oxygen concentrations regulate VEGF production. Hypoxia stimulates the binding of hypoxia-inducible factor (HIF) to the VEGF promoter, promoting VEGF gene transcription and mRNA stability [438].

The pressure of hypoxic environment not only induces the production of VEGF, but also aids the selection of apoptosis-resistant tumour cells. These cells are the P53 mutant-type cells, and that explains the phenomenon of the increased amount of cells harbouring this phenotype in higher stages of cancer[439, 440]. IL-8 is another mediator, which has shown angiogenic abilities. In the tumour microenvironment, IL-8 is produced by macrophages in a state of chronic inflammation [441, 442]. It has been demonstrated that IL-8 is mitogenic and chemotactic for HUVECs and angiogenic in rat cornea [443].

It also has the effect of increasing the expression and activity of MMP2 [444]. Considering the important role of angiogenesis in the growth of tumour cells, inhibition of this process has been one of the major focuses in anti-cancer biology and therapeutic research [445-448]. For example, Bevacizumab, a FDA-approved monoclonal antibody against VEGF has been successfully used in combination with chemotherapy agents in clinical trials [449-452]. Bevacizumab was able to inhibit endothelial sprouting and normalize the architecture of vessels, enhancing drug uptake of a tumour [453-455]. Since then, targeting the VEGF pathway was the focus of anti-angiogenesis developments. However, several groups described that these drugs might actually accelerate metastases formation. Therefore, other targets also need to be considered [456].

miRNAs can control endothelial cell function as angioregulatory switches in tumour angiogenesis. Since a single miRNA can regulate a variety of endothelial functions by targeting multiple mRNAs, miRNA targeted therapy could greatly influence endothelial cell behaviour. In this regard, miRNAs, especially those that are involved in endothelial cell biology, have attracted attention for targeted anti-angiogenesis therapy. Of note, in anti-cancer therapies, cellular senescence has an important role. Numerous miRNAs are engaged in the regulation of cellular senescence of endothelial cells. A recent

study evaluated the expression of miR‑34a in primary endothelial cells and demonstrated that base line expression increases during cell senescence [457].

As noted thoroughly in this review, miR‑34a regulates proliferation and differentiation of many cell types. Similarly, miR-34 controls the cycle in endothelial cells. It decreases SIRT1 levels and increases acetylation of p53[334]. Mammalian SIRT1 functions as a metabolic regulator by deacetylation of histones and large numbers of proteins including protein 53 (p53), Ku70 protein, nuclear factor κβ (NF‑κβ), and peroxisome proliferator‑activated receptor γ [342].

It has been shown that miR-34a expression is downregulated in highly angiogenic endothelial cells (endothelial cells overexpressing Bcl2) as compared to normal human endothelial cells[458]. In one study, miR-34a expression was analysed in HNSCC cell line and 15 tumour samples of the oral cavity, oropharynx and larynx. Bhavna and the team demonstrated that miR-34a could regulate tumour angiogenesis through down-regulation of key proteins including E2F3, SIRT1, survivin and CDK4 whereby the function of the endothelial cell was directly inhibited. E2F3a and E2F3b are an important family of transcriptional factors that play a pivotal role in cell proliferation and differentiation and cell cycle regulation.

Bhavana also studied the correlation of VEGF expression to miR-34a as the main player in angiogenesis process and demonstrated that overexpression of miR-34a, downregulated the upstream proteins of VEGF expression such as E2F3, Myc and c-met in both of HNSCC cell line and tumour samples The expression of VEGF was significantly reduced in cell lines over-expressing miR-34a. Interestingly, they have shown that miR-34a has direct effects on the proliferation and migration of endothelial cells and tube formation was inhibited *in vitro* [459].

MicroRNA-34 family, mechanisms of action in cancer: a review

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Paper 03: MicroRNA-34 family, mechanisms of action in cancer: a review

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2.4.1 The role of miR-34 in cancer cell proliferation

Cell proliferation is an essential and fundamental process in embryogenesis and tumourigenesis. The process is also important in metastasis, as small tumours with a balance between cell proliferation and programmed cell death (apoptosis) provide dormant tumour reservoirs for metastasis in cancer patients. Metabolic activity of various kinds plays a stimulatory role for signalling cell proliferation. Consequently, the metabolic state of cancer cells can be a significant factor in their cell proliferation. Naturally, many studies have focused on cell proliferation inhibitor mechanisms for cancer biology studies and eventually treatment. Since the miR-34 family is known to repress the expression of several important genes in proliferation pathways, it has been among the microRNAs studied in cancer development.

In an interesting study, the expression of liver miR-34a was found to be upregulated in response to DNA damage and oxidative stress created by Methapyrilene HCL (MP HCL), 2-acetyl aminofluorene (2-AAF) and two non-damaging hepatocarcinogens with unknown modes of action, monuron (MON) and chlorendic acid (Ch1.Ac). In this study, it was shown that miR-34a blocked ectopic cell proliferation in male fisher rats [460]. Using nicotinamide and etoposide as therapeutic agents, cell proliferation was inhibited in leukemic cells via activation of the p53-tumour suppressor pathway and modulation of its target genes including p21, NOXA, Mcl-1 and BAX, all of which occurred due to upregulation of miR-34a function [461].

In an elegant study, Pramanik *et al.*, 2011, have reported that cell proliferation was inhibited in subcutaneous and orthotopic xenografts of pancreatic cancer in mouse models by miR-34a upregulation. They used a miR-34a nanovector and systemic intravenous delivery method to inhibit pancreatic cancer. This study also reported that enhanced miR-34a induced downregulation of SIRT1 and CD44 in mice models [462]. A different study demonstrated that upregulation of miR-34a inhibits human pancreatic cancer stem cells and cancer cell lines by targeting the anti-apoptotic factor Bcl2, as well as CDK6 and SIRT1 when used in combination with chemotherapeutic agents such as 5-Aza-Dc and SAHA [463]. Wang *et al.* found that the pre-transfection of lung cancer cell lines A549 and SBC-5 with miR-34a inhibits cancer cell proliferation in a p53-independent manner by increasing sensitivity to the chemotherapy drug cisplatin (DDP) and in part by downregulation of SIRT1 [464].

The importance of miR-34 in regulating cellular proliferation lies in the pathways targeted by the microRNA. Induced miR-34a upregulation by Adriamycin, a DNA damaging agent, has been found to downregulate cell proliferation by inhibition of the E2F pathway in human colon cancer cell lines (HCT116 and RKO) [465].

Kumar *et al.* reported that in head and neck squamous cell carcinoma (HNSCC), ectopic expression of miR-34a inhibits cancer cell proliferation by downregulation of its target genes including transcription factor E2F3 and survivin [459].

Definitive evidence showed that forced expression of miR-34a by the CCAAT enhancer binding protein alpha (C/EBP α-p42) in AML patients and granulopoiesis due to a mutation in the CEBPA gene, inhibited the ability of myeloid cells to proliferate via restraining the expression of E2F3 and E2F1 [466]. Transient transfection of miR-34a is also able to inhibit cell proliferation by targeting the hepatocyte growth factor receptor (c-Met) in glioma and medulloblastoma cell lines [467].

Similarly, in another study, downregulation of the hepatocyte growth factor receptor (also called MET, MNNG HOS transformation gene or c-Met) by miR-34b overexpression inhibited non-small cell lung cancer (NSCLC) proliferation. This has subsequently altered the downstream targets, phospho S392 p53 and Mdm2 which in turn leads to apoptosis in human NSCLC cell lines (A549 and SPC-A-1) [468]. A study from Kasinski's group confirmed that ectopic expression of miR-34a using a lentivirus expression system suppresses cell proliferation in both a *kraslsl-G12D/+; Trp53LSL-R172H/+* lung cancer mouse model and *kraslsl-G12D/+; Trp53LSL-R172H/+* lung adenocarcinoma epithelial cells in culture. It does this via targeting the B-cell lymphoma 2 (Bcl2) and c-Met genes and to some extent by suppressing c-Myc and cyclin-dependent kinase 4 (CDK4) [469].

In addition, Delta-like1, Notch1, c-Met and Ezh2 have been identified as being regulated by both miR-34b and miR-34c in response to DNA damage and mitogenic signals. Further functional analysis by Corney *et al.*, indicated that cooperation of both miR-34b and miR-34c in neoplastic cell lines OSN1 and OSN2 suppresses cell proliferation and colony formation by mediating function of p53 protein [470]. In addition, suppression of the expression of miR-34a and miR-34c has been found to be induced by p63, a family member of p53. This p63 repression of miR-34 and miR-34c was found to control cancer cell proliferation via removing miR-34 targeting of the cell cycle regulators Cdk4 and cyclin D1 in primary keratinocytes and embryonic skin cells [471].

An *in vivo* and *in vitro* study on glioblastoma revealed that miR-34a expression suppresses cell proliferation in proneural malignant gliomas through directly targeting the PDGF receptor (PDGFRA) [472]. Ramaiah *et al* used anthranilamide-pyrazolo [1, 5-a] pyrimidine compound to activate the p53 pathway by phosphorylation at serine residues

15, 20 and 46, resulting in downregulation of MYCN and Mdm2 as oncogenic proteins. Subsequent to the effect of that compound, increased expression level of miR-34a/c were also noticed which in turn lead to inhibition of cell proliferation via activation of apoptotic pathway in neuroblastoma cell lines Neuro-2a, SK-N-SH and IMR-32 [473]. Furthermore, reconstitution of miR-34 expression in p53 mutant human epithelial ovarian cancer (EOC) restrained cell proliferation by downregulation of Cdk4 and in part inversely correlated with Met expression [474]. Intriguingly, it has been demonstrated that induction of miR-34c by BMP2 during osteoblast differentiation directly provokes the proliferative effect of Notch1/2 and Jag1 at the post–transcriptional level in C2C12 osteoblast cells and osteoblast progenitors. This mechanism has been seen to play a pivotal role in osteosarcoma pathogenesis [475]. Concepcion *et al.* showed a correlation between expression level of the miR-34 family and cell proliferation in mice with deletions in miR-34 family target genes. Slight elevation of cell proliferation has been observed in mice with reduced or absent miR-34 expression. Interestingly, no increase in tumourigenesis, as initiated by c-Myc, was noted in these mice. The findings reported by Concepcion *et al.* imply that p53 function remains intact in miR-34 deficient mice, perhaps through other feedback systems maintaining its expression [476]. In a recent in vitro study by Iqbal et al, it has been shown that ectopic expression of p19 (Arf), a tumour suppressor gene product independently but similar to p53 in cell development, induces cell proliferation in eye-specific mouse embryo fibroblasts (MEFs) lacking p53, with indirect effects on platelet-derived growth factor receptor beta (PDGFRβ). Through the introduction of ectopic and inhibitor of miR-34a in the Arf-driven suppression of PDGFRβ, this study has also identified a link that Arf, independent to p53 effect, correlated to miR-34a level [477]. Additionally, Tanaka *et al.* reported that the downregulation of miR-34 family members induced cancer cell proliferation, resulting in

an oncogenic phenotype in non-malignant human mesothelial cell lines, peritoneal mesothelial cells (LP-9) and primary-cultured mesothelial cells (HPMCs). This was accompanied by upregulation of Bcl2, c-Met and phospho-c-Met proteins. Results demonstrated that miR-34 downregulation plays a pivotal role in the transformation of human non-malignant mesothelial cells to malignant mesothelial cells [478].

Most researchers have focused on the role of miR-34 family members in cell proliferation and expression in tissue, and there are few reports of circulating levels of miR-34 in cancers. Nugent *et al.*, however, performed a study on this and indicated that in colorectal and breast cancer patients, regardless of stage or grade of tumours, the expression level of circulating miR-34a was considerably reduced and could be considered as an accurate biomarker for prognosis and outcome of treatment [479].

Clonogenic cell growth has been prevented by re-expression of miR-34a in human p53-deficient pancreatic cancer miapaca2 and bxpc3 cell lines via downregulation of Notch1 and Notch2 and Bcl2, resulting in inhibition in the G1, and G2/M phases of the cell cycle [480]. In line with this report, other research has indicated that the restored expression of miR-34a inhibits cell growth in pancreatic ductal adenocarcinoma (PDAC), specifically in twenty-one pancreatic cancer cell lines [481]. It has been shown that genistein causes upregulation of miR-34a. Investigations on pancreatic cancer lines AsPC-1 and MiaPa-2 showed that this upregulation of miR-34a by genistein inhibits cell growth by downregulation of Notch1 [482]. Overexpression of miR-34a has been associated with inhibition of cell growth and certain morphological features in prostatic cancer cell lines (VCaP, lNCaP, CWR22rv1 and C4-2B cells) through downregulation of Notch1, androgen receptor (AR) and prostate-specific antigen (PSA) [483].

Furthermore, use of 5-aza-Dc and BR-DIM in the treatment of prostate cancer cell lines LNCaP and C4-2B and in tissue specimens with higher Gleason scores, led to

demethylation of the miR-34a promoter. Over-expression of miR-34a due to this demethylation inhibits cancer cell growth with downregulation of AR, PSA and Notch1, as in other prostate cell line studies [484].

In order to increase miR-34a expression in the breast cancer cell line (184A1 and MCF-10 A), Li *et al* used T-VISA-miR-34a as a miR-43a expression vector in an orthotopic breast cancer mouse model. Robust expression of miR-34a was thus indicated to be involved with cell growth suppression through protein level downregulation of SIRT1, CD44 and E2F3 as target genes of miR-34a. Intravenous injection of T-VISA-miR-34a markedly suppressed tumour growth without the creation of systemic toxicity [485]. Accumulating evidence indicates that induced expression of miR-34b/c in SCLC lines (H1048 and SBC5) can inhibit cancer cell growth by downregulating the protein expression levels of the miR-34a target genes CDK6 and c-Met [486].

Furthermore, in human pancreatic cancer cell lines and human pancreatic cancer stem cells, the upregulation of miR-34a caused by the therapeutic intervention of 5-Aza-Dc and SAHA agents, has been shown to inhibit cancer cell growth and cell cycle progression by downregulation of SIRT1, CDK6 and Bcl2 [463]. Induced expression of miR-34a by Delta-tocotrienol, was found to inhibit cancer cell growth in human NSCLC lines A549 and H1650 via downregulation of Notch1 and additional downstream targets including Hes-1, Bcl2, cyclin D1 and survivin [487]. It is worth noting that downregulation of miR-34a has been reported to relate with cell growth inhibition in glioblastoma multiform (GBM) cell lines U373MG and SHG44 and GBM specimens by directly modulating expression of Notch1[488]. Ji and co-workers confirmed that miR-34s is a *bona fide* miRNA for cell arrest in the G1 phase and inhibition of cell growth in human gastric cancer Kato ш cells via targeting Bcl2, HMGA2 and Notch1. Moreover, it

is possible that restoration of p53 function in p53-deficient human gastric cancer cells may be induced by miR-34 functions [489].

Roy *et al* reported that difluorinated curcumin (CDF), a synthetic analogue of curcumin and 5-aza—2'-deoxycytidine, a methyltransferase inhibitor, are two reliable agents for promoter demethylation which can cause upregulation of miR-34a/c. As expected, they also showed that upregulation of miR-34a/c in colorectal cancer (CRC) cell lines (SW620, HCT116), inhibited cell growth in both chemo-sensitive and resistant CRC through targeting of Notch1 [490].

Bommer *et al.* observed that enhanced expression of miR-34 family members inhibits cancer cell growth by downregulation of their target protein effector Bcl2 in NSCLCs [491]. A study from Li 's group confirmed that expression levels of miR-34a inversely correlate to c-Met levels. In that study, re-expression of miR-34a powerfully suppressed cell growth in human glioma and medulloblastoma cells by downregulation of c-Met and glioma cells by downregulation of Notch1/2 and CDK6 protein expression. More *in vivo* investigation has shown the accelerated inhibition of glioma xenograft growth by transient expression of miR-34a [467].

2.4.2 The role of miR-34 in cancer invasion and metastasis

Metastasis is the dissemination of cancer cells to other organs in both local and distant sites in the final stages of cancer disease progression and thus has crucial importance in prognosis and cancer-related mortality. Recent reports indicate that microRNAs have implications in the modulation of cancer metastasis processes.

The deregulation of miR-34a has been proposed as a biomarker of metastasis in prostate cancer by prostatic cancer xenografted lines in NOD and SCID mice models. Such studies have shown that downregulation of miR-34a has a direct correlation with metastatic capacity in these cancer xenograft lines [389].In addition, miR-34a has been

found to be significantly downregulated in metastatic breast cancer tissues, compared to non-metastatic breast cancer conditions, which are significantly associated with upregulation of miR-34a. It is thus possible that the expression level of miR-34a may be useful as a biomarker of metastasis [492]. miR-34a/b has also been determined to play a crucial role in small cell lung cancer (SCLC) pathogenesis. Reduced expression of miR-34a/b due to promoter methylation has been revealed to correlate with invasion capacity of the SCLC lines, H1048 and SBC5 cell. Also, overexpression of miR-34b/c inhibits cancer cell invasion by targeting c-Met and CDK6 in SCLC [486]. In a striking study, it has been shown that restoration of miR-34 expression in a human pancreatic cancer cell lines, MiaPaCa2 and BxPC3, inhibited invasion by targeting Bcl2, Notch 1 and Notch2 [480].

Li *et al*, in 2012, demonstrated that prolonged expression of miR-34a using T-VISA-miR34a as a highly accurate expression vector of miR-34a could inhibit invasion in breast cancer cell lines (184A1, MCF-10A), by downregulation of miR-34a target genes including SIRT1, CD44 and E2F3 [485]. This pattern of gene expression showed a correlation in tissues, using a series of 22 human primary breast cancers. Also, following treatment with the chromatin remodelling agents 5-Aza-Dc and SAHA, in human pancreatic cancer stem cells from primary tumours and cell lines, an increase in miR-34a expression was observed. This was associated with inhibition of invasion through targeting of Bcl2, CDK6 and SIRT1 [463].

In support of these findings, further study showed that lower expression level of miR-34b in (A549 and SPC-A-1) cell lines is associated with higher lymph node metastasis in non-small cell lung cancer (NSCLC) [468]. The c-Met gene has been known to be involved in migration and cell scattering in hepatocellular carcinoma cells (HCC), and it has been observed that upregulation of miR-34a in the HepG2 cell line inhibits the

expression of c-Met via targeting the extracellular single-regulated kinase 1 and 2 (ERK1/2) pathway [493]. In agreement with this observation, c-Met protein expression and it's 3’ UTR reporter activity have been shown to be inhibited by miR-34a in medulloblastoma and glioma cell lines [467]. The upregulation of miR-34a also inhibits cancer cell invasion in glioma cells lines through targeting protein expression of Notch-1/2, confirmed by reductions in their 3’ UTR reporter activity [486].

Ectopic expression of miR-34a is associated with inhibition of cell invasion, metastasis and pro-migratory cytoskeletal structure formation in human lung carcinoma cells and human breast carcinoma cells through inhibition of the *Arbgap1* gene, which is responsible for the production of the RHO GTPase protein [494]. In a recent study, it has been indicated that Fra-1, a new target gene of miR-34a, induces cell invasion in colon cancer cell lines (HCT116 and RKO). The overexpression of Fra-1 is influenced by MMP-2 and MMP-9 expression in invasive cells and can be inhibited by modulatory functions of p53 in a miR-34a-dependent manner. Emphasis on dissection of correlation between miR-34a and Fra-1 has unveiled that upregulation of miR-34a directly inhibits Fra-1 expression via targeting it's 3’ UTR [495]. In cervical carcinoma and choriocarcinoma cell lines, miR-34a expression plays a role in the reduction of invasion capacity by inhibition of Upa (urokinase plasminogen activator), which in turn, is mediated by Notch1 and Jagged1 [496].

Recently, some research has identified a correlation between CD44, an adhesion molecule associated with cancer stem and progenitor cells, in occurrence of cancer metastasis in colon [497], head and neck [498], ovary [499], liver [500], stomach [501] and bladder [502] cancers. CD44 is also implicated as a predisposing factor for cancer stem cell invasion. Interestingly, CD44 has been found to be a target of miR-34a. It has been revealed that over-expression of miR-34a inhibits cancer stem cell invasion and

metastasis by negative regulation of CD44 in a tumourigenic CD44+ prostate cancer cell line. Furthermore, accumulating evidence has shown the major role of miR-34a, as a modulator of p53, in negative regulation of CD44 via non-canonical p53 binding sites in the CD44 promoter [503].

Along the same lines, miR-34s can influence the re-activation of p53 as a tumour suppressor gene in p53 function deficient human pancreatic cancer cell lines [480]. Beltran and co-workers demonstrated a further connection of miR-34a to invasion and metastasis, through the mapsin protein in breast tumours in immunodeficient mice and the breast cancer cell line MDA-MB-231. When expression of the mapsin gene was induced through the use of an artificial transcription factor, the over-expression of maspin lead to inhibition of tumour metastasis. This occurred through upregulation of miR-34a and other metastasis suppressor genes such as CARNS1, DACT3 and SLC8A2 [504].

Chang and co-workers demonstrated that overexpression of miR-34a inhibits cancer cell invasion in hepatocellular carcinoma HepG2 cells [505]. Siemens *et al* showed that ectopic expression of miR-34a suppresses cancer cell invasion and metastasis by inhibition of Snail and TGF-β, which also play important roles in epithelial-mesenchymal-transition (EMT) induction and enhancement of mesenchymal-epithelial-transition (MET) [506]. In addition, forced downregulation of miR-34s increased the invasion ability of cell by upregulation of their target proteins c-Met and Bcl2 in human non-malignant mesothelial cell lines LP-9 (peritoneal mesothelial cell) and human primary-cultured mesothelial cells (HMPCs) [478], implying that the expression level of miR-34a correlates with increased survival in non-small lung cancer. *In vitro* investigation also reported that the overexpression of miR-34a inhibits distant metastasis by regulation of the Ax1 protein as an induced receptor of invasion and metastasis in H1299, MDA-MB-231 and Rko cell lines [507]. Pogribny *et al* found that exposure to

tamoxifen in female fisher F344 rats was an effective hepatocarcinogen drug and that overexpression of miR-34a could induce the downregulation of cell proliferation by inhibition of Notch1 protein [508].

Kim *et al* demonstrated that EMT-related invasion is associated with upregulation of Snail 1, a zinc finger transcriptional repressor, which increases due to a loss of p53 function and subsequently low expression of miR-34a. They showed that upregulation of miR-34a inhibits cancer cell invasion by targeting Snail1 and molecules that regulate Snail1 function such as LEF1, Axin2 and β-catenin [509]. The p53-dependent expression level of miR-34a has been found to inhibit distant and nodal metastasis by affecting EMT through a feedback loop, resulting in downregulation of IL-6R/STAT3/miR-34a [510]. Moreover, p53-dependent overexpression of miR-34a that targets MET, a key regulator of invasive growth, has been shown to inhibit cancer cell invasion and motility in OSE cell lines (OSN1 and OSN2)[511].

There is also definitive evidence that the expression of miR-34a suppresses cell invasion in the human renal carcinoma cell lines 769P and A498. In addition, miR-34a has been found to indirectly suppress RhoA transcription through inhibition of c-Myc-skp2-Miz1 transcriptional complex assembly as well as the c-Myc-P-TEFb transcriptional elongation complex and also Suppression of RhoA activity was associated with suppression of invasion in this study [512].

2.4.3 The role of miR-34 in cancer apoptosis and cell survival

The term apoptosis refers to the process of programmed cell death that provides a pivotal defence mechanism against tumour formation and progression. Targeting apoptosis pathways presents an attractive strategy for cancer treatment virtually in all cancers. It has recently been demonstrated that ectopic expression of several microRNAs, in particular, miR-34b, is associated with apoptosis.

An independent study showed that down-regulation of miR-34a is implicated in apoptosis resistance, impaired DNA damage response and chemotherapy refractory disease through inhibition of Bax and p21, especially in chronic lymphocytic leukaemia patients. It is particularly notable that irradiation stimulates miR-34a over expression that consequently regulates the expression of p53 target genes including Bax and p21, although not Puma [513]. Loss of expression of miR-34 (due to loss of 1p36 as the genomic region containing it) has frequently been reported in pancreatic cancer. Re-expression of miR-34a in non-transfected pancreatic ductal epithelial cancer cell lines (HPNE and HPDE) induces apoptosis via targeting p53-dependent mechanisms [514]. The restoration of miR-34 family members has been shown to stimulate apoptosis and chemosensitization to Doxorubicin by down-regulation of several target genes such as Bcl2, Notch1 and high mobility group AT-hook 2 (HMGA2) in p53-deficient human gastric cancer Kato ш cells [489].

Upregulation of miR-34a in chronic lymphocytic leukaemia (CLL), in which the expression of SIRT1 commonly is increased, can induce apoptosis through targeting of p53-dependent genes such as p21, NOXA, Mcl-1 and BAX. This can occur in cooperation with nicotinamide and etoposide which have been identified as SIRT1 inhibitors [514].

In support of the significant role of miR-34a in cancer cell apoptosis, a further study showed that the CD95 death receptor (APO-1/Fas), which is a p53 target gene, plays a critical role in stimulating cells to commit to apoptosis. Altered expression of CD95 promotes p53 activation, which results in regulation of miR-34 expression levels to respond to genotoxic stress and DNA damage [515]. Interestingly, Hi *et al.* reported that induced miR-34 expression by irradiation and Adriamycin increases apoptosis via targeting CDK6, E2F3, cyclin E2 and Bcl2 in a p53-dependent manner in human

osteosarcoma cell lines U2OS and SAOS-2 [516]. Another study showed links between miR-34 and apoptosis following treatment with 3,6-dihydroxyflavone (3,6-DHF), a chemopreventive flavonoid anticancer drug, which induced apoptosis in 1-methyl-1-nitrosouresa (MNU)-induced transplanted tumour growth in rats and BALB/C nude mice using MDA-MB-453 and MDA-MB-231 human breast cancer cell lines [517]. This research showed that this apoptosis was accompanied by an increase in miR-34a, indicating a potential link between the two events. As another example, Ji *et al* reported that treatment with Delta-tocotrienol, a non-toxic miR-34a stimulator, has significant correlation with apoptosis induction and increases in p53 activity in human NSCLC cell lines (A549 and H1650) through downregulation of Notch1 and its concomitant downstream targets such as Hes-1, Bcl2, survivin and cyclin D1 [487].

Upregulation of miR-34a due to stimulation by genistein, which is a natural compound and non-toxic activator of microRNAs, enhances cancer apoptosis through downregulation of Notch-1 in pancreatic cancer cell line aspc-1 and miapa-2 [482]. Wang et al observed that the upregulation of miR-34a in the HT29 colon cancer cell line is associated with enhanced cancer apoptosis by downregulation of some of its targets that are involved in apoptosis process, such as Bcl2. This enhanced apoptosis is also associated with upregulation of the DROSHA enzyme and increasing the binding between nuclear factor kappa beta (NFkB) and prostate apoptosis response protein 4 (par-4), inhibiting NFkB transport from cytoplasm to the nucleus. Prostate apoptosis response protein 4(par-4) is also able to increase the sensitivity of colon cancer to the presence of 5-fluorouracil (5-FU) via binding nuclear factor kappa beta (NFkB) in the cytoplasm. This potential synergy with a known treatment compound is a potential avenue for the use of miR-34 family members in developing new targeted treatments in cancers [518]. Forced expression of miR-34a in human glioma and medulloblastoma cells inhibits cell

survival by downregulation of Notch1/2, CDK6 and c-Met expression [467]. A study in chronic lymphocytic leukaemia (B-CLL) showed that overexpression of miR-34a promotes apoptosis by upregulation of PUMA and p21, which are canonical targets of p53 [519].

This study also examined the correlation of miR-34a and single nucleotide polymorphism 309 (SNP309) in the promoter region of ubiquitin ligase murine double minute z-protein (MDM2), a tight regulator of p53, and revealed that different genotypes of that SNP have a significant shortage of expression in miR-34a. Authors finally concluded that miR-34 have a strong therapeutic potential in malignancies [519]. Ebnar *et al.* reported that forced expression of miR-34a and miR-34b inhibited the anti-apoptotic proteins Fkbp8 and Vcl in Hela cells [520].

In an exception to the usual state of miR-34 expression in cancer cells, it has been found that the expression of miR-34a is severely elevated in a TCL1-mouse model of chronic lymphocytic leukemia (CLL) as well as in human CLL. Forced expression of miR-34a induces apoptosis in HCT-116 cell lines with intact p53, and this response is removed when p53 itself was knocked down in these cells, providing further evidence of the link between p53 and miR-34a [521].

Yamakuchi *et al* indicated the tumour suppressor function of miR-34a could regulate apoptosis in p53 wild type human colon cancer cells (HCT116) by SIRT1 protein inhibition which subsequently augmented the expression of PUMA and p21 and increased acetylation of p53 [427]. Kastl *et al* recently found that resistance to Docetaxel, a chemotherapy drug that induces apoptosis, in breast cancer cell lines MCF-7 and MDA-MB 231 is correlated with overexpression of miR-34a. It has been hypothesised that this

connection occurs via targeting miR-34a's downstream target proteins Bcl2 and cyclin D1 [522].

Downregulation of PDGFR-α/β by induced expression of miR-34a/c has been found to induce apoptosis in lung cancer cell lines A549, H460, H1299 and H1703 by increasing sensitivity to TNF-related apoptosis inducing ligand (TRAIL)-induced cell death [523]. Some efforts have been made to manipulate the miR-34 system to manipulate cancer cell behaviour. The application of a thioacetamido modified nucleic acids that specially binds with an antisense oligonucleotide of miR-34a, has been shown to modulate cancer cell apoptosis in part through the oncogenes V-Myc and N-Myc in a human embryonic kidney cell line (HEK293T) and mouse neuroblastoma cell line (Neuro 2a) [524].

Balca-Silva *et al* observed that over expression of miR-34a inhibits cancer cell survival and increases radiosensitivity in the NSCLC line A549 by downregulation of Bcl2 [525]. It has also been shown that exogenous expression of miR-34a in the retinoblastoma cell lines Y79 promotes cancer cell apoptosis by downregulation of target genes such as CCND1, CNNE2, EMP1, CDK4, MDMX, SIRT1 and E2F3 [526].

In pancreatic cancer stem cell self-renewal, the restoration of miR-34 by lentiviral miR-34-MIF transfection induces apoptosis by downregulation of the target genes Bcl2 and Notch1 [480]. miR-34a has also been reported to induce apoptosis in the pancreatic cancer cell line (MiaPaC-2) by downregulation of SIRT1, aldehyde dehydrogenase and CD44. This study also demonstrated the potential therapeutic ability of this approach in pancreatic cancer xenograft mouse models, in both intra-pancreatic (orthotopic) and subcutaneously injected tumour forms [462].

Hu *et al* have facilitated the penetration of miR-34a into pancreatic cells using a cationic nanovector that is comprised of bifunctional CC9 (CRGDKGPDC) peptides. Upregulation of miR-34a due to penetration with the nanovector induced apoptosis through decreased expression of Bcl2 and cyclin D1 in the PANC-1 cell line [527]. Re-expression of miR-34a in p53-null PC3 cell lines has shown that the miR-34a apoptosis inducing effect in chemoresistant cell lines occurs via dowregulation of SIRT1 mRNA and its protein [528]. Additionally, ectopic expression of miR-43a has found to be accompanied with sensitivity to Doxorubicin and induces apoptosis by dowregulation of E2F3 and Notch1 in the human breast adenocarcinoma cell line MCF-7 [529].

Members of the miR-34 family have been found to increase in expression due to captothecin, an inhibitor of DNA topoisomerase ı ,in p53-dependent and p53-independent manners by targeting Noxa, caspase-2, Puma and Bax in Bows melanoma cell lines [530]. Sarveswaran *et al* revealed that selenite, an anticancer nutritional supplement, could increase the rapid transactivation of p53 and upregulate miR-34b and miR34c but not miR-34a in LNCaP human prostate cancer cells through upregulation of p53 targets gene including p21, Bax, PIG-3 and DR5 [531].

However, it is important to mention the physiologic role of miR-34 and its relation to apoptosis in non-cancer tissues. For the first time Niederer and co–workers indicated that the expression of miR-34a modulates apoptosis in rheumatoid arthritis synovial fibroblasts (RASFs) via directly targeting XIAP. They also showed that enforced expression of miR-34 correlates with FASL and TRAIL rates that in turn play mediatory role in RASFs apoptosis [532]. The expression of miR-34a in cardiomyocytes and induced apoptosis appear to be related with regulatory function of bone marrow-derived mononuclear cells (BMCs) secreting insulin-like growth factor-1(IGF-1) [533].

Apoptosis in spermatogenesis is highly regulated and it recently has been shown that over expression of miR-34c in germ cells upregulates germ cell specific genes such as SPATA17, GOPC, KLHL10 and SPEF2 by targeting TGIF2 and Notch2 in a p53-independent manner [534].

2.4.4 The roles of miR-34 in cell cycle progression

Forced expression of miR-34a in prostate and pancreatic carcinoma cell lines induced cell senescence and G1 cell cycle arrest in part via targeting CDK6. This finding also showed that forced expression of miR-34a is an effective tumour suppressor which is commonly inactivated through CpG methylation in promoter region [535].

In another study, Fujita and co-workers demonstrated that ectopic expression of miR-34a can dramatically decrease cell growth, enhance cell cycle arrest and eventually increase chemosensitivity to the anti-cancer drug camptothecin by targeting SIRT1 in prostatic cancer PC3 cells as well as in DU145 cells [528].

It is tempting to speculate that combinations of miRNAs, which act on the same pathways, could be more effective at suppression of cancer growth than each miRNA alone. Following this idea, Bandi and co-workers showed that miR-15a/16 in combination with miR-34a could cooperatively induce cell cycle arrest in the G1 and G0 phases. The combined treatment decreased the protein level of retinoblastoma and cyclin E1 in NSCLC cell lines A549, H1299, H358 and H2009. It also downregulated the downstream targets of retinoblastoma such as E2F1, E2F2, E2F3, E2F7, WEE1, CARD10 and CHK1 [536].

A study from Li ‘s group confirmed that expression levels of miR-34a inversely correlate to c-Met levels. In that study, re-expression of miR-34a powerfully supressed cell growth and cell cycle progression in human glioma and medulloblastoma cells by downregulation of c-Met and in glioma cells by downregulation of Notch1/2 and CDK6

protein expression. More *in vivo* investigation has shown the inhibition of glioma xenograft growth by transient expression of miR-34a [467].

Recent studies have implied that miR-34a could have a "cell-fate determinant" function in early-stage division of colon cancer stem cells (CCSCs) by regulating of its downstream effector Notch1 [537]. Transient transfection of miR-34a in hepatocellular carcinoma HepG2 cells inhibits cell cycle in G1 phase and accumulation of cells reduce in both S and G2 phase through downregulation of its downstream target proteins including MACF1, TPM4 and CTSD. Along this theme, proteomic approaches have indicated that key target proteins of miR-34a in hepatocarcinogenesis include LMNA, ALDH2, LOC100129335 and MACF1 [505]. Reintroduction of miR-34a in neuroblastoma cell lines SK-N-AS, Kelly, SK-N-BE and NGP decreases cell cycle progression by downregulation of E2F3 (E2F transcriptional factor 3 protein) [538]. Gene inactivation of miR-34s and p53 in prostate epithelium of mice has been found to accompany an increase in the prostate stem and progenitor cell compartment by stimulating MET-dependent growth and self–renewal [539].

Zhao et al reported that miR-34a induces inhibition of cancer cell proliferation in p53 –positive and in p-53 negative cells. Irrespective of p53 status, miR-34a inhibited cancer cell proliferation by upregulation of p21 (CIP1/WAF1), a cell cycle–dependent kinase inhibitor, through dowregulation of histone deacetylase 1 (HDAC1) which is a direct target of miR-34s. This finding provides an attractive therapeutic strategy for cancer patients [540].

Chen and co-workers indicated an inverse correlation between expression level of miR-34c and its target gene cyclin E2 in nasopharyngeal carcinoma (NPC) tissue. Thereby dysregulation of miR-34c can coordinately regulate G1-S cell cycle progression in NPC tissue via targeting cyclin E2 level [541].

2.4.5 The role of miR-34a family in cell senescence (aging)

In vitro and *in vivo* investigations by Tazawa *et al* indicated that re-expression of miR-34a induces a senescence-like phenotype in human two human colon cancer cell lines (HCT116 and RKO) by E2F signalling pathway downregulation and p53-pathway upregulation [465]. He *et al* reported that overexpression of miR-34b/c inhibits growth by downregulation of EMT expression, CDK4 and CCNE2 and continuing in this theme, substantially accelerated cellular senescence in a human fetal lung cell line (IMR90) and p53-deficient mouse embryonic fibroblasts (MEFs) by enhanced expression of SA-β-Gal [542].

The hippocampus is a centre of memory formation that can be influenced by aging and during Alzheimer's disease (AD) and other neurodegenerative dementias. *In vivo* and *in vitro* investigation revealed that upregulation of miR-34c has been implicated in disruptions of hippocampal function such as AD and age-associated memory impairment in the aging hippocampus of APPPS1-21 mice models by downregulation of hippocampal SIRT1 levels [543].

Interestingly, miR-34 family members, as part of the p53 pathway display a subtle role in senescence. Upregulation of miR-34a, miR-34b and miR-34c in NHF-hTEFT cells (normal human fibroblast cells) by Nutlin-3 (an MDM2 inhibitor) induce senescence pathways in a p53 dependent manner [544]. Christoffersen *et al* have shown a novel miR-34a expression pathway where miR-34a is regulated independently of p53 effects with miR-34a upregulation transcriptionally induced by ETS family transcription factor and ELK1 downstream of B-RAF oncogene activation. In this novel pathway, induced upregulation of miR-34a enhanced cellular senescence in primary human TIG3 fibroblast cells through repression of the proto-oncogene MYC [545].

Yamkuch *et al* reported that there is a positive feedback loop between miR-34a, p53 and SIRT1 in the senescence process. The SIRT1 gene has been considered to be involved in longevity limitation and cellular senescence. Expression of miR-34a has been induced by modulatory functions of p53 and induction of miR-34a downregulates the expression of SIRT1, in turn elevating p53 activity [546], suggesting that miR-34 family upregulation can modulate degeneration which is dependent on aging. This process was specifically observed in the organ of corti in C57BL/6J and CBA/J mice strains [547]. The expression level of miR-34 family members has been indicated to modulate age-related function in the brain by downregulation of E74A, a transcriptional factor of the ETS domain that plays a critical role in steroid hormone pathways, can ablate damage caused by polyglutamine disease protein (ployQ) and affects aging and neurodegeneration in drosophila [548]. In the study of Yang *et al* it was demonstrated that the expression of miR-34a is enhanced during aging in rat tissue. Additionally, it was noted that in *Caenorhabditis elegans*, a deletion mutation of miR-34a extends lifespan (reducing senescence) and age-related physiological functions by inhibition of atg9 (an autophagy-related protein) expression [549].

Inactivation of miR-34a by CpG methylation of its promoter is associated with increasing senescence in many cancer types including prostate carcinoma cell lines as well as breast, lung, colon, bladder, kidney and melanoma cell lines and primary melanomas [535]. Ito *et al* showed that over expression of miR-34a induces cellular senescence in primary human umbilical cord vein endothelial cells (HUVEC) as well as heart and spleen cells of older mice, though suppression of SIRT1 protein [550]. In order to assess the role of miR-34a expression level in NSCLC line A549, Sun and co-workers indicated that overexpression of miR-34a induces cellular senescence by targeting G1 cell cycle arrest via suppressing cyclin-dependent kinase 6 (CDK6) and cyclin D1 [551].

Overexpression of miR-34a induces cellular senescence via downregulation *hdmx* gene transcription in part by effects on p53 and Ras signalling pathways in human tumour cell lines and primary human diploid fibroblasts [552, 553].

2.4.6 The role of miR-34 family in cancer cell migration

A wide array of migration mechanisms is utilized by cancer cells to allow them to disseminate to other locations, which are to some degree independent on their invasive capacity. Recently, numerous studies have demonstrated that dysregulation of microRNAs is involved in migration of cancer cells.

Induced expression of miR-34a by the using a T-VISA-miR-34a expression plasmid in breast cancer cell lines (184A1 and MCF-10A), resulted in persistent expression of miR-34a and suppressed cancer cell migration, demonstrating the therapeutic potential of miR-34a via targeting of E2F3, CD44 and SIRT1 [485]. In support of the tumour suppressive role of miR-34b/c in small cell lung cancer cell lines (H1048 and SBC5) Tanaka *et al* indicated that re-expression of miR-34b after 5-aza-2'-deoxycytidine treatment, significantly supressed cancer cell migration by downregulation of two target genes c-MET and CDK6 [486]. *In vivo* investigation has shown that expression of miR-34a partially downregulated Fra-1 as new target of miR-34a, in human colon cancer cell lines (HCT116 and RKO) and also expression of miR-34a supresses cancer cell migration by downregulation of MMP-2 and MMP-9, two enzymes that are implicated in cell migration and invasion which can be rescued by Fra-1 overexpression. This subsequently implies that cancer cell migration and invasion are miR-34a-dependent manners [495].

In addition, upregulation of miR-34a has been observed to inhibit cancer cell migration and cancer cell scattering in hepatocellular carcinoma cell (HCC) tissue and HepG2 cell lines through downregulation of c-Met mRNA and protein and reducing phosphorylation of extracellular single-regulated kinases 1 and 2 (ERK1/2) [493].

Emerging evidence suggests that in the breast tumour-initiating cells (BT-ICs) of MCF-7 and SK-3rd cells (breast cancer cell lines enriched for BT-ICs), ectopic expression of miR-34c inhibits cancer cell migration via targeting Notch4 [554]. In addition, miR-34a overexpression restrained the ability of hepatocellular carcinoma HepG2 cells to migrate [505]. *In vivo* and *in vitro* investigation, reported that re-expression of miR-34a restrained the migration ability of the human giloblastoma multiform cell line U251 by downregulation of cyclin-A1, cyclin-B1, cyclin-D1, cyclin-D3, cyclin-dependent kinase, epithelial growth factor receptor (EGFR) as well as Yin Yang-1, (an expression stimulator of EGFR) and upregulation of p21 and p27 proteins, which are both cyclin kinase inhibitors [555].

Another report suggests that overexpression of miR-34a inhibits cell migration and energy production by downregulation of 136 neural progenitor genes that are commonly involved with cell motility, electron transportation, oxidative phosphorylation based ATP synthesis in Wharton's jelly matrix of human umbilical cord cells (WJ-MSCs) and bone marrow mesenchymal stem cells (BM-MSCs) [556]. The ectopic expression of miR-34a in colorectal cancer cell lines SW480 and DLD-1, supresses cancer cell migration and increased chemo-sensitivity to anti-metabolite 5-fluoracil (5-FU) through downregulation of c-Kit and disruption of SCF/c-Kit axis, respectively [557]. Chondroblast migration has also been reported to be negatively controlled by miR-34a expression levels and its relevant target epha5 during chondrogenesis, in particular in chick limb mesenchymal cells [558].

2.4.7 The role of promoter methylation in miR-34 activity

Recently, it has been shown that CpG methylation in the promoter region of microRNAs and resultant expression silencing is associated with cancer occurrence in many human tissues. Along this theme, promoter hypermethylation of miR-34 family is

a common cause for the loss of their expression in cancers and has been noted in many cancers.

The expression of miR-34a is known to regulate the expression of androgen receptor (AR) and prostate specific antigen (PSA), both associated with poor outcomes in prostate cancer, and consequently the loss of miR-34a due to hypermethylation of its promoter has been identified in progression of prostate cancer tissue specimens. Thus, the demethylation of the miR-34a promoter and restoration of its expression provides a potentially effective avenue for treatment of prostate cancer [559].

Lodygin *et al* showed that aberrant DNA methylation of miR-34a promoter is associated with downregulation of miR-34a in many cancer types such as prostate carcinoma cell lines (Du 145, LAPC-4 and PC3) breast, lung, colon, bladder, kidney, melanoma cell lines and primary melanomas and is a likely cause of transcriptional silencing of miR-34a in these cancers. Indeed, loss of miR-34a expression due to promoter CpG methylation, has been identified in almost eighty percent of primary prostate carcinoma [535].

It has been recently reported that aberrant hypermethylation of mature miR-34b and miR-34c promoters are directly associated with recurrence, disease-free survival as well as poor survival in stage I (T1-T2N0) non-small cell lung cancer (NSCLC). Hence, investigation of miR-34b/c methylation or expression could thus be a potentially useful prognostic marker for lung cancer. This could be especially useful, as approximately 30-40% of patients with stage I NSCLC die because of recurrence of lung cancer [560].

Investigation of the interaction of miR-34 family members and p53 mutation indicates that methylation of miR-34 promoter region preferentially occurs in tumours expressing wild-type p53 and inactivation of miR-34 family genes by hypermethylation may substitute for loss of p53 function in colorectal cancer [561]. Single

hypermethylation in the promoter region of miR-34c is associated with its down regulation in breast cancer cells which in turn simulates self-renewal and induce epithelial mesenchymal transition (EMT) [554].

Nalls *et al* indicated that re-expression of miR-34a inhibits epithelial mesenchymal transition and self-renewal in pancreatic cancer stem cells by downregulation of the Notch pathway and N-Cadherin and upregulation of E-Cadherin [463]. Wong *et al* found that promoter hypermethylation of miR-34a b/c is found to occur in multiple myeloma cells and that re-expression of miR-34b but not miR-34c by using 5-Aza-2'-deoxycytidine inhibits myeloma proliferation and increases apoptosis in bone marrow sample of myeloma patients [562].

In line with this report, Toyota *et al* demonstrated the hypermethylation of the miR-34b/c promoter CpG island and B-cell translocation gene 4 (BTG4) in the colorectal cancer cell line HCT116. This region was indicated to be a bidirectional promoter, which regulates the expression level of both BTG4 and miR-34b/c. Therefore, there is a compelling rationale for the possibility that miR-34b/c and BTG4 play dual tumour suppressor roles in colorectal cancer [563].

Moreover, Siemens *et al* identified the inverse correlation between miR-34a promoter hypermethylation and elevated expression level of its targets including c-Met, β-catenin in liver metastases of colon cancer. Furthermore, the development risk of distant metastases has been identifying in patients with primary colon tumours that were positive for miR-34a promoter hypermethylation in combination with c-Met and β-catenin expression levels. This finding brings a new horizon to the management of the diseased patient and might be used as a prognostic tool to avoid unnecessary chemotherapy in colon cancer [564].

There is a very clear link between loss of miR-34 family expression and development/progression of many human cancers (Figure 1). The miR-34 family also has potential to be used as additional or even surrogate markers for prognosis diagnosis of human cancers. Interestingly, there are many known compounds that can up-regulate miR- 34 family expression levels, which in turn could serve to improve cancer treatment and inhibit growth of cancer cells.

Therefore, as discussed, specific delivery strategies to the site of ongoing tumour angiogenesis are vital. Besides there can be different approaches: delivery of anti-angiogenesis miRNAs to sites of tumour which could directly 'switch off' the angiogenesis process (current approach in this research study) or inhibition of the activity of those pro-angiogenesis miRNAs by using introduction of antago-miRs (Inhibitor of the miRNAs expression) as a novel class of chemically engineered oligonucleotides that prevent pro-angiogenesis miRNAs.

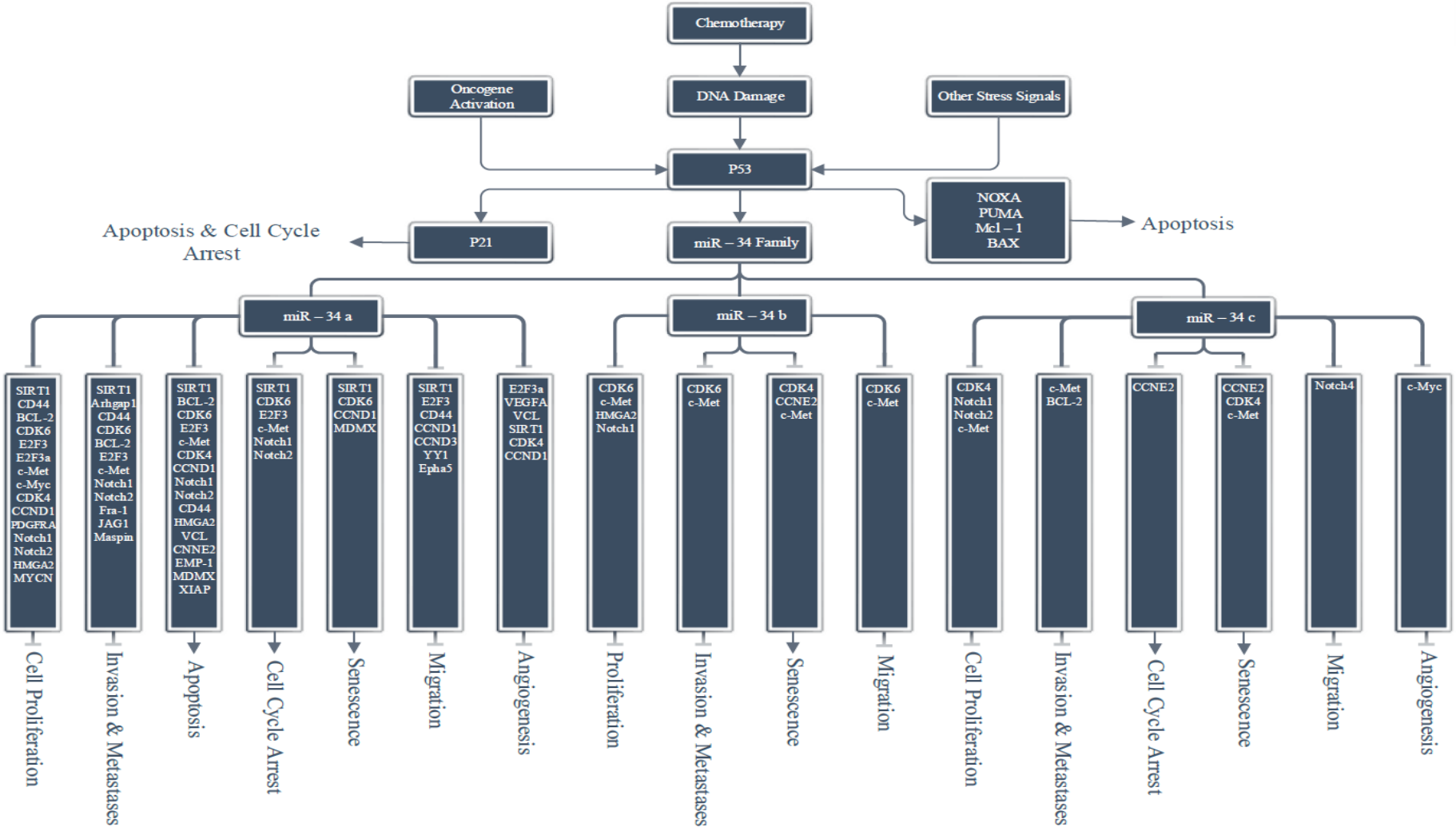

Figure 1. The diverse roles of miR-34 family in the mechanism of cancer. The members of the miR-34 family act on different process in the body through different genes. The disruption of these pathways will lead to cancer. The miR-34 family members are downstream factors in the p53 pathway

Chapter 3: Methodology

3.1 Experimental methods

In this research, first we aimed to investigate the expression of miR-34b in different thyroid cancers cell lines and tissue compared with their normal immortalized cells and investigate the major vascularization regulatory genes (Bcl2, VEGF-A, and Notch1) and their protein expression using different experimentations including qPCR, Immunofluorescent and western blot. Our second plan was to suppress Bcl-2, VEGF-A, and Notch1 expression by transient introduction of miR-34b mimic in thyroid cancer cell lines. This experiment was performed using Immunofluorescent, western blot, ELISA and checked the effect of this suppression on thyroid cancer cell proliferation status. Then we determined the effect of miR-34b (PEGlyated-miR-34b) on thyroid cancer angiogenesis *in vitro* and *in vivo*.

We checked the expression level of mir-34b in thyroid cancer cell lines after the introduction of PEGlyated-miR-34b.We evaluated the influence of PEGlyated-miR-34b on VEGF-A protein expression and secretion in thyroid cancer cell lines and cell culture medium using western blot and VEGF-A ELISA method, respectively. I investigated the effect of this manipulation on overall cell proliferation and apoptosis using cell cycle, apoptosis, wound healing, CCK-8 assays. To investigate the angiogenesis and proliferation inhibitory effect of PEGlyated-miR-34b *in vivo*, thyroid cancer cell lines were subcutaneously injected in right flank of mice and intravenous injection of PEGlyated-miR-34b were performed through the permitted and suggested time intervals. Histopathology method was performed to make the final confirmation to these finding. All experiments performed in this section involving human samples were in accordance with the ethical standards of Griffith University (MED/01/17/AEC) and with the 1964 Helsinki declaration and its later amendments or comparable to the ethical standards All the experimental methods are described below.

3.2 List of all the materials used for the study

All materials used in this study are listed below.

Table 3: Mammalian cell lines.

Cell line	Description	Obtained from/supplier
K1	Human papillary thyroid carcinoma	European Collection of Cell Cultures (ECACC)
B-CPAP	Metastasizing papillary thyroid carcinoma	German Collection of Microorganisms and Cell Cultures (DMSZ)
8505C	Undifferentiated carcinoma and histologically a largely papillary adenocarcinoma with some spindle, polygonal and giant cells	German Collection of Microorganisms and Cell Cultures (DMSZ)
MB-1	Undifferentiated thyroid carcinoma	German Collection of Microorganisms and Cell Cultures (DMSZ)
BHT-101	Undifferentiated thyroid carcinoma from lymph node metastasis	German Collection of Microorganisms and Cell Cultures (DMSZ)
Immortalized normal thyroid cell (Nthy-ori3-1)	Human thyroid follicular epithelial cell	European Collection of Cell Cultures(ECACC)

Table 4: Materials used for cell culture.

Product	Catalogue No.	Company
Rosewell Park Memorial Institute medium (RPMI)	1185	Life technologies
Dulbecco's Modified Eagles Medium (DMEM)	11995	Life technologies
Ham's F-12 Nutrient Mix	11765054	Life technologies
Fetal Bovine serum (FBS)	10099	Life technologies
MCDB 105	M6395	Sigma Aldrich
Penicillin and streptomycin solution	15140122	Life technologies

Trypsin -EDTA	15400-054	Life technologies

Table 5: List of all the antibodies used in this study.

Antibody	Catalogue No.	Company
Bcl-2 (N-19), Rabbit polyclonal IgG	Sc-492	Santa Cruz
VEGFA (A-20), Rabbit polyclonal IgG	Sc-152	Santa Cruz
Notch1 (H-131), Rabbit polyclonal IgG	Sc-99170	Santa Cruz
ß-actin (C4), Mouse monoclonal IgG1	Sc-4778	Santa Cruz
HRP conjugated antibodies		
Goat anti – Rabbit IgG	Sc-2030	Santa Cruz
Goat anti- Mouse IgG	Sc-2031	Santa Cruz
epitope antigen retrieval 2 (ER2)	AR9640	Leica Biosystems
Precision protein streptactin	1610381	BIO-RAD
Fluorescence conjugated secondary antibody		
Texas Red ® goat anti—rabbit IgG (H+L)	T-2767	Thermo Fisher Scientific
Ki-67 Rabbit polyclonal	M7240	Dako
Horseradish peroxidase conjugated rabbit secondary antibody	K1009	Dako
Eosin Y solution	17372-87-1	Sigma Aldrich
Hematoxylin	517-28-2	Sigma Aldrich
Xylene	1330-20-7	Sigma Aldrich

Table 6: List of all the kits used in this study.

Name	Catalogue No	Company
Total DNA, RNA and protein isolation kit	740933.10	MACHEREY-NAGEL
iScript™ Advanced cDNA Synthesis Kit	172-5037	BIO-RAD

Protein Quantification assay kit	740967.50	MACHEREY-NAGEL
Clarity™ Western ECL Substrate	170-5061	BIO-RAD
miScript II RT Kit (50)	218161	QIAGEN
Human VEGF ELISA Kit	KHG0111	Life technologies
miScript SYBR Green PCR Kit	218073	QIAGEN
Membrane permeability/Dead Cell Apoptosis Kit	V13243	Invitrogen
iQ™ SYBR ® Green Supermix kit	170-8880	BIO-RAD
Cell Counting Kit – 8 (CCK-8)	96992	Sigma Aldrich
high pH 50x Dako EnvisionTM Flex	K-802421-2	Dako

Table 7: Primers and Molecular markers used in this study.

Name	Catalogue No	Company
Hs_miR-34a_ 1	MS00003318	QIAGEN
Hs_miR-34b*_ 2	MS00031780	QIAGEN
Hs_miR-34b_ 2	MS00008190	
Hs_miR-34c_ 1	MS00003332	QIAGEN
Hs_RNU6B_2	MS00033740	QIAGEN
Precision Plus Protein™ Dual Colour Standards	161-0374	BIO-RAD

Table 8: List of reagents used in this study for blotting and biochemical assays.

Name	Catalogue No	Company
Paraformaldehyde	P6148	Sigma Aldrich
Triton™ X-100	9002-93-1	Sigma Aldrich
Goat serum	G9023	Sigma Aldrich
Fluoroshield™ with DAPI	F6057	Sigma Aldrich
NP40 Cell Lysis Buffer	FNN0021	Invitrogen
PMSF	329-98-6	Sigma Aldrich
Protease inhibitor cocktail	P2714	Sigma Aldrich
Immun-Blot PVDF membrane	162-0174	BIO-RAD
4–20% Mini-PROTEAN® TGX™ Precast Protein Gels	4561093	BIO-RAD
10x Tris/Glycine/SDS	161-0772	BIO-RAD
TWEEN® 20	P1379	SIGMA-ALDRICH
Blotto, non-fat dry milk	sc-2325	Santa Cruz

Restore™ PLUS Western Blot Stripping Buffer	46430	Thermo Fisher Scientific
HiPerFect Transfection Reagent	301704	QIAGEN
MiScript miRNA Mimic	MSY0000685	QIAGEN
Propidium iodide solution	P4864	Sigma Aldrich
Laemmli sample buffer (4x)	161-0747	BIO-RAD
18:1 TAP (DOTAP)	890890P(1g)	Avanti Polar
18:1 (Δ9-Cis) PE (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine	850725P	Avanti Polar
cholesterol (plant derived)	700100P	Avanti Polar
C16 PEG2000 Ceramide, N-palmitoyl-sphingosine-1-succinyl- methoxy (polyethylene glycol)2000.	880180P	Avanti Polar
tert-Butanol	360538	Sigma Aldrich
SUCROSE, RNASE & DNASE FREE	0335	AMRESCO

3.3 Cell Culture

The thyroid cancer cell lines used in this study, were purchased from DSMZ (German collection of microorganisms and cell culture) and European Collection of Cell Cultures (ECACC). B-CPAP, BHT-101, MB-1 and 8505C, were purchased from DSMZ and K1 thyroid cancer cell lines and an immortalized normal thyroid cell line (Nthy-ori3-1) were purchased from European Collection of Cell Cultures (ECACC). K1 cell line was cultured in DMEM: Ham's F12: MCDB 105 (2:1:1) + 2mM Glutamine + 10 % foetal Bovine Serum (FBS). B-CPAP and 8505C were cultured in 90% RPMI medium 1640 (Life Technologies, Carlsbad, CA, USA) + 10% of heat inactivated FBS (Life Technologies, Pty Ltd). Nthy-ori3-1 was cultured in 90% RPMI medium 1640+2mM Glutamine (Life Technologies, Carlsbad, CA, USA) + 10% FBS (Life Technologies, Pty Ltd). BHT-101 was cultured in 80% DMEM Dulbecco's modified Eagle's medium, Life Technologies, Carlsbad, CA, USA) + 20% of heat inactivated FBS (Life Technologies, Pty Ltd). MB-1 was cultured in 80% RPMI medium 1640+2mM L-Glutamine (life

technologies, Carlsbad, CA, USA) + 20% heat inactivated FBS (life technologies, Pty Ltd).

Protocol 1: Thawing and recovering the thyroid cancer cells.

1. The cryo-vial was collected from liquid nitrogen and immediately placed into a VWR water bath (Houston, TX, US) at 37°C, during this temperature, medium was thawed in 60 seconds with constant agitation of cells.
2. Vial was wiped with 70% ethanol before transferring to cell culture fume hood.
3. Washing the cells: (two steps):

   Step 1: thyroid cell lines was transferred into a sterile centrifuge 15ml falcon tube that contained 2ml of pre-warmed complete medium contains heat inactivated FBS 20% (foetal Bovine Serum) and then centrifuge for 4min at 1400 rpm Sigma Centrifuge (Sigma, St. Louis, MO, USA) and in room temperature. Then the supernatant was discarded.

   Step 2: 2ml PBS was added into falcon tube with pellet, and gently resuspended, then centrifuge for 4min at 1400 rpm in Sigma Centrifuge and in room temperature, then the supernatant was discarded. The cells were washed with PBS and fresh medium to remove residual DMSO.
4. The cell pellet was slowly resuspended into 5ml of medium containing FBS and transferred to a T75 culture flask containing the 10ml of medium and incubated at 37°C and 5% concentration of CO2 for overnight incubation.
5. The culture after 24 hours was microscopically checked to ensure that the cells attached the flask, then medium changed after overnight incubation or as necessary.
6. The medium was changed after 3 days and the culture was kept in medium containing FBS until cell lines was re-established.

7. The medium was changed every 3 days until the cells reached 85% confluence.

Protocol 2: Subculture and trypsinzing of thyroid cancer cell lines.

1. All cell lines were checked with an invert microscope to ensure all cell lines have enough confluence and absence of bacterial and fungal contamination was confirmed.
2. With a sterile pasture pipet, all medium from primary culture was removed and cells were washed twice with 3 ml of PBS.
3. 3ml 0.25% trypsin /EDTA solution was added to the T75 cell culture flask, then was rotated to ensure all cells covered with 0.25% trypsin /EDTA.
4. The flask was returned to the incubator and incubated at 37°C for 3 min, until the cells were detached, then examined the OLYMPUS inverted microscope (Shinjuku, Tokyo, Japan) to ensure all cells were detached.
5. 5ml of complete medium was added into the flask and then was drawn the cell suspension into the 15ml falcon tube to inhibit trypsin activity. To separate the trypsin and medium from cells, centrifugation was done for 4min at 1400 rpm. Cells were washed two times with PBS. The supernatant was discarded and the 3ml fresh medium was the added into the falcon tube and cells gently suspended.
6. The equal (1ml) volume of cell suspension was added to the three T75 fresh flasks containing 12 ml fresh medium and incubated at 37°C and 5% concentration of CO2.

Protocol 3: Preserving thyroid cancer cell lines.

1. All medium from each flask was discarded. 3 ml of trypsin were then added. Flasks placed in the incubator for 3 min. Cells were checked under an inverted microscope to ensure all cells detached.

2. The resuspended cells were transferred into the 15 ml centrifuge tube and then were centrifuged for 4 min at 1400 rpm and room temperature.
3. The supernatant was removed and then cells were thoroughly washed twice with warm PBS**.**
4. 3 ml of medium was added into centrifuge tube, then the cell number was counted, and the viability of cells ware checked using trypan blue staining and haemocytometer.
5. Cells suspended in freezing media containing cell medium, 20% of PBS and 10% DMSO. Cell suspension was prepared in a concentration of 1.000.000 cells per 1 ml.
6. Cryo-vials were immediately placed on wet ice for one hour, and transferred to a Mr. Frosty freezing container (Thermo Scientific) and kept for 24 hours at -80ºC for slow freezing then, cryo-vials transferred to a liquid nitrogen for long-term storage.

Protocol 4: Mycoplasma Detection.

Mycoplasma is a type of bacteria without a cell wall. Many common antibiotics such as penicillin and other beta-lactam antibiotics are effective on synthesis of the cell wall. Many cellular functions such as metabolism and cell proliferation and chromosome aberration can be affected by mycoplasma cell culture contamination.

DAPI staining (DNA Stainer) is an easy way to confirm the presence of mycoplasma in cell cultures.

The Present or absence of mycoplasma contamination in cytoplasm of cultured cells was identified by using the below protocol:

1. Cells cultured in glass bottom dish, medium removed, and cells were incubated in 4% PFA (Paraformaldehyde) for 15 min at room temperatures.

2. The cells washed twice with 1X PBS, then the cells were incubated with DAPI solution for a minute.
3. The process was continued with the washing of the cells 3 times with 1X PBS and then cells were kept in PBS.
4. The Olympus FV10i confocal microscope was used to identify the presence of mycoplasma in cells cytoplasm.

3.4 Immunofluorescent staining

Indirect immunofluorescence assay (IFA) was used to confirm the results of the western blot. This technique was performed to visualize how much of the Bcl2, VEGF-A and Notch1 RNA expression detected by PCR method has been translated into the final product. In this regard, cells were stained using the immunofluorescence method and quantified using ImageJ software (rsbweb.nih.gov/ij/) as follows:

Protocol 5: Immunofluorescent staining.

1. First, 100.000 cells were seeded with the 1 ml media onto the 12-well plate and cultured overnight at 37ºC.
2. Cells were washed one time with 1X PBS Buffer.
3. To achieve better results, fixation of the cells was performed using cold 4% Formaldehyde Solution in PBS and 400 µl of 4% paraformaldehyde Solution was then added onto each well for 20 min at 4 ºC and then 5 min at room temperature.
4. This process was followed by washing the cells two times with 1X PBS Buffer.
5. For Permeabilization, 400 µl of Triton X-100 was added onto the wells for 10 min at room temperature. The Triton X-100 was used to improve the antibody penetration.
6. The cells were rinsed with 1X PBS Buffer and then PBS was discarded.

7. For blocking, 400 µl of 5% Goat serum solution (Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA), was applied for 45 min at room temperature and then the 5% Goat serum solution was discarded (without wash).
8. Then, 400 µl of diluted primary antibodies (for BCL2, VEGF-A and Notch1 was 1:100) in 0.2% BSA was added into each well at 4 ºC overnight.
9. The cells were washed with 1X PBS Buffer three times (5 min each time) at room temperature.
10. The second blocking was performed with 1% BSA (400 µl) for each wells for 30 min at room temperature.
11. The cells were incubated with secondary antibodies, Texas Red ® goat anti—rabbit IgG (H+L) (Life technology, Grand Island, USA), that was diluted at 1/1000 (for BCL2, VEGF-A and Notch1), for 2 h at room temperature. Next step was performed in a darkroom to create appropriate protection for the slides from the light sources.
12. Cells were washed with 1000 µl of 1X PBS Buffer five times (5 min each time) at room temperature.
13. One drop of DAPI was added into each well for 1 min and the followed by mounting coverslip.

For negative control, all staining processes were performed without a primary antibody. The immunofluorescence image was captured using confocal laser-scanning microscopy (Eclipse Ti-E, Nikon) and NIS-Elements imaging software platform, version 4.13 with following setting: image Size 2,048 x 2,048 and 16 bits; Pixel/dwell of 25.2 µs; Pixel Size 0.31 µm; laser power 10%; Master gain 600–1,000. The imaging results were converted into TIFF files.

3.5 Immunohistochemical (IHC)

Formalin-fixed paraffin-embedded (FFPE) tissue samples that participated in this study were used to investigate the expression level of Ki-67 proteins as a target for miR-34b-5p. A high pH 50x Dako EnvisionTM Flex (Dako, Glostrup, Hovedstaden, Denmark) was used for the immunohistochemical analysis of both proteins.

Protocol 6: Microtome and slides preparation.

Formalin fixed paraffin embedded tissue blocks from the 27 cases which were used in this study were sectioned to 3µm thickness and placed on positively charged slides by using the microtome (Leica Biosystems, Wetzlar, Hesse, Germany). To examine Ki-67 protein. The slides left to dry overnight at 37 ˚C in order to be ready for immunostaining.

Protocol 7: Immunohistochemical (IHC) study to detect the expression profiles of Ki-67 proteins from thyroid cancer tissue samples.

1. Deparaffinised and rehydrated sections (3µm) were first processed for epitope antigen retrieval 2 (ER2) (Leica Biosystems, AR9640, Biosystems, Wetzlar, Germany) with target retrieval solution high pH 50x Dako EnvisionTM Flex (Dako, Glostrup, Hovedstaden, Denmark) in a pre-treatment module PTlink (Dako, Model PT 10130).
2. Deparaffinised and rehydrated sections continued at pH: 9.0 for 20 minutes in a microwave oven at 94˚C and then blocked with peroxidase blocking reagent for 8 minutes.
3. Tissues were incubated with Primary monoclonal mouse antibody anti-human KI67 (Clone MIB-1, M7240; Dako) at a dilution rate of 1:50 for 60 minutes at room temperature (25˚C).

4. Immunohistochemistry was performed using an automated stainer (BOND-III, Leica, Biosystems, Wetzlar, Germany) by the peroxidase-indirect- polymer method (K8000, Dako) for Ki-67.
5. Tonsil was used as positive control and for negative controls, the primary antibody was omitted during the staining.
6. A thyroid carcinoma tissue that showed strong nuclear staining was used as a positive control in each run of the experiment. Negative control sample was prepared with the same procedure, except that tissue was not incubated with the primary antibody.
7. Nuclear staining was quantitated by counting the number of Ki-67-positive cells in a total of 500 tumour cells in at least six randomly selected fields at high power (400×) under a standard light microscope. A grading scale ranging from 0 to 3 was used for this assessment, where 0 represented a negative staining, 1 represented weak staining (1-30%), 2 represented moderate staining (31 to 70%) and 3 represented strong staining (>70%). Assessment of the slides according to the extent of positive Ki-67staining was also taken in consideration. Pale yellow indicating weak-positive staining (1); brown yellow indicating moderate-positive staining (2) and nut-brown yellow indicating strong-positive staining (3).

Protocol 8: Hematoxylin and Eosin Stain.

1. Slides were incubated at 65 ˚C for 35 minutes.
2. Immersed slides in xylene (1330-20-7, Sigma Aldrich) for 35 minutes twice.
3. Immersed slides in ethanol 100% for 10 minutes twice.
4. Immersed slides in 90% ethanol for 5 minutes, 70% ethanol for one minute, 50% ethanol for 5 minutes, then 30% for one minute.
5. Rinsed with PBS for 5 minutes and wiped away wax from around the tissue.

6. Stained with 200 µl Hematoxylin (517-28-2, Sigma Aldrich) for 5 minutes and incubated at the room temperature (25˚C) for 5 minutes.
7. Stained with 400 µl of Eosin Y solution (17372-87-1, Sigma Aldrich) for 30 seconds.
8. Rinsed with PBS for 5 minutes and dehydrated in absolute ethanol, two changes for 2 minutes each.
9. Cleared in xylene and two changes for 10 minutes each and mounted the slide.

3.6 Western Blot

The following protocols were used for all western blotting experiment included in this dissertation.

Protocol 9: protein extraction.

The total protein was extracted from all cell lines using NP40 Cell Lysis Buffer (Invitrogen, Grand Island, NY) using the following protocol:

1. Centrifugation was performed to collect all the cell pallet in PBS. The cells were then rinsed twice with cold PBS.
2. The supernatant was discarded and then cell pallet was collected.
3. The cell lysis buffer was prepared for 10^{8} cells as ratios below of NP40, PMSF (1:100) and PIC (protease inhibitor cocktail) (1:10). 1 ml of prepared lysis buffer was used for 10^{8} cells and then the cell pallet was lysed in a prepared lysis buffer for 30 minutes, on ice, with overtaxing at 10-minute intervals.
4. The extract was transferred to a microcentrifuge tube and was centrifuged at 13,000 rpm for 10 minutes at 4ºC, then the clear lysate was aliquot to clean microfuge tubes and the lysates were stored at -80ºC.

Protocol 10: Protein quantification

The quality of extracted protein was assessed using a Macherey-Nagel kit (MACHEREY-NAGEL GmbH & Co. KG., Germany) and was estimated using FLUOstar Omega Multi-mode microplate reader (BMG LABTECH GmbH, Allmendgruen Ortenberg, Germany) according to follows protocol:

1. To prepare 1mg/ml BSA stock solution,1 mg of BSA was added to 1ml of protein solving buffer PSB. Number seven reaction tubes were considered to prepare BSA dilution series, Tube Number 1 contained only BSA solution (reference protein) and Tube Number 7 contained only 50 µl PSB (protein solving buffer). To tubes # 2-#7, 50 µl PBS was added, and the BSA solution was added to tubes #2- #6.
2. 20 µl of each dilution series (#1- #7) was then dispensed into microplate wells.
3. 20 µl of protein samples were added to the empty wells and then 40 µl of PSB was added to dilution series and protein samples to make a final volume of 60 µl.
4. Then, 40 µl of Quantification Reagent QR was added to each well (dilution series and protein samples), the microplate was gently shaken until a colour change from blue to yellow occurred (avoided from bubble formation as light scattering had impact on the measurement).
5. The microplate was incubated for 30 minutes at room temperature.
6. The light extinction was measured photometrically at 570 nm using a FLUOstar Omega Multi-mode microplate reader.
7. Eventually, the protein concentration of samples was calculated in relation to the BSA dilution series.

Protocol 11: Western blotting.

After determining the concentration of protein, an equilibrium amount 20 µg of each protein sample was considered for Western blot analysis. The precast Gel and

polyvinylidene difluoride transmembrane (PVDF) blot were purchased from BIO-RAD (Hercules, CA, USA).

The primary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The ladder (Precision Plus Protein™ Western C™ Standards) was purchased from BIO-RAD (Hercules, CA, USA) and the secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ß-actin was used as a positive control. The image software imager instrument (Versa Doc, Bio-Rad, Hercules, CA, USA) was recruited to measure the intensity of the protein bands. Each antibody was optimised, and Western Blot was done according to the protocol below:

1. First, the protein concentration was calculated to obtain an equal amount of each protein sample. The selected amount of protein concentration was 20 µg/µl for each well and total volume in each well was 20µl. Samples were prepared as follow: 0.05µl of ß-mercaptoethanol was added to 4.5µl of Laemmli sample buffer (4x) and depending on the protein concentration ,14 µl of each protein sample was diluted in ddH2O (double distilled water).
2. Prepared samples were heated to 95ºC for 8 minutes.
3. The concentration of SDS- PAGE running buffer was diluted from 10x Tris/Glycine/SDS Buffer to 1x. The comb and strip at the bottom of the Gel were removed, then the Gel was soaked by pouring the SDS- PAGE running buffer onto it.
4. The Gel was placed in the prepared frame and the SDS- PAGE running buffer was poured into the tank and the gap between the frames was filled with running buffer. Wells were loaded with 20 µl of each sample and the last well was loaded by 3 µl of ladder as a control.

5. The Gel was run at 100V (voltage). After Gel electrophoresis, the Stacking Gel was cut off with razor blade from the glass plates and soaked in the double distilled water for a few minutes, then Gel was carefully placed on the top side of the PVDF membrane. transferred to the membrane using the Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA). Air bubbles were removed then the turbo transfer blot was set up in 0.5 A, 25 V for 8 minutes.

6. The proteins band was transferred to the PVDF membrane and PVDF membrane which was then removed and washed twice with PBS-T (0.1% Tween 20 in PBS) for 10 minutes each time on the shaker interval.

7. Protein blocking was performed by incubating of membrane in a cold low fat 5% milk (Non-Fat Dry Milk-Santa Cruz, CA, USA) solution (5% milk in PBS-T) and was placed on the shaker for 1 hour. The 5% milk solution was discarded and the membrane was washed one time with PBS-T for 10 minutes.

8. Protein blocking was followed by incubation of membrane with a diluted primary antibody in 5% milk solution and placed on the shaker at 4º C overnight. VEGF-A, Notch1, Bcl2 and ß-actin were diluted in the ratios of 1:200 and 1:700 respectively.

9. The process was followed with membrane washing 3 times in PBS-T for 10 minutes each time. The membrane was incubated with secondary antibody diluting in PBS for 1 hour. The secondary antibody for VEGF-A, Notch1, Bcl2, ß-actin and Ladder were diluted in 1:5000 ,1:2000 and 1:10000 respectively.

10. The washing step was performed with PBS-T, 3 times, each time for 10 minutes, the final wash was performed twice with PBS, each for 10 minutes to remove any residual of Tween 20.

11. The Final step was incubating the membrane in chemiluminescence reagent (Clarity western ECL subtract, Bio-Rad, Hercules, CA, USA) which was a mixture of 1.5 ml of Luminol-Enhancer Solution and 1.5 ml of a Peroxide Solution. Incubation times for VEGF-A, Notch1, Bcl2 and β-actin were 5 minutes and 1 minute respectively. A VersaDoc Imaging System (Bio-Rad, Hercules, CA, USA) was used to capture the membrane image.

3.7 Quantitative Real Time PCR (qPCR)

The following protocol were used for Quantitative Real Time PCR (qPCR) experiment included in this dissertation.

Protocol 12: RNA purification.

Total RNA was extracted from cultured cancer cell lines using NucleoSpin® TriPrep Kits (Macherey- Nagel Company) as follow:

1. 5×10^6 of cultured cancer cells were collected by centrifugation and were lysed by adding 350 µl of RP1 buffer and 3.5 µl of β-mercaptoethanol (β-ME).
2. Reduced viscosity and lysate clearing were performed by using a NucleoSpin ® Filter (violet ring), which was placed in collection tube (2ml) and then the mixture from step 1 was added followed by centrifugation for 1minutes at 11,000 × g.
3. The NucleoSpin ® Filter was discarded and 350 µl of ethanol (70%) was added to homogenized lysate, then the mixture was mixed by pipetting up and down 5 times.
4. The lysate was loaded into a NucleoSpin ®TriPrep column that was placed in a collection tube (2 ml) and then centrifugation was done for 30 s at 11.000 × g.
5. The lysate was washed twice by 500 µl DNA wash to the NucleoSpin ®TriPrep column and centrifugation for 1 minute at 11.000 × g.

6. The NucleoSpin ®TriPrep column was placed into a 1.5 ml microcentrifuge tube with an open lid for 3 minutes.

7. 100 µl DNA elute was directly added onto the membrane and incubated for 1 minutes and DNA was eluted by centrifugation for 1 minute at 11.000 × g.

8. For digestion of residual DNA on-column, rDNase reaction mixture was prepared by adding 10 µl of reconstituted rDNase to 90 µl reaction buffer for rDNase and then 95 µl rDNase reaction mixture was directly added onto the column, then incubated at room temperature for 15 minutes.

9. Silica membrane was washed and dried in 3 steps:

9.1 200 µl buffer RA2 was added to the NucleoSpin ®TriPrep column and centrifugation was done for 30 S at 11.000 × g, then NucleoSpin ®TriPrep column was placed into a new collection tube (2ml).

9.2 600 µl buffer RA3 was added to the NucleoSpin ®TriPrep column and centrifugation was done for 30 minutes at 11.000 × g then flow-through was discarded.

9.3 250 µl buffer RA3 was added to the NucleoSpin ®TriPrep column, followed by centrifugation for 2 minutes at 11.000 × g to completely dry the membrane, then NucleoSpin ®TriPrep column was pleased into a 1.5 ml RNase-free collection tube.

10. RNA was eluted in 60 µl RNase-free H_2 O. Centrifugation was performed at 11.000 × g for 1 minutes to provide the Highly pure RNA.

Protocol 13: Purification of total RNA from Thyroid tissues.

Total RNA extraction was done by using QiagenmiRNeasy Mini Kit for purification the total RNA including miRNA from fresh frozen tissues (Qiagen GmbH9D. 40724 Hilden).

1. The procedure involved adding 700 µl QIAZOL Lysis reagent to the collecting tube which was contain the tissue slices, this step enhanced disruption and homogenization of the tissue.
2. homogenization of the of disrupted tissue was done using TissueLyser II for 40 second.
3. The tube containing homogenate placed on the benchtop at room temperature (25 ºC) for 5 minutes.
4. 140 µl chloroform was added to the homogenate and mixed vigorously for 20 seconds and paced at room temperature (25 ºC) for 3 minutes.
5. Centrifugation was done for 15 minutes at 8000 ×g at 4 ºC.
6. Upper aqueous phase was transferred to a new collection tube and 1.5 volume of 100% ethanol was added to the sample and mixed thoroughly by pipetting up and down several times.
7. 700µl of the sample was pipetted to RNeasy Mini spin column in a 2ml collection tube, followed by adding the 500 µl washing buffer and the flow-through was discarded.
8. 500 µl buffer RPE was then added into the RNeasy Mini spin column and centrifuged for 2 minutes at 8000 ×g to dry the spin column membrane. The flow-through and the collection tube discarded.
9. Placed the RNeasy Mini spin column into a new 2 ml collection tube. Centrifuged for 5 minutes at 8000 ×g at room temperature (25 ºC).
10. Placed the RNeasy Mini spin column into a new 1.5 ml collection tube and pipetted 14 µl RNase-free water onto the spin column membrane. Then centrifuged for 1 minute at 8000 ×g to elute the miRNA-enriched fraction.

11. RNA quality was quantified by using a NanoDrop™ 1000 Spectrophotometers (Thermo Fisher Scientific, Massachusetts, United State). Purity of RNA was obtained by checking the optical density (OD) 260/280 ratio. Concentration of RNA was also noted in ng/µl.

Protocol 14: cDNA preparation.

MiScript II Reverse Transcription kit (Qiagen, Hilden, NRW, Germany) was used for cDNA conversion of miRNA, according to recommended manufacturer protocol as described below:

1. Total extracted RNA containing miRNA was used as the starting material.
2. Thawed total RNA on ice.
3. Thawed RNase –free water, 10X miScript Nucleices Mix and 5X miScript HiSpec at room temperature (25 ºC).
4. Mixed each solution by flicking the tubes.
5. Centrifuged briefly to collect residual liquid from the sides of tubes and then stored on ice.
6. Prepared the revers-transcription reaction on ice according to table 7.

Table 9: Revers- transcription reaction components.

Component	Volume / Reaction
5x miScript HiSpec Buffer	4 µl
10X miScript Nucleices Mix	2 µl
RNase –free water	Variable
miScript reverse transcriptase mix	2 µl
Template RNA	1 µg
Total volume	**20 µl**

7. Added templated RNA to each tube containing revers-transcription master mix. Solution gently mixed, briefly centrifuged and then placed on ice.
8. Incubated for 60 minutes at 37ºC and then incubated for 5 min at 95ºC to inactivate miScript Reverse Transcriptase Mix and placed on ice.
9. Transferred undiluted revers-transcription reaction prior to real-time PCR to a -20 ºC Freezer.

Protocol 15: Quantitative Real Time PCR (qPCR).

1. sets of primers for evaluation of expression of miR-34a (Hs_miR-34a_1 miScript Primer Assay), miR-34b* (Hs_miR-34b_2 miScript Primer Assay) and miR-34c (Hs_miR-34c_1 miScript Primer Assay) were purchased from Qiagen (Hilden, NRW, Germany). Human RNU6B RNA was purchased from Qiagen (Hs_RNU6B_2 miScript Primer Assay) was used as ubiquitous control gene and purchased from Qiagen. iQ™ SYBR ® Green Supermix kit was purchased from BIO-RAD (Hercules, CA, USA) for performing Quantitative Real Time PCR (qPCR).
2. 30 ng/µl cDNA was diluted to obtained final concentration of 1 ng/µl for each sample.
3. Thawed iQ™ SYBR ® Green Supermix, template RNA, RNase-free water and primers. Mixed the individual solutions, briefly centrifuged and kept on ice.
4. Prepared a total volume of 20 µl reaction mixture according to table 10.

Table 10: Reaction setup for Quantitative Real Time PCR (qPCR).

Component	Volume/reaction	Final concentration
iQ™ SYBR ® Green Supermix (2x)	10µL	1x
miScript Universal Primer	1 µl	500 nM
miScript Primer Assay	1 µl	500 nM
cDNA template	2 µl	1 ng/µl
RNase-free water	6 µL	-
Total volume	**20 µL**	

5. Mixed the reaction master mix thoroughly, and then dispensed 18 µL of prepared master mix, except cDNA template into each qPCR tubes.
6. Added 2 µl the cDNA template containing reaction master mix (table 7), sealed tubes with flat caps, and then vortexed for 20 second to removed air bubbles and mixed reactions.
7. Programmed the IQ5 Multicolour Real-Time PCR detection system (Bio-Rad, Hercules, CA, United States of America) according the program outlined in table 9.

Table 11: Real time PCR program.

Cycle Type	Time	Temperature	Cycle
Initial Denaturation	5 minutes	95°C	1
Denaturation	10 Seconds	95°C	40
Annealing	30 Seconds	64°C	
Extension	30 Seconds	72°C	
Final extension	5 minutes	72°C	
Melting curve analysis	Increase in, 0.2°C increments with a hold time of 1 sec for each read	45-95°C	

8. Placed the PCR tubes into IQ5 Multicolour Real-Time PCR instrument. PCR were performed in triplicate to improve the reliability of the results and 2 µl of RNase-free water was added instead of cDNA template in the last tube of each test. Human RNU6B RNA was considered as control.
9. Performed the data analysis as described below.

Protocol 16: PCR efficiency and Data analysis.

For evaluation of PCR efficiency, a collection of known cDNA concentration of universal human reference RNA (Stratagene, Cedar Creek, TX, USA) was prepared and a standard curve was made. To determine the ΔCt for miR-34a, miR-34b and miR-34c and RNU6B for each concentration, the best gradient line was calculated from a scheme with situation of log of total cDNA on the X axis and ΔCt on the Y axis. The efficiencies of miR-34a, miR-34b and miR-34c and RNU6B genes were identified to be comparable. Analysis of miR-34a, miR-34b and miR-34c and RNU6B genes expression was normalized using the following method:

$$\Delta Ct = Ct \text{ miR-34a, miR-34b, 34c [sample]} - Ct \text{ RNU6B [sample]}$$

After obtaining the normalized values for each triplicate sample, the main value ware calculated to create the usable final data. In addition, the fold change in the target genes for quantitative amplification results was computed for each sample using $2^{-\Delta\Delta Ct}$ method as follow:

$$\Delta\Delta Ct = (Ct_{\text{miR34a, miR-34b, 34c}} - Ct_{\text{RNU6B}})_{\text{CANCER}} - (Ct_{\text{miR34a, miR-34b, 34c}} - Ct_{\text{RNU6B}})_{\text{NORMAL}}$$

Ratios (=Mean $Ct_{\text{miR34a, miR-34b, 34c}}$ [sample]/Ct_{RNU6B3} [sample]) were also used to demonstrate the miR-34 family expression results.

In order to determine whether there was a significant difference of miR-34a, miR-34b and miR-34c expression level between thyroid cell lines, the one-way Analysis of Variance (ANOVA) was recruited to analyse normalised final data (ΔCt).

Resulting data was entered into computer database and all statistical analysis was done using the Statistical Package for Social Sciences for Windows (version 21.0, SPSS Inc., Chicago, IL, USA). P-value <0.05 (P<0.05) was considered to display significant level.

3.8 Transient Transfection of miRNA-34b

The following transient transfection protocol was used for all experiment included in this thesis.

Protocol 17: Transient transfection of miR-34b.

1. 5×10^{4} cells per well of a 24- well plate was seeded, and then 500 µl of appropriate culture media with serum and antibiotic were added onto the each well. Cells incubated under normal growth condition (at the 37°C and 5% concentration of CO2) for overnight.
2. Based on the qPCR results, the 1.5 µl of miScript miRNA-34b Mimic (Qiagen, Hilden, NRW, Germany) from 2µM stock was diluted in 100 µl culture medium without serum to obtain final concentration of 5nµ after adding complexes in to cells. then, 5 µl HiPerFect Transfection reagent (Qiagen, Hilden, NRW, Germany) was added in diluted miScript miRNA-34b Mimic and gently mixed by vortexing. The complex was placed for 15 minutes at room temperature to allow the completely formation of transfection reagent (specific amount of miScript miRNA-34b Mimic and HiPerFect Transfection reagent is mentioned in each individual experiment later).

3. media was removed and to provide a uniform distribution of transfection reagent, first the 500 µl of fresh media was added and then complex was applied drop-wise onto the cells.
4. Cells were incubated with transfection reagent under the normal growth condition (at the 37°C and 5% concentration of CO2) for 48 hours. media changed as required.

3.9 Cell proliferation and migration assays

For All cell proliferation assays performed in this thesis, the following protocols were used.

Protocol 18: CCK-8 protocol.

1. A cell proliferation assay kit-8 (Sigma–Aldrich) was used to determine the changes of proliferation in miR-34b-5p transfected cell lines.
2. Plated out at the density of 10.000 cells in 96 well-plate in triplicate.
3. Transfected the cells with miR-34b for 48 hours following *protocol 12* mentioned e earlier.
4. Tawed the CCK-8 on bench top for 30 minutes.
5. Added 10µl of CCK-8 kit to each well and plate incubated for 4 hours.
6. Measured the absorbance at 450 nm using FLUOstar Omega Multi-mode microplate reader (BMG LABTECH GmbH, Allmendgruen Ortenberg, Germany) once a day for three days.

Protocol 19: wound healing assay.

1. A wound healing assay was performed to determine the migration capacity of cells after transfection with PEGlylated-miR-34b-5p.
2. Plated out at the density of 200.000 cells in 6 well-plate in triplicate.

3. Transfection was performed at a final concentration of 5nM when cells grew up to 70% confluency as a monolayer.
4. 48 hours later, scratches were made in PEGlylated-miR-34b-5p, PEGlylated-miR-1 treated cells and control cells with a 200-ml pipette tip across the centre of culture plates.
5. Media was added to the culture after the cells were washed three times with PBS.
6. Images were then taken under inverted microscope at 0,24, 48 and 72 hours after the wounding.
7. The mobility of cells in different days were measured and compared with ImageJ 1.48 software.

Protocol 20: Cell cycle analysis.

1. A Propidium Iodide Staining protocol (BD Biosciences, San Jose, CA, USA) for cell cycle analysis was used to determine the cell distribution in different phase of cell cycle.
2. Plated out 1.000.000 cells in 12 well-plate
3. Transfected the cells with for 48 hours following *protocol 12* mentioned earlier.
4. Harvested with 100 µl of trypsin/EDTA (2mM) and neutralised with medium.
5. Centrifuged to precipitate cells at 2000 rpm for 3 minutes.
6. Aspirated the medium and incubated with 1ml of 70% cold ethanol for 1 hour at -20 °C. added drop-wise to cell pellet while vortexing to minimise clumping.
7. Centrifuged at 1500 rpm for 5 minutes and then washed twice with PBS.
8. Discharged PBS and added 50 µl RNase A solution directly to pellet and then added 200 µl propidium iodide (PI) to cells in RNase A solution.
9. Incubated for 40 minutes at 37 °C and analysed flow cytometry in PI/RNaseA solution with cell cycle instrument setting.

Protocol 21: Apoptosis assay.

1. A membrane permeability/Dead Cell Apoptosis Kit (Invitrogen) was used to measure the apoptotic cell.
2. Plated out 1.000.000 cells in 12 well-plate.
3. Transfected the cells with for 48 hours following *protocol 12* mentioned earlier.
4. Cell were trypsinised and washed twice with ice-cold PBS and resuspended in PBS at a concentration of 25×10^{4}/ml cells in total volume of 1 ml.
5. 1 µl of YO-PRO®-1 and 1 µl of PI were then added.
6. Samples were kept in the dark for 20 minutes at room temperature.
7. The numbers of apoptotic cells were analysed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) and calculated with FlowJo single-cell analysis software (FLOWJO, LLC, Ashland, OR, USA).

3.10 Enzyme-linked immunosorbent (ELISA) assay

Protocol 22: ELISA assay.

1. A Human VEGF ELISA kit (Life technologies, St. Louis, MO, USA) was used to measure the VEGF-A secretion in thyroid cancer cell media.
2. Plated out 1.000.000 cells in 12 well-plate.
3. Transfected the cells for 48 hours following *protocol 12* mentioned earlier.
4. Cell media was collected and kept in -80 °C for a short time until analysis.

Standard dilution preparation

5. Reconstituted Hu VEGF Standard to 10,000 pg/ml with Standard Diluent Buffer.
6. To ensure complete reconstitution, swirled or mixed gently and allow the contents to sit for 10 minutes.

7. Added 90 µl of the reconstituted standard to a tube containing 510 µl Standard Diluent Buffer. Labeled as 1500 pg/ml Hu VEGF. Used the standard within 1 hour of reconstitution.

8. Added 300 µl Standard Diluent Buffer to each of 6 tubes labeled as follows: 750, 375, 188, 93.8, 46.9, and 23.4 pg/ml of Hu VEGF.

9. Serial dilution mixed thoroughly between each steps of preparation and made a serial dilution of the standard as described below in the dilution diagram.

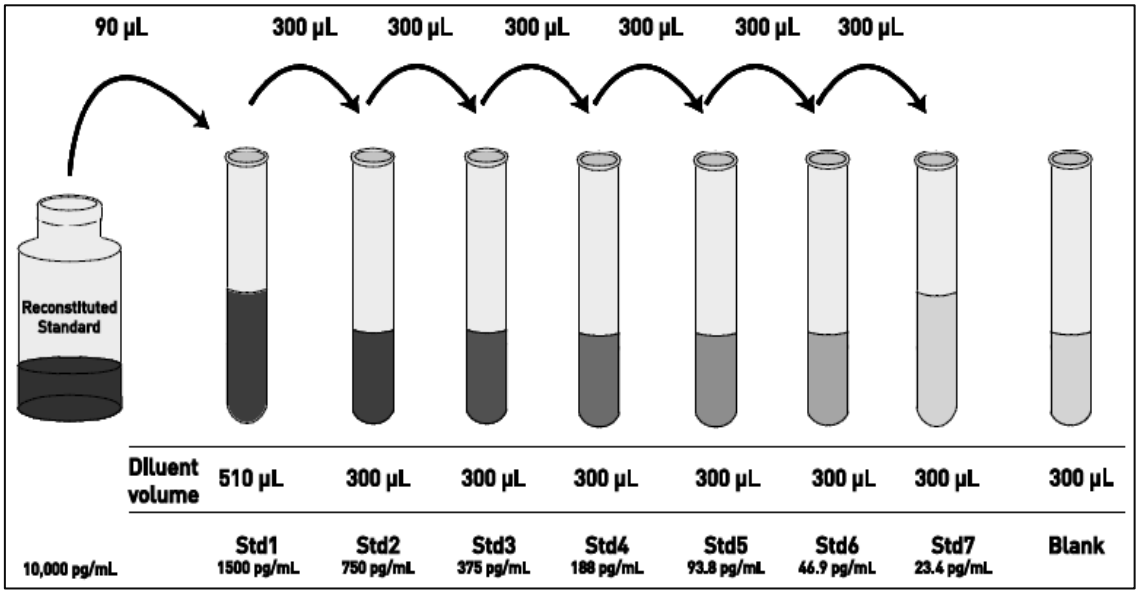

Figure 2. Dilution diagram of a stock solution preparation to produce a dilution series.

Preparation of Streptavidin-HRP solution

10. For each 8-well strip used in the assay, 10 µl of Streptavidin-HRP (100X) solution, was added into a tube containing 990 µl of HRP Diluent and mixed thoroughly.

Bind antigen

11. Added 50 µl of the Incubation Buffer to all wells except chromogen blanks.

12. Added 100 µl of standards to the blank wells.

13. For samples and controls, added 50 µl of Standard Diluent Buffer to each appropriate well followed by 50 µl of sample or controls.

14. Covered the plate with plate cover and incubated for 2 hours at room temperature.
15. Thoroughly aspirated the solution and washed wells 4 times with diluted Wash Buffer.

Detector antibody

16. Added 100 µl Hu VEGF Biotin Conjugate solution into each well except chromogen blanks.
17. Covered the plate with plate cover and incubated for 1 hour at room temperature.
18. Thoroughly aspirated the solution and washed wells 4 times with diluted Wash Buffer.

Streptavidin-HRP

19. Added 100 µl Streptavidin-HRP into each well except the chromogen blanks.
20. Covered the plate with plate cover and incubated for 30 minutes at room temperature.
21. Thoroughly aspirated the solution from the wells and washed wells 4 times with 1X Wash Buffer.

chromogen

22. Added 100 µl Stabilized Chromogen to each well.
23. Covered the plate with plate cover and incubated for 30 minutes at room temperature in the dark and then added 100 µl stop solution to each well and gently mixed Taped. All samples were run in triplicate.

3.11 Liposome Preparation

Protocol 23: Formulation of lipid nanoparticles.

The monophase dehydration-rehydration method was based on the preparation of a monophase solution containing phospholipids, nucleic acids, tertiary butanol and

distilled water in the presence of sucrose; the solution was snap-frozen and lyophilised before rehydration with water, where liposomes were formed spontaneously as follows:

1. Warmed up tert- butanol to 37°C in water bath.
2. Measured out the required amount of lipids in a ratio of in flasks the overall molar ratio of lipids was 50:35:5:10.
3. Dissolved the lipids using tert-butanol in the hood.
4. volume calculated by multiplying actual amount of mg weighed out by 0.125 ml butanol and divided by mg per dose.

Table 12: Volume calculation table for preparing one dose of lipid nanoparticles.

Lipid	**1 Dose**	**× Doses required**	**Actual**	**Volume needed**	**Total**
Dotap	0.352 mg	0.352x = ------- mg	----- mg	= actual x 0.125/0.352	------- ml
Cholesterol	0.136mg	0.136x = ------ mg	----- mg	= actual x 0.125/0.136	------- ml
DOPE	0.037 mg	0.037x =------- mg	----- mg	= actual x 0.125/0.037	------- ml
PEG Ceramide	0.263 mg	0.263x =------- mg	----- mg	= actual x 0.125/0.263	------- ml

5. Warmed solutions to 37 °C to fully dissolve and placed in incubator and swirl every 5 minutes.
6. Aliquoted each of the dissolved lipid/butanol solutions into a round bottomed flask (0.125ml/lipid/dose).

7. Prepared sterile 55.5mg/ml sucrose solution and filtered before use. Added 0.5ml per dose containing 40ug/dose miR-34b-5p mimic to the 0.5ml lipid mixture and mixed well by gentle shaking.
8. Prepared dry ice/ethanol and then snap-freezed lipid mixture by swirling mixture to form a thin frozen layer.
9. Loaded samples onto the lyophiliser and slowly turned on vacuum when the pressure was below 0.1mbar and kept in dry ice/ethanol bath until pressure returns to 0.1mbar.
10. Kept samples to dry overnight on the lysophiliser for 3 days.
11. Rehydrated liposomes in 300ul of sterile filtered Diethyl pyrocarbonate (DEPC) water for each dose and reconstituted for at least 2 hours before use and/or particle analysis.

Protocol 24: Nanoparticle characterization.

1. Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) was used for measurement of size, polydispersity, and zeta potential of the resultant liposomes.
2. diluted 150 ul of liposome in distilled water to a final concentration of 20 μM.
3. Loaded into the cuvette and pipetted 5 times to remove bubbles.
4. Measurements were carried out at room temperature and two size measurements were performed with 10 runs per measurement undertaken.

3.12 In vivo study

Protocol 25: Subcutaneous Injection of Tumour Cells.

1. Removed growth medium from cells and wash with 5 ml of PBS.
2. Aspirated PBS, add 2 ml of trypsin and incubate until cells have detached, at 37 °C.
3. Notarised trypsin by adding at least 5 ml of 10% FBS containing medium.

4. Pelleted cells by centrifugation for 5 minutes at 1400 rpm and 37 °C.
5. Aspirated medium, washed cells with 10 ml sterile PBS, mix well with pipette and saved 50 µl aliquot of cells for counting.
6. Pelleted cells by centrifugation for 5 minutes at 1400 rpm and 37 °C.
7. While cells are spinning, added 50 µl of trypan blue to save aliquot, mix well and counted cells using a hemocytometer.
8. Aspirated PBS, resuspend cells in fresh PBS to a concentration 2×10^6 cells/100 µl and transfer cells to a sterile Eppendorf tube.
9. Slowly pulled up 100 µl of cells using an insulin syringe.
10. Inject 2×10^6 cells into the right flanks of NU/NU nude mouse of 6-8 weeks old mice, pinched the skin of the mouse between index finger and thumb and pulled the skin away from the body of the mouse.
11. Injected cells slowly and evenly into the pouch created by fingers, created a single bubble of cells beneath the skin and avoided too much spread of the cells.
12. Anesthetized the mice using isoflurane to make the injection process significantly less stressful for the both the mice and the researcher.

Protocol 26: Tail Vein Injections.

1. Placed the mouse in a cage under the heat lamp to warm up the mouse, turned on 'low', for 15 minutes show the tail veins clearly. Ensured the animal does not overheat.
2. Placed the mouse in the restraining device.
3. Swabbed the tail with a gauze dampened with alcohol to increase the visibility of the vein.
4. Located one of the two lateral tail veins in the middle third of the tail.

5. Restrained the tail while occluding the vein with your non-dominant hand. With the bevel of the needle facing upward and the needle almost paralleled to the vein, slide the needle into the tail vein. Confirmed the location by gently applying negative pressure to the plunger.
6. Released the vein occlusion proximal (closer to the animal) to injection site. Slowly pressed the plunger to inject PEGlyated-miR-34b into the vein.
7. Removed the needle from the vein and applied slight pressure to the puncture site with a dry piece of gauze until the bleeding has stopped.
8. Removed the animal from its restrainer and placeed it in the cage. Monitored the animal for 5-10 minutes to ensure hemostasis (bleeding has stopped).

Protocol 27: Carbon Dioxide (CO_2) and Cervical Dislocation for mouse Euthanasia.

1. placed mouse in an empty chamber and a CO_2 delivery lid is placed on its home cage.
2. Started The flow of CO2 from the gas cylinder at a rate that displaced 10-30% of the cage or chamber volume per minute (8 litters/minute) and gas levels raised to 40-50%, unconsciousness occurred
3. At this point, the flow of the CO_2 increased to decrease the time to death. CO2 flow maintained for at least one minute after respiratory arrest.
4. Placed the mouse in a normal standing position on the firm, and grasp the base of the tail firmly with one hand.
5. Placed the thumb and first finger of the other hand against the back of the neck at the base of the skull.
6. To produce the dislocation, quickly pushed forward and down with the hand while pulling backward with the hand holding the tail base.

7. The effectiveness of dislocation checked by feeling for a separation of cervical tissues.

Chapter 4: *miR-34b* has influence on expression of major angiogenic regulator genes and proteins in cancer biology and moderates' cancer proliferation and survival through angiogenic events in thyroid cancer *in vitro*

The roles of microRNA-34b-5p in angiogenesis of thyroid carcinoma

This section includes a co-authored published paper. The bibliographic details of co-authored paper, including all authors, is:

Hamidreza Maroof, Farhadul Islam, Armin Ariana, Vinod Gopalan, Alfred King-Yin Lam.

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First author contribution to the submitted paper involved: Literature review, Experimental design, laboratory performance, data collection, data analysis, categorisation of the data into a usable format and providing direction on the scope and structure of the analysis, drafting the manuscript and revision.

Other authors contribution to the submitted paper: Critical review and revisions were conducted by King-Yin Lam and Vinod Gopalan.

(Signed) _____ (Date) 19-09-2017

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Paper 04: The roles of microRNA-34b-5p in angiogenesis of thyroid carcinoma

Hamidreza Maroof**,** Farhadul Islam, Armin Ariana, Vinod Gopalan, Alfred King-Yin Lam

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4.1 Introduction

Papillary thyroid carcinoma is the most common thyroid cancer, and patients with cancer have relatively good prognosis [8]. In some instances, papillary thyroid carcinoma could progress to an aggressive type of thyroid cancer, anaplastic thyroid carcinoma [565]. Angiogenesis is an essential process for growth and metastases of cancer mediated by vascular endothelial growth factor (VEGF) [566, 567]. Thyroid carcinomas are good models to study angiogenesis of cancer as they are vascular [8]. Angiogenesis has substantial clinical impacts in the pathogenesis and progression of thyroid cancer mediated by VEGF [568-570]. Angiogenesis is activated in the hypoxic microenvironment of cancer[571]. This angiogenic switch directly leads to the secretion of angiogenic factors such as VEGF and indirectly activates proliferating genes such as *B-cell lymphoma 2* (*Bcl-2*) and *Notch homolog 1* (*Notch1*) [43, 182, 184].

VEGF-A has been identified to play a significant role as a proangiogenic factor involved in thyroid cancer [567]. Therapies targeting the VEGF have become a promising tool in some cancers such as lung and colorectal cancers [572]. However, many cancers are resistant to these therapies[572-577]. Therefore, recognition of new angiogenesis regulators could improve the effectiveness of these therapeutic strategies.

The mature *miR-34* family is a part of the *p53* tumour suppressor network [578, 579]. In thyroid cancer, Yip and colleagues have shown the role of miR-34b in predicting

the aggressiveness of papillary thyroid carcinoma[580]. Also, negative expression of VEGF, Notch1 and Bcl-2 proteins correlated with the presence of miR-34b-5p. Furthermore, these studies showed that the 3' UTR of VEGF, Notch1 and Bcl-2 mRNA contain miR-34b-5p binding site and they can be regulated by miR-34b-5p [356, 581-584].

There is no previous study on the role of miR-34b on VEGF, Notch1 and Bcl-2 in thyroid carcinoma. Thus, the aim of this study is to determine the function of miR-34b-5p in the pathogenesis of thyroid carcinoma. Furthermore, this study also investigated the role of miR34b-5p in the modulation of angiogenesis initiation process by examining the expression of VEGF-A, Bcl-2 and Notch1 in thyroid carcinoma cells.

4.2 Materials and Methods

4.2.1 Human tissue samples

We have collected cancer tissue from 65 patients operated for papillary thyroid carcinoma. Ethics approval for the use of human tissue samples was obtained from Griffith University (MED/19/08/HREC). Seven non-neoplastic thyroid tissues from patients diagnosed with nodular hyperplasia of thyroid were used as controls in this study (Figure 3).

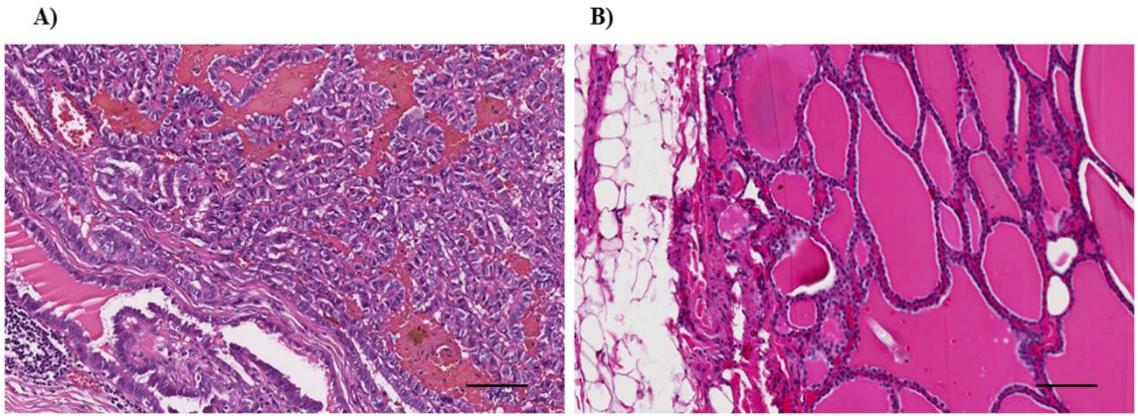

Figure 3. Selection of material for detection of miR-34b-5p expression. A. Papillary thyroid carcinoma tissue was selected with more than 80% of the area occupied by the carcinoma (haematoxylin and eosin x

15); B. Non-neoplastic thyroid tissue was selected from a relatively normal area in patients with nodular hyperplasia (haematoxylin and eosin x 15 and scale bar 20 μm).

4.2.2 Cell culture

Five thyroid carcinoma cell lines were used in this study. Four of the cancer cell lines were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmBH-German Collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany). They were B-CPAP (from metastasizing human papillary thyroid carcinoma), 8505C (from metastatic human anaplastic thyroid carcinomas in a lymph node with primary papillary thyroid carcinoma), MB-1(from anaplastic thyroid carcinoma) and BHT-101 (from metastatic anaplastic thyroid carcinoma in the lymph node). The remaining cancer cell line was a human papillary thyroid carcinoma cell line (K1) which was obtained from European Collection of Cell Cultures (ECACC). A non-neoplastic thyroid follicular cell line (Nthy-ori 3-1, from ECACC) was used as a control. The cell lines were authenticated in the standard protocol (using multiplex polymerase chain reaction of mini-satellite markers for DNA fingerprinting and identification of short tandem repeats of cell lines and cytogenetics), and the passage number of these cell lines was less than nine. All the cell lines were maintained according to the suppliers and were cultured in a humidified incubator in an atmosphere of 5% CO_2 and 95% air at 37°C.

4.2.3 Quantification of miR-34b-5p expression

The miR-34b-5p expression level was quantified by real-time quantitative polymerase chain reaction (qRT-PCR) using Hs_miR-34b*_2 miScript Primer Assay (Qiagen, Venlo, Limburg, Netherlands) following the suggested protocol. Samples were normalised using the housekeeping gene RNU6B RNA (Hs_ RNU6B_2 miScript Primer Assay, Qiagen). Total RNA from cells and tissue samples was extracted using NucleoSpin® miRNA Kit (MACHEREY-NAGEL, Duren, Germany) and Qiagen

miRNeasy FFPE Kits (Qiagen Pty. Ltd., Hilden, NRW, Germany), respectively, with a DNase supplementary step. Then, cDNA was synthesised using miScript II RT Kit (Qiagen) according to the manufacturer instructions. Amplification, detection and analysis were performed with an IQ5 multicolour Real-Time PCR detection system (BIO-RAD, Hercules, CA, USA).

Real-time PCR amplifications were performed in a 20 µl reaction volume consisting of 10 µl QuantiTect SYBR Green PCR Master Mix (Qiagen), 1 µl miScript Primer Assay (Qiagen), 1 µl of miScript Universal Primer (Qiagen), and 5 µl of cDNA template at 2 ng/µl stock and 3 µl RNase- free water. All qRT-PCR reactions were carried out in triplicates with non-template controls as previously published protocol [585]. Expression of miR-34b-5p was presented as the ratio between miR-34b-5p and RNU6b. The $2^{-\Delta\Delta ct}$ method was used to calculate the fold changes of miRNA in each sample group. Less than 0.5-fold changes were considered as low expression. Fold changes between 0.5 and 2 were considered as normal expression whereas fold changes of more than two were considered as high expression.

4.2.4 miR-34 mimics transfection

Exogenous miR-34b-5p (mimic) (Syn-hsa-miR-34b-5p) and HiPerFect transfection reagent were purchased from Qiagen. Mature miR-34b mimic sequence (guide strand) was 5' UAGGCAGUGUCAUUAGCUGAUUG-3′. Transfection was performed according to our previously published protocol[586, 587]. Briefly, thyroid cancer cells were transfected immediately after being seeded at a density of 25×10^{4} cells / well in 6 well plate with miR-34b-5p mimics and with a non-targeting control, positive control (miR-1; miR-1 is only expressed in muscle cells and is not expressed in other cell types. As hsa-miR-1 is not expressed in thyroid cell lines used in this study, then transfection of this mimic and subsequent analysis of its target can be used as a positive

control experiment) and negative control siRNA (scramble control) (Qiagen), using the HiperFect transfection reagent (Qiagen) for overexpression of miR-34b-5p. A final concentration of 5nM, as well as 48-hours incubation period, was selected for both transfections.

4.2.5 Immunofluorescence

In the immunofluorescence analysis, the cells were transfected as described above after being cultivated on a glass culture slide (BD Biosciences, San Jose, CA, USA). The cells were fixed in 4% cold paraformaldehyde/phosphate buffer saline (PBS) for 25 minutes, permeabilised in 0.4% triton X-100 for 8 minutes. They were blocked in 5% normal goat serum/PBS (Sigma–Aldrich, St. Louis, MO, USA) for 45 minutes. Then, they were incubated with antibodies against Bcl-2 (N-19, 1:200; Santa Cruz Biotechnology, Dallas, TX, USA), VEGF-A (A-20, 1:200 dilution; Santa Cruz Biotechnology), Notch1 (H-131, 1:300 dilution; Santa Cruz Biotechnology) overnight at 4ºC. Afterwards, the cells were incubated with Texas Red-labelled secondary antibody (1:1000 dilution; Life technologies, St. Louis, MO, USA) for 1 hour at room temperature. As a negative control, all staining was performed without the primary antibody. After being counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich), confocal laser scanning microscopic images were captured with an Eclipse Ti-E microscope (Nikon Instruments, Inc., Melville, NY, USA) using a plan apochromat 60×/1.40 objective and NIS-Elements imaging software platform (Nikon).

4.2.6 Western blot analysis and antibodies

After transfection, the cells were lysed in Cell Lysis Buffer NP40 (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1% Nonidet P40 and 0.02% NaN3) (Invitrogen Carlsbad, CA, USA) supplemented with protease inhibitor cocktail (Sigma-Aldrich), phenylmethanesulfonyl fluoride solution (PMSF) (Sigma) and

phosphatase inhibitor cocktail (Cell Signaling, Danvers, MA, USA). Then, protein lysates were prepared, and protein concentration was quantified using the Macherey-Nagel protein assay kit (MACHEREY-NAGEL GmbH & Co.KG, Düren, Germany).

Equal quantities of 20 µg protein samples were loaded on 4–15% precast polyacrylamide gel (Mini-PROTEAN TGX Precast Gel, Bio-Rad) and were transferred onto polyvinylidene-difluoride membranes (Trans-Blot Turbo Mini PVDF Transfer Packs, Bio-Rad) using the blotting instrument (Trans-Blot Turbo Transfer Starter System, Bio-Rad). Blocking was performed with 5% non-fat milk in TBST (Tris buffered saline-Tween 20: 120 mmol/l Tris–HCl, pH 7.4, 150 mmol/l NaCl, and 0.05% Tween 20) for 1 hour at room temperature. The membrane was incubated with anti-Bcl-2 (Sc-492), 1:500 dilution; anti-VEGF-A (Sc-152), 1:200 dilution; anti-Notch1 (Sc-9170), 1:200 dilution and anti-ß-actin (Sc-4778), 1:1000 dilution (Santa Cruz Biotechnology) overnight at 4˚C. Following the manufacturer's protocol, blots were washed six times with TBST, incubated for one and half hour with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000 dilution) (Santa Cruz Biotechnology) at room temperature. The blots were then developed and visualised as previously published protocol [588].

4.2.7 Enzyme-linked immunosorbent (ELISA) assay

Cells transfected with miR-34b-5p mimic were cultured at a density of 2×10^5 cells/well in six-well plates. After 48 hours, the media was collected and quantified for secreted VEGF using a Human VEGF ELISA kit (Life technologies, St. Louis, MO, USA), according to the manufacturer's instructions.

4.2.8 Cell cycle analysis

After 48 hours transfection, cells were harvested by trypsinization and centrifugation at 1500 rpm for 5 minutes. Then, the supernatant media was discarded. After that, the cells were washed with ice-cold PBS and fixed in 70% ice-cold ethanol at

-20 ˚C for one hour. Subsequently the cells were centrifuged again at 1500 rpm for 5 minutes and were washed twice with PBS. These cells were then stained with propidium iodide (PI) (50 mg/ml in PBS), RNase (50 mg/ml) and Triton X-100 (0.1%) and incubated for 40 minutes at 37°C and analysed using a FACS Calibur flow cytometer (BD Biosciences). Data were analysed to calculate the percentage of the cell population in each phase using the FlowJo single-cell analysis software (FLOWJO, LLC, Ashland, OR, USA).

4.2.9 Apoptosis assay

A membrane permeability/Dead Cell Apoptosis Kit (Invitrogen) was used to measure the apoptotic cell. Briefly, after 48 hours of transient transfection, cells were trypsinized, washed twice with ice-cold PBS and resuspended in PBS at a concentration of 25×10^{4} /ml cells in a total volume of 1 ml. After that, 1 µl of YO-PRO ®-1 and 1 µl of PI were added. All the samples were kept in the dark for 20 minutes at room temperature. Finally, the numbers of apoptotic cells were analysed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) and calculated with FlowJo single-cell analysis software (FLOWJO, LLC, Ashland, OR, USA).

4.2.10 Data analysis

Results were analysed using GraphPad Prism 7.0 (Graph Pad Software, SanDiego, CA, USA) and were expressed as means ± SD (standard deviation). All of the *in vitro* experiments were performed at least three times. Statistical comparisons between groups were conducted using one-way ANOVA. A P value of < 0.05 was considered statistically significant and individual P-value was shown in the figures of this manuscript.

4.3 Results

4.3.1 miR-34b-5p expression is lower than other miR-34 family member in thyroid carcinoma cell lines.

Downregulation of miR-34 family have been reported in different type of cancer including thyroid cancer [578, 579]. It pointed out for the first time that overall expression of miR-34b-5p is much lower than miR-34b-3p (A) miR-34a and miR-34c (B) in all thyroid carcinoma cell lines. Therefore, miR-34b was chosen for further investigation in this study (Figure 4). Our result is in consistency with other research which represents the effect of miR-34b downregulation in thyroid cancer aggressiveness [580]. Hongbo and et al, also summarized literature evidence of expression of these microRNAs in glioblastoma [589]. Furthermore, study conducted on mouse spermatogenesis, investigated expression level of miR-34b-5p and -3p [590].

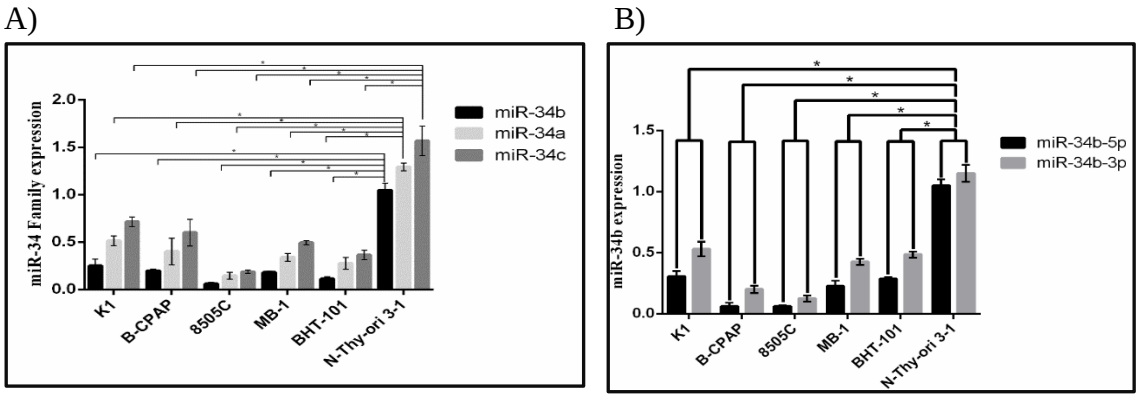

Figure 4. Expression of miR-34 family (A) and miR-34b-5p and -3p (B) in thyroid cancer cell lines and control. There is a significant downregulation of miR-34 family in all type of thyroid cancer cell lines. the expression level of miR-34b-5p ($P < 0.05$) was lower when compared with the miR-34a, miR-34c, miR-34b-3p and control. (K1: human papillary thyroid carcinoma; B-CPAP: Metastasizing human papillary thyroid carcinoma; 8505C: human undifferentiated thyroid carcinoma from a papillary thyroid carcinoma origin; MB-1: human undifferentiated thyroid carcinoma; BHT-101: human undifferentiated thyroid carcinoma metastases in lymph node), Nthy-ori 3-1: human normal thyroid follicular cell line. RNU6B was used to normalize the mRNA level. An asterisk (*) indicates $p < 0.05$, when compared to Nthy-ori-3-1. The quantitative values were expressed as means ± SD of triplicate measurements. They are representative of three separate experiments.

4.3.2 miR-34b expression is altered in human thyroid carcinoma tissues and cell lines.

To investigate changes in the miR-34b-5p expression level in human thyroid carcinomas, we examined a series of human thyroid carcinoma tissues, cell lines (K1, B-CPAP, 8505C, MB-1, BHT-101) and a non-neoplastic immortalised thyroid cell line (Nthy-ori3-1). miR-34b-5p showed significant downregulation in thyroid carcinoma cells when compared to the Nthy-ori-3-1cell line ($P < 0.05$). The lowest level of miR-34b-5p was noted in carcinoma cell lines derived from more aggressive thyroid carcinoma- anaplastic thyroid carcinoma (8505C, MB-1 and BHT-101) (Figure 5A). Notable reduced expression of miR-34b-5p in cancer tissues was noted when compared to that of non-neoplastic thyroid tissue samples (Figure 5B). The fold changes of miR-34b in thyroid cancer tissues samples was noted in the range of 0.000909 to 10.858 (0.487±1.585). The alignment of Bcl-2, Notch1 and VEGF-A with the miR-34b-5p have been shown in figure 5C.

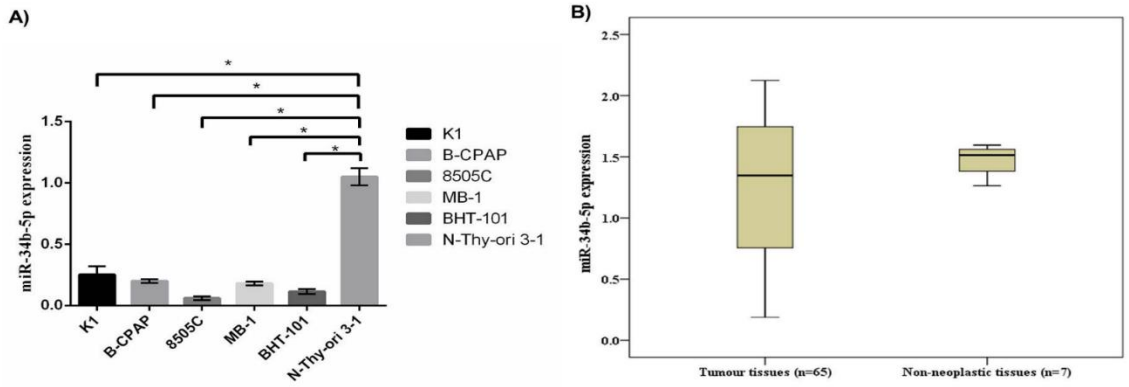

Figure 5. Expression in thyroid cancer cells and tissues of miR-34b-5p and targets interaction sites:

A) Expression of miR-34b in thyroid carcinoma: The miR-34b-5p decreased significantly in all thyroid carcinomas ($p < 0.05$). miR-34b-5p expression was lower in 8505C (anaplastic thyroid carcinoma from a papillary thyroid carcinoma origin) and BHT-101 (anaplastic thyroid carcinoma metastases in lymph node) than K1 (papillary thyroid carcinoma), B-CPAP (metastasizing papillary thyroid carcinoma) and MB-1(anaplastic thyroid carcinoma) when compared with the Nthy-ori 3-1 (non-neoplastic thyroid follicular cell line) ($p < 0.05$). RNU6B was used to normalize the mRNA level. An asterisk (*) indicates $p < 0.05$, when compared to Nthy-ori-3-1. The quantitative values were expressed as means ± SD of triplicate measurements. They are representative of three separate experiments.

B) Expression of miR-34b-5p in cancer and non-neoplastic thyroid tissue samples after normalization with internal control RNU-6B. Bar represent the standard deviation of the values in each group.

C) Alignment of Bcl-2, Notch1 and VEGF-A with the miR-34b-5p: The transcript is positioned and matched with miR-34b-5p between 190-210, 166-184 and 845 – 867 base pairs of Bcl-2, Notch1 and VEGF-A respectively. microRNA.org predicated mirSVR of -0.2530, -1.357 and -0.1480 for Bcl-2, Notch1 and VEGF-A respectively (http://www.microrna.org/). Genes that have a score of -1.0 or lower, corresponding to the top 7% of predictions, have more than a 35% probability of having a (Z-transformed) log expression change of at least -1 (downregulation by at least a standard deviation in terms of log expression changes) and better than 50% probability of a log expression change of at least -0.5. Thus, mirSVR scores can be converted to a probability of downregulation. This can be used as a guide for selecting a meaningful cut-off for reporting target sites. (Betel, et al., 2010) [591].

On tissue level, 88% (57/65) of the patients with thyroid carcinoma had shown downregulation of miR-34b expression whereas 7% (5/65) of the patients showed high miR-34b expression (Table 12). Approximately 5% (3/65) of the patients with thyroid carcinoma exhibited no change in miR-34b expression when compared to non-neoplastic thyroid tissues. In addition, miR-34b downregulation was associated with T-stage of the thyroid carcinoma (Table 12). In thyroid carcinomas of advanced T stages (3 or 4), all had either low or normal expression whereas, in thyroid carcinomas of earlier T stages (1 or 2), 91.8% had either low or normal expression (p=0.042). Given that expression of miR-34b-5p was notably downregulated in thyroid carcinoma cell lines and tissues ($p < 0.05$), we chose to restore miR-34b-5p in thyroid carcinoma cell lines to study its various cellular and biological effects.

Table 13: Expression of miR-34b and its correlation with clinicopathological parameters of patients with thyroid cancer.

Parameters	Total No.	High expression	Normal expression	Low expression	p values
Sex					
Male	19 (29.2%)	1 (5.3%)	0 (0%)	18 (94.7%)	0.249
Female	46 (70.8%)	4 (8.7%)	3 (6.5%)	39 (84.8%)	
Age					
< 55 years	47 (72.3%)	2 (4.2%)	3 (6.4%)	42 (89.4%)	0.118
≥ 55 years	18 (27.7%)	3 (16.7%)	0 (0%)	15 (83.3%)	
Size					
< 40 mm	50 (76.9%)	5 (10.0%)	2 (4.0%)	43 (86.0%)	0.240
≥ 40 mm	15 (23.1%)	0 (0%)	1 (6.7%)	14 (93.3%)	
T-stage					
T1 or T2	54 (83.1%)	5 (9.2%)	1 (1.9%)	48 (88.9%)	**0.042**
T3 or T4	11 (16.9%)	0 (0%)	2 (18.2%)	9 (81.8%)	
Lymph node invasion					
Positive	34 (52.3%)	3 (8.8%)	1 (2.9%)	30 (88.3%)	0.576
Negative	31 (47.7%)	2 (6.4%)	2 (6.5%)	27 (87.1%)	
Stage					
Stage 1	55 (84.6%)	4 (7.3%)	3 (5.4%)	48 (87.3%)	0.581
Stage 2	10 (15.4%)	1 (10.0%)	0 (0%)	9 (90.0%)	

4.3.3 miR-34b-5p regulates markers of cell proliferation

As a potent target for miR-34b-5p, the expression of VEGF, Notch1 and Bcl-2 proteins can be suppressed due to their affinity to bind miR34b (Figure 5C). To gain an insight into the mechanism by which miR-34b-5p inhibits Bcl-2 and Notch1, we identified the miR-34b-5p binding site in the Bcl-2 and Notch1 mRNA 3′ UTR region (Figure 5C). Figure 5C reveals the sequence matches between miR-34b-5p and the *VEGF, Notch1* and *Bcl-2* genes.

After a 48 hours transfection, Western blot analysis showed that Bcl-2 and Notch1 protein expressions were markedly reduced in miR-34b-5p transfected cell lines when compared with miR-1 transfected and scramble control (Figure 6 and 7) ($P < 0.05$).

Immunofluorescence analysis confirmed the Western blot results. After 48 hours of transfection, the immunofluorescence analysis of the Bcl-2 and Notch1 revealed that miR-34b-5p overexpression potently suppresses Bcl-2 and Notch1 protein expression in 8505C (Figure 8A) and BHT-101 (Figure 8B) when compared with miR-1 transfected and scramble control ($P < 0.05$). Fewer immunofluorescence distributions were noted in the nucleus and cytoplasm for Bcl-2 and in the cytoplasm for Notch1 when compared with miR-1 transfected and scramble control (Figure 9). This finding suggested that Bcl-2 and Notch1 are direct targets of miR-34b-5p, consistent with the data from the Western blot analysis.

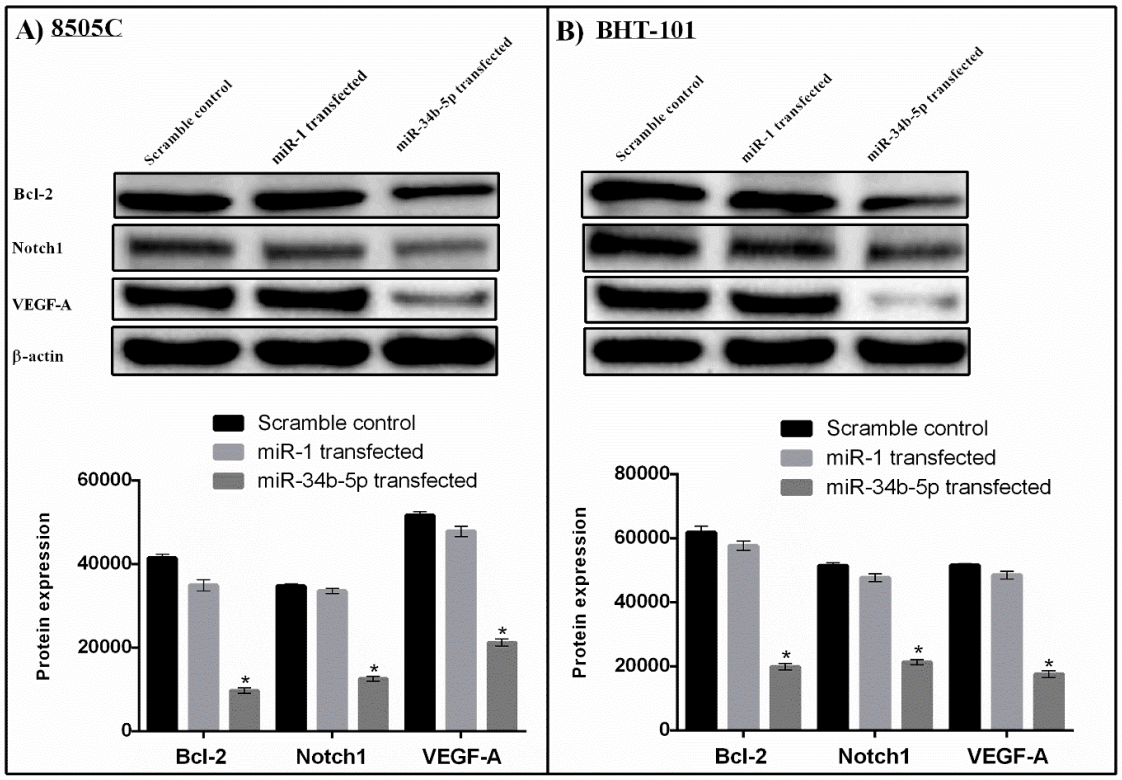

Figure 6. miR-34b-5p decreased the expression of Bcl-2, Notch1 and VEGF-A in 8505C and BHT-101 detected by Western blotting: In 8505C (A) and BHT-101 (B) cells, expression of Bcl-2, Notch1 and VEGF-A were significantly reduced in the miR-34b-5p mimic transfected groups when compared to miR-1 transfected and scramble control groups. β-actin was used as a sample loading control; y-axis comparison diagrams shows Bcl-2, Notch1 and VEGF-A protein expressions based on signal absorption. Results were representative of three independent experiments. Results presented as mean ± SD; and (*) implies as probability value $P < 0.05$ when compared to control (8505C: anaplastic thyroid carcinoma from a papillary thyroid carcinoma origin; BHT-101: metastatic anaplastic thyroid carcinoma in lymph node).

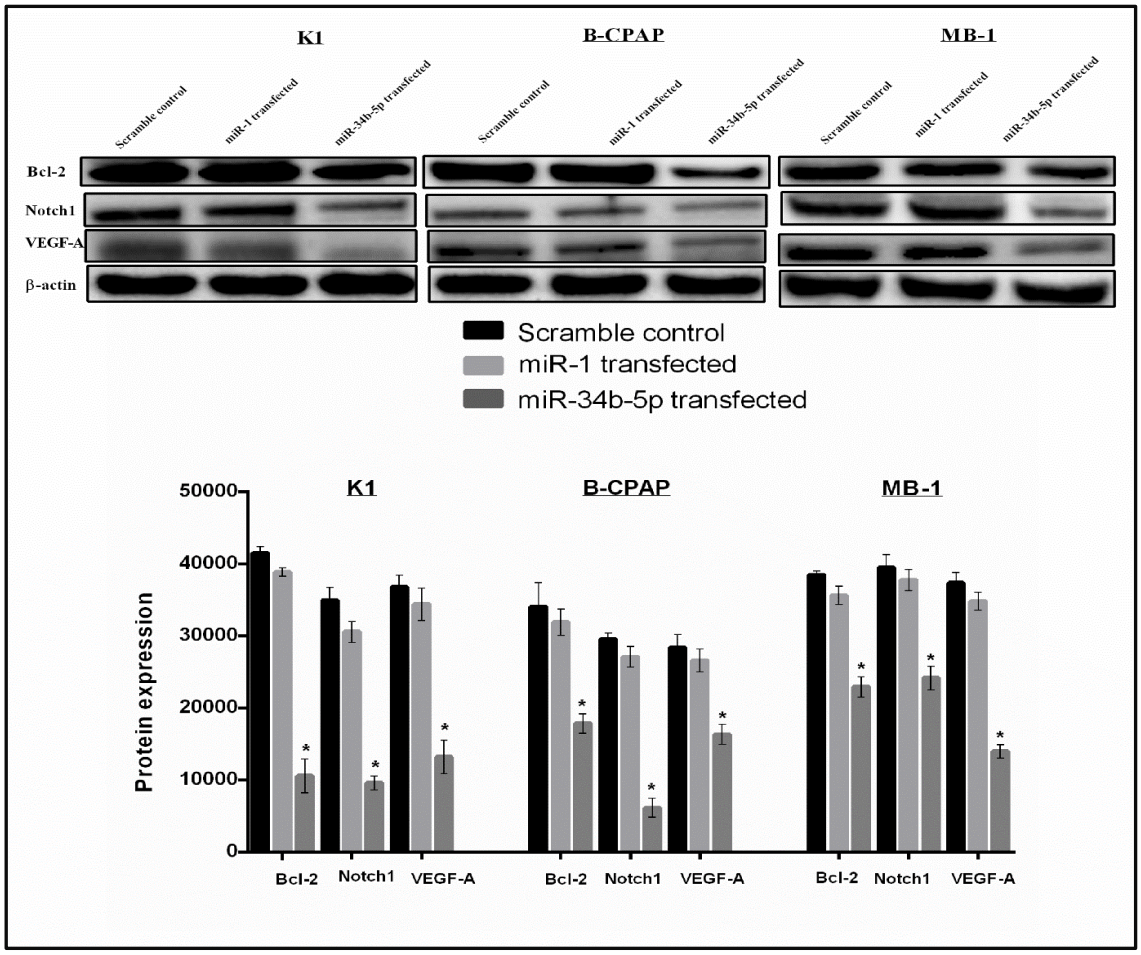

Figure 7. miR-34b-5p is also involved in repressing Bcl-2, Notch1 and VEGF-A levels in K1, B-CPAP and MB-1 detected by Western blotting: After 48 hours transfection with miR-34b-5p mimic, decreased pattern of Bcl-2, Notch1 and VEGF-A protein expressions were noted in K1, B-CPAP and MB-1 cells when miR-34b-5p mimic transfected group compared with miR-1 transfected and scramble control groups. β-actin was used as a sample loading control; y-axis comparison diagrams shows Bcl-2, Notch1 and VEGF-A protein expressions based on signal absorption. Results were representative of three independent experiments. Results presented as mean ± SD; and (*) implies as probability value $p < 0.05$ when compared to control (K1: papillary thyroid carcinoma; B-CPAP: metastasizing papillary thyroid carcinoma; MB-1: anaplastic thyroid carcinoma).

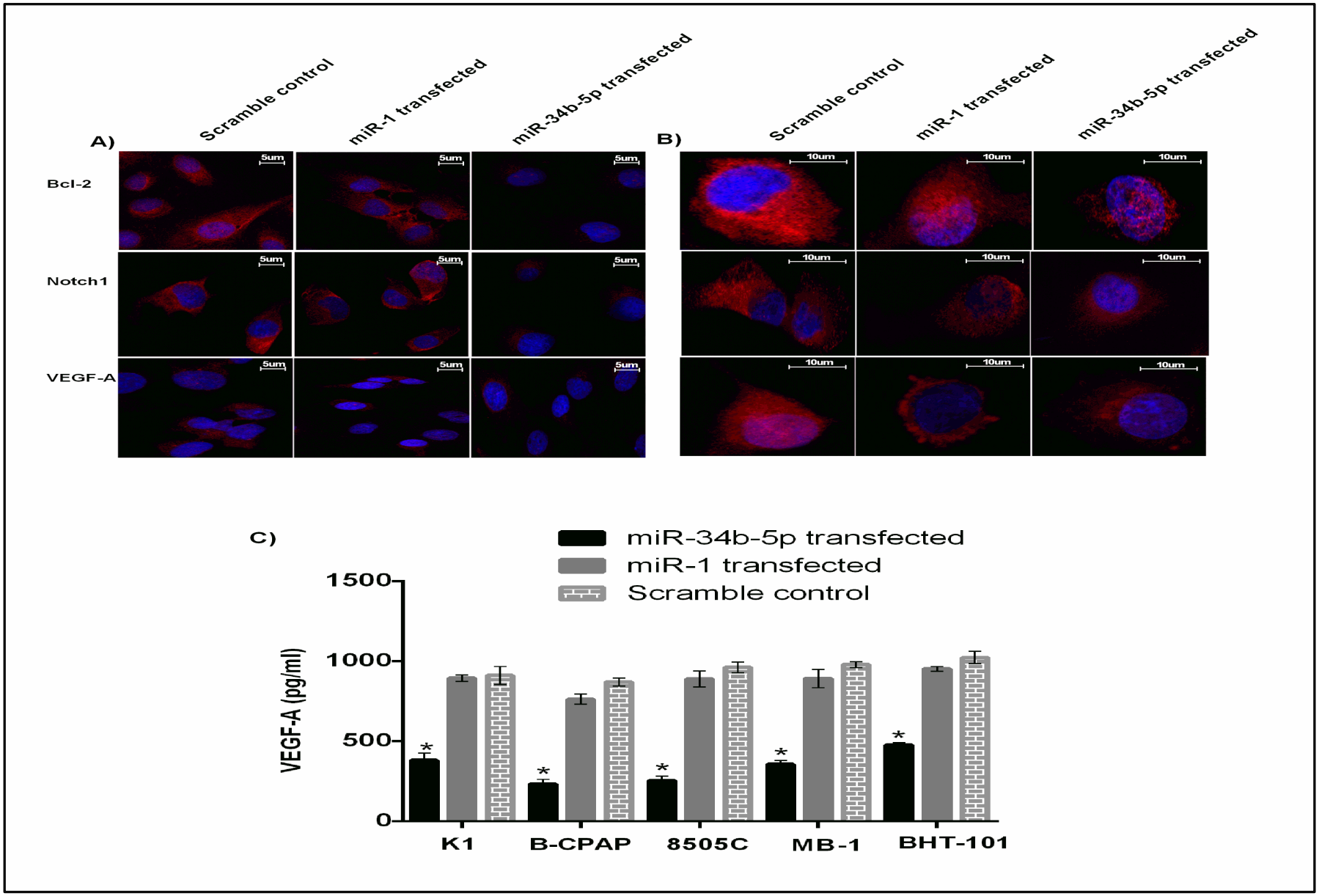
A)
Scramble control
miR-1 transfected
miR-34b-5p transfected
Bcl-2
Notch1
VEGF-A
5um
B)
Scramble control
miR-1 transfected
miR-34b-5p transfected
10um
C)
miR-34b-5p transfected
miR-1 transfected
Scramble control
VEGF-A (pg/ml)
1500
1000
500
0
*
K1
B-CPAP
8505C
MB-1
BHT-101

Figure 8. Confirmation of miR-34b-5p mediated alteration of targets proteins (Bcl-2, Notch1 and VEGF-A) in 8505C and BHT-101 cells via immunofluorescence microscopy and ELISA: Similar to the Western blot analysis, miR-34b-5p restoration significantly reduced the expression level of Bcl-2, Notch1 and VEGF-A proteins in 8505C (A) and BHT-101 (B) cells when miR-34b-5p mimic transfected group, miR-1 transfected and scramble control groups were compared. Immunofluorescence images were captured by a Nikon A1R+ confocal microscope using 60× objective with immersion oil and Bcl-2, Notch1 and VEGF-A are stained red, and nuclei are stained blue; Scale in the immunofluorescent images shows 5 and 10 μm. In Western blotting, β-actin was used as a sample loading control. 8505C: anaplastic thyroid carcinoma from a papillary thyroid carcinoma origin; BHT-101: metastatic anaplastic thyroid carcinoma in lymph node.

C) Following transfection of K1, B-CPAP, 8505C, MB-1 and BHT-101thyroid carcinoma cells with miR-34b-5p mimic for 48 hours, supernatants were collected for quantification of VEGF protein. ELISA assay data revealed that when compared to control cells, VEGF expression levels were inhibited in the supernatant fractions of thyroid carcinoma cells, transfected with miR-34b-5p. An asterisk (*) indicate statistically significant differences ($P < 0.05$, Student's t-test) when compared to control cells.

4.3.4 miR-34b-5p downregulates endogenous VEGF-A expression as a hallmark of angiogenesis in thyroid cancer cell lines.

To investigate the hypothesis that miR-34b-5p negatively regulate VEGF-A expression, the thyroid carcinoma cells were overexpressed with miR-34b-5p mimic (5nM) for 48 hours, and the VEGF-A protein level was determined afterwards. Western blot analysis showed a significant reduction in VEGF-A protein expression levels following miR-34b-5p overexpression when compared to miR-1 transfected and scramble control groups (Figure 6 and 7) ($P < 0.05$). Downregulation of VEGF-A by miR-34b-5p mimic in thyroid carcinoma has been confirmed by immunofluorescence analysis which showed decreased expression of VEGF-A protein. A remarkable reduction in the expression of VEGF-A protein was noted in the thyroid carcinoma cells in 8505C (Figure 8A) and BHT-101 (Figure 8B) when compared with miR-1 transfected and scramble controls ($P < 0.05$). Other thyroid carcinoma cells (K1, B-CPAP and MB-1)

showed only slight changes in the expression of VEGF-A protein when compared with miR-1 transfected and scramble control (Figure 9).

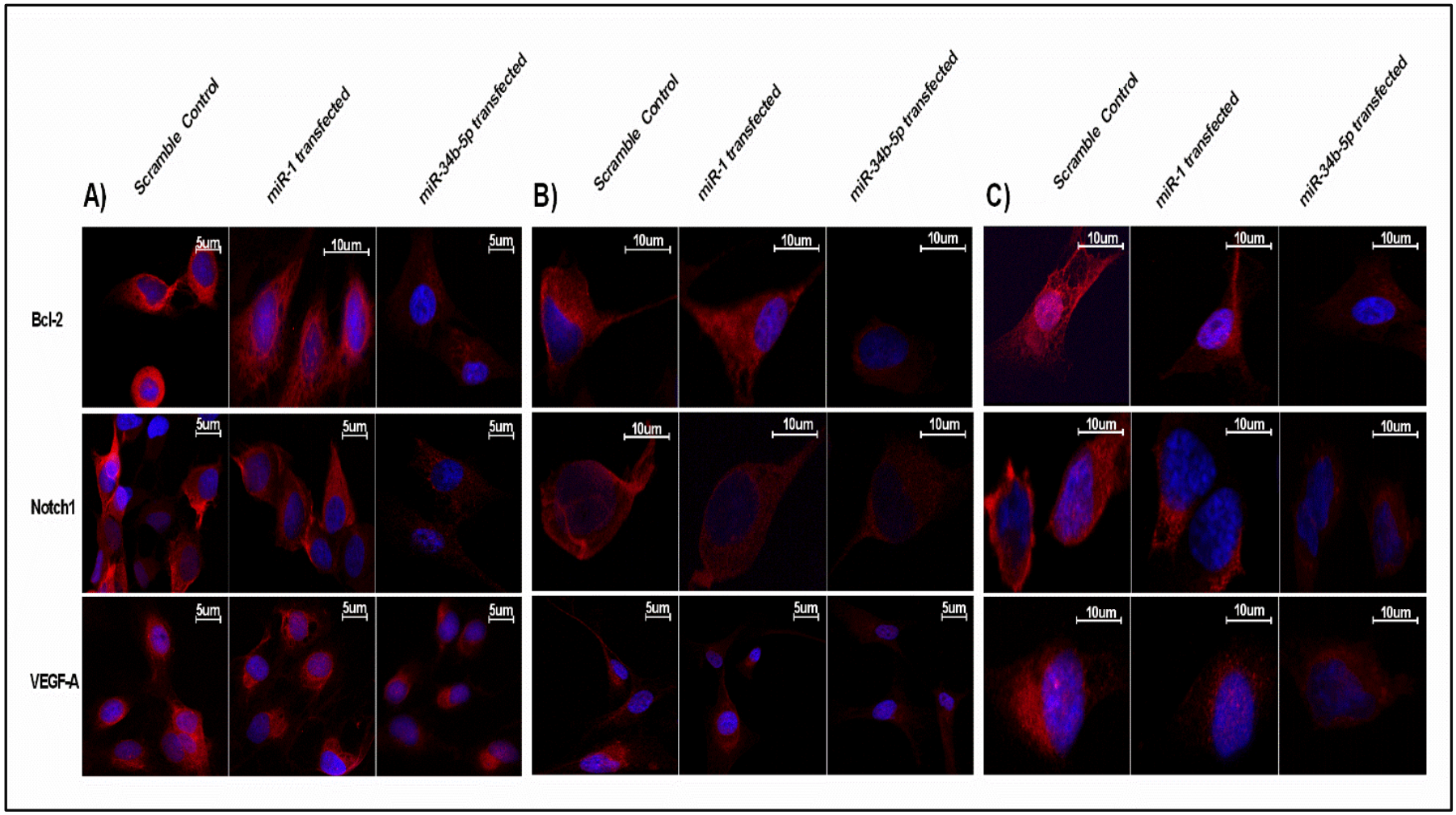

Figure 9. K1 (A), B-CPAP (B) and MB-1 (C) cell lines showed only slight changes in expression of Bcl-2, Notch1 and VEGF-A proteins in immunofluorescence microscopy images when compared with miR-1 transfected and scramble controls.

4.3.5 Overexpression of miR-34b-5p inhibits VEGFexpression in thyroid cancer cell lines supernatant.

ELISA results showed that when compared with the non-neoplastic immortalised thyroid cell line, the expression of VEGF in supernatant fractions of the thyroid carcinoma cells (KI, B-CPAP, 8505C, MB-1 and BHT-101) transfected with miR-34b-5p was inhibited by 54%, 72%, 69%,58% and 45%, respectively (Figure 8C) ($P < 0.05$). The above data revealed that overexpression of miR-34b-5p resulted in downregulation of Bcl-2, Notch1 and VEGF-A.

4.3.6 miR-34b-5p caused cell cycle arrest in thyroid cancer cells.

The concentration of miR-34b-5p was raised to 15 nM. After 48 hours of tranfection, the cells were studied by flow cytometry. Cell cycle arrest was observed at this time point. miR-34b-5p showed significant statistical differences in thyroid cancer cell cycle changes compared to miR-1(15nM) and control groups ($P<0.05$). Following miR-34b-5p overexpression, there was a significant increase in the accumulation of cells in G0-G1 phase as well as cell number reduced in the synthetic or S phase in all selected five thyroid cancer cell lines, especially in the thyroid carcinoma cells from B-CPAP and BHT-101 (Figure 10) ($P < 0.05$).

In the K1 (papillary thyroid carcinoma) cells, mimic transfection of miR-34b-5p (15nM) showed a significant arrest in the G0-G1 phase (11.36% ± 5.91) and a significant drop in S phase and G2/M phase after 48 hours' transfections when compared with miR-1(15nM) transfected and scramble control groups ($P < 0.05$) (Figure 10A). After 48 transfections, the percentage of cells in the G0-G1 phase in the B-CPAP (metastasizing papillary thyroid carcinoma) cell line transfected with miR-34b-5p (15nM) mimic was also significantly increased by day 2 of transfection to 35.06% ± 18.33 with a significant

drop in the S phase and G2-M phase compared with miR-1(15nM) transfected and control groups (Figure 10B) ($P < 0.05$). The introduction of miR-34b-5p (15nM) mimic into the 8505C cell line (anaplastic thyroid carcinoma from a lymph node with primary papillary thyroid carcinoma) displayed its anti-growth abilities with a significant accumulation of cells in the G0-G1 phase (20.1% ± 9.12). There was also a significant drop in the S phase and G2-M phase, compared with miR-1(15nM) transfected and control groups ($P < 0.05$) (Figure 10C). Anaplastic thyroid carcinoma cells (MB-1 and BHT-101) showed a similar trend with significant arrest in the G0-G1 of 20.53 % ± 8.74 and 23.83 % ± 11.37, respectively, followed by a significant drop in the S phase and G2/M phase ($P<0.05$) (Figure 10D and 10E). Collectively, a significant increase in the G0-G1 phase along with a reduction of cells in the G2-M phase were noted in the five different types of thyroid carcinoma cells when compared to miR-1 (15nM) and control groups.

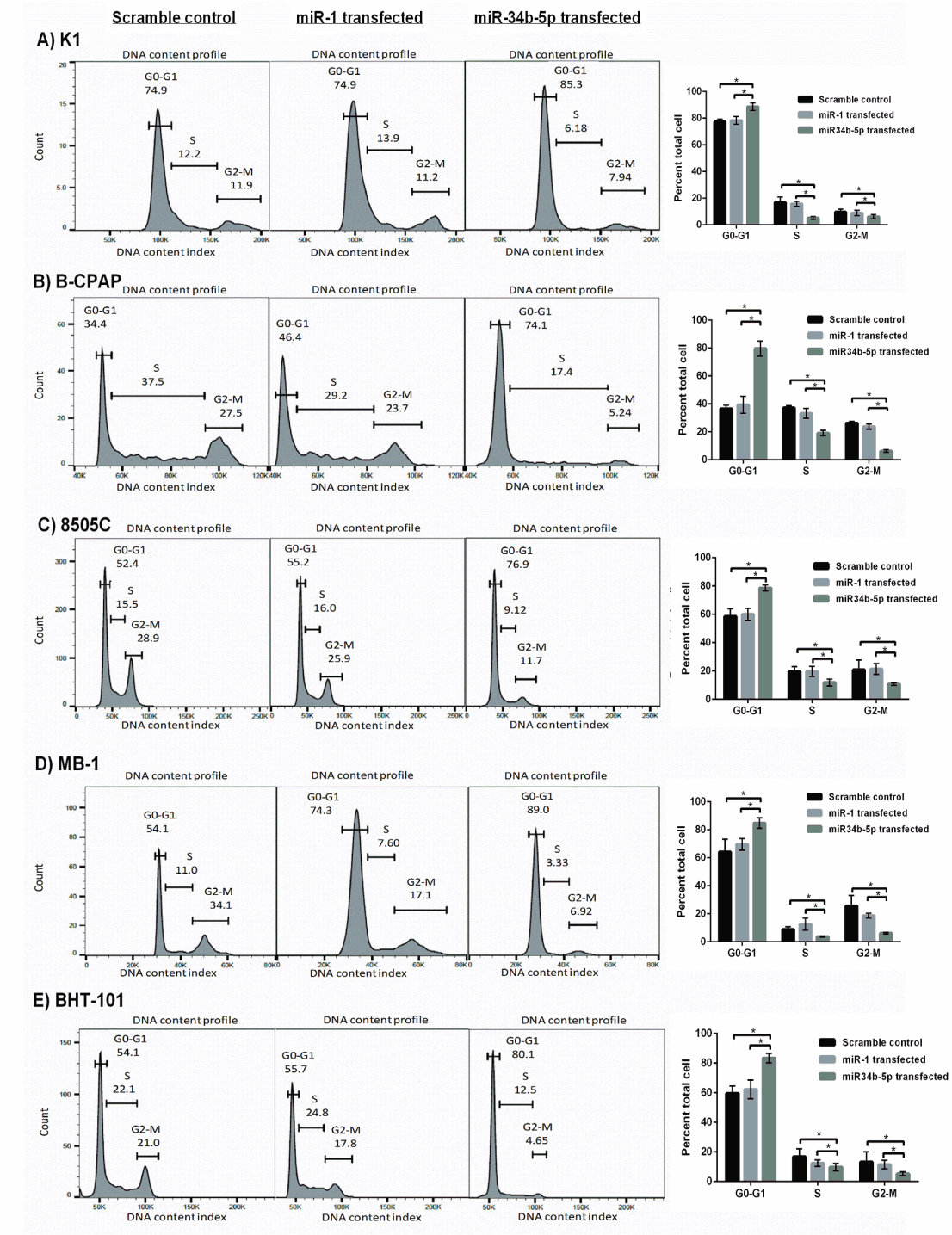
Scramble control
miR-1 transfected
miR-34b-5p transfected
A) K1
DNA content profile
G0-G1 74.9
S 12.2
G2-M 11.9
G0-G1 74.9
S 13.9
G2-M 11.2
G0-G1 85.3
S 6.18
G2-M 7.94
Count
DNA content index
Percent total cell
Scramble control
miR-1 transfected
miR34b-5p transfected
G0-G1
S
G2-M
B) B-CPAP
G0-G1 34.4
S 37.5
G2-M 27.5
G0-G1 46.4
S 29.2
G2-M 23.7
G0-G1 74.1
S 17.4
G2-M 5.24
C) 8505C
G0-G1 52.4
S 15.5
G2-M 28.9
G0-G1 55.2
S 16.0
G2-M 25.9
G0-G1 76.9
S 9.12
G2-M 11.7
D) MB-1
G0-G1 54.1
S 11.0
G2-M 34.1
G0-G1 74.3
S 7.60
G2-M 17.1
G0-G1 89.0
S 3.33
G2-M 6.92
E) BHT-101
G0-G1 54.1
S 22.1
G2-M 21.0
G0-G1 55.7
S 24.8
G2-M 17.8
G0-G1 80.1
S 12.5
G2-M 4.65

Figure 10. miR-34b-5p induces the cell cycle arrest in thyroid carcinoma cell lines:

Following transfection of K1, B-CPAP, 8505C, MB-1 and BHT-101 thyroid carcinoma cells with miR-34b-5p mimic or miR-1 for 48 hours, nuclei of the carcinoma cells were stained with propidium iodide (PI) solution and analysed for DNA content by flow cytometry. Data were shown as mean ± SD of three independent experiments and represent percentage cells in different phases of the cell cycle with miR-34b-5p related to miR-1 and scramble treatments. Flow cytometry results indicated the cell number increased in G0-G1 phase and decreased in S phase when compared with miR-1 transfected and control groups. An asterisk (*) indicate statistically significant differences ($P < 0.05$, Student's t-test) when compared to control cells (K1: papillary thyroid carcinoma; B-CPAP: metastatic papillary thyroid carcinoma in a lymph node; 8505C: anaplastic thyroid carcinoma from a papillary thyroid carcinoma origin; MB-1: anaplastic thyroid carcinoma; BHT-101: metastatic anaplastic thyroid carcinoma in a lymph node).

4.3.7 Expression of miR-34b-5p leads to increased cell death in thyroid cancer cells

Inhibition of growth of cell could be resulted from apoptosis induced by overexpression of miR-34b-5p. Herein, we further investigated the miR-34b-5p induced apoptotic changes of thyroid cancer cells. The increase in the G0-G1 phase induced by miR-34b-5p mimic suggests that high expression of miR-34b-5p can lead to the induction of cell death (Figure 11). A few late apoptotic cells were observed in the control group. The early apoptotic rate in thyroid carcinoma cells treated with miR-34b-5p mimic was significantly different from the control group. However, in the miR-34b-5p-treated group, a large number of apoptotic cells was found (Figure 11) ($P < 0.05$).

The early and late apoptosis rates of K1 (primary papillary thyroid carcinoma) transfected with miR-34b-5p mimic (15 nM) was significantly increased after 2 days of transfection (9.56 % ± 5.14) when compared with transfected with miR-1 (15 nM) and the control groups (Figure 11A) ($P < 0.05$).

The percentage of early and late apoptosis events were slightly increased in the B-CPAP cells (metastasizing papillary thyroid carcinoma) after 2 days of transfection with miR-34b-5p mimic, compared with mock transfected and scramble controls (2.69 % ± 1.46) (Figure 11B). Similar trends were also detected in the 8505C cells (metastatic anaplastic thyroid carcinomas from a lymph node with primary papillary thyroid carcinoma) with a slight increase in early and late apoptosis events after 2 days of transfection when compared to the mock and scramble control transfected (1.42 % ± 0.69) (Figure 11C). In anaplastic thyroid carcinoma (MB-1 and BHT-101), the early and late apoptotic features were also noticed to be increased significantly up to (6 % ± 3.91) and (4.99% ± 3.31) respectively (Figure 11D and 11E) ($P < 0.05$).

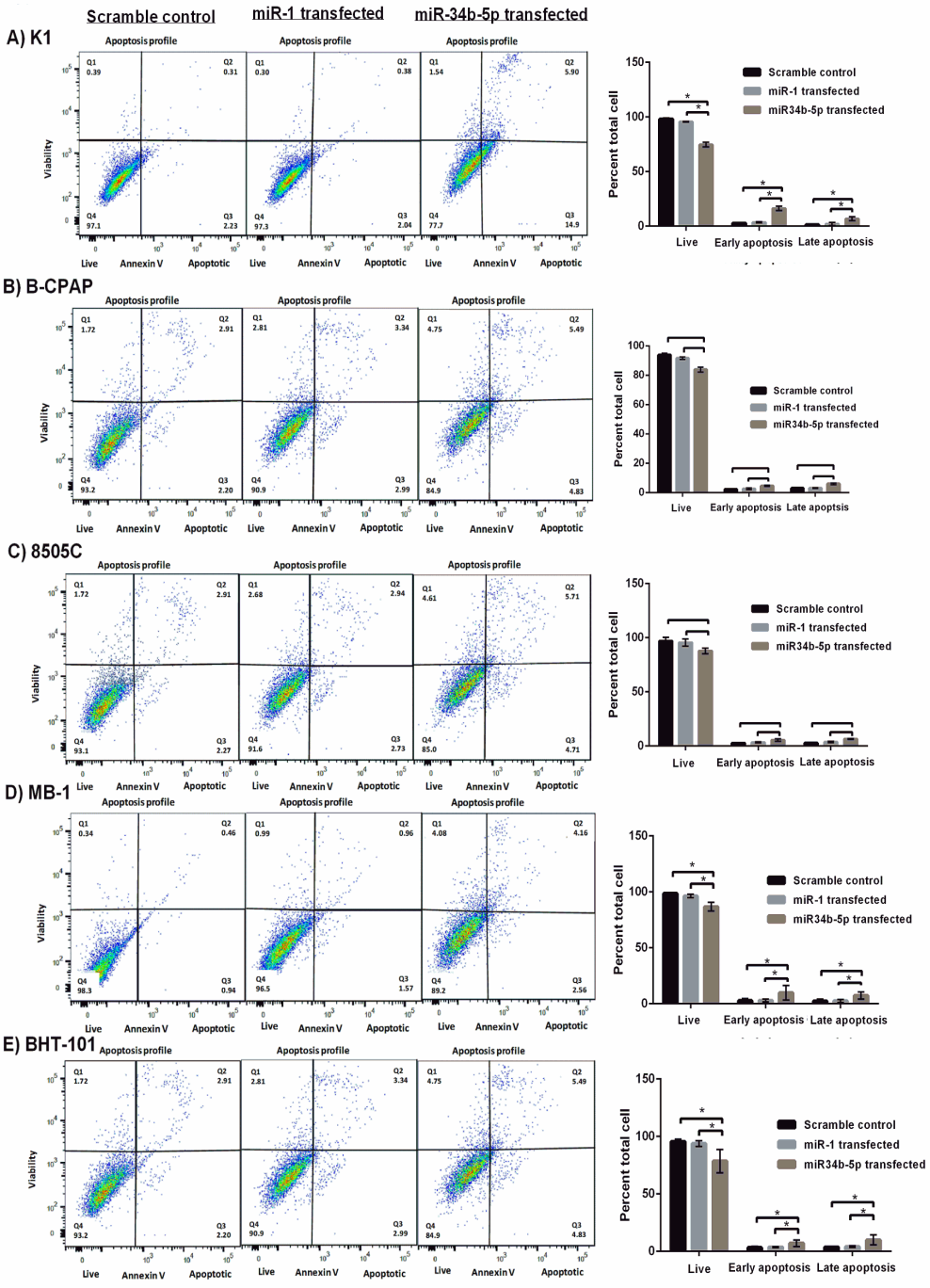
Scramble control
miR-1 transfected
miR-34b-5p transfected
A) K1
B) B-CPAP
C) 8505C
D) MB-1
E) BHT-101
Apoptosis profile
Viability
Live
Annexin V
Apoptotic
Percent total cell
Scramble control
miR-1 transfected
miR34b-5p transfected
Early apoptosis
Late apoptosis
Late apoptsis

Figure 11. Re-expression of miR-34b-5p enhances apoptosis in thyroid carcinoma cell lines: Thyroid carcinoma cells transfected with either miR-34b-5p or miR-1 and scramble miRNA for 48 hours. After 48 hours, cells were subjected to Annexin V/propidium iodide (PI) staining and flow cytometry analysis. Data were shown as mean ± SD of three independent experiments. The data represented percentage of AnnexinV-positive cells with miR-34b-5p related to treatment (miR-1) and scramble control groups. The percentage of dead cells (Q1; upper left quadrant), live cells (Q4; lower left quadrant), late apoptosis cells (Q2; PI+/Annexin V+; upper right quadrant) and early apoptosis cells (Q3; PI-/Annexin V+; lower right quadrant) were indicated. Asterisks indicate statistically significant differences (* $P < 0.05$, Student's t-test) when compared to control cells (K1: papillary thyroid carcinoma; B-CPAP: metastatic papillary thyroid carcinoma in a lymph node; 8505C: anaplastic thyroid carcinoma from a papillary thyroid carcinoma origin; MB-1: anaplastic thyroid carcinoma; BHT-101: metastatic anaplastic thyroid carcinoma in a lymph node).

4.4 Discussion

In the present study, we have demonstrated the biological effects and tumour suppressive effects of miR-34b-5p for the first time in thyroid cancer tissues and cell lines. Altered expression levels of miR-34b-5p and its correlation with pathological T-stage in patients with thyroid carcinoma imply its potential regulatory effects in the initiation and progression of thyroid carcinoma. Our results are in agreement with finding by others in thyroid cancer tissue [580, 592, 593]. In addition, this study has noted multiple cellular effects of miR-34b-5p by modulating its downstream targets such as Bcl-2 and Notch1 proteins. Thus, it can be hypothesised that miR-34b-5p suppresses the tumour growth through this downstream effect in thyroid carcinomas.

Overexpression of VEGF-A often noted in tissues from patients with thyroid carcinoma and this overexpression correlated with the pathogenesis of thyroid carcinoma[594, 595]. In the current study, VEGF-A protein expression decreased significantly following miR-34b-5p overexpression. Thus, deregulation of miR-34b-5p has a potent effect on tumour growth and progression by regulating angiogenesis in thyroid carcinoma cells. Further functional assays, as well as in-vivo studies, are required to confirm miR-34b-5p's angiogenic regulation in thyroid carcinomas.

We noted pro-apoptotic and anti-proliferative effect for miR-34b-5p in cells from the five thyroid carcinoma cell lines, which is in agreement with previous studies on miR-34b in other cancers[578, 596, 597]. Previous studies have also pointed out the similar roles for miR-34a and miR-34b, particularly in the suppression of cancer cell cycle, mainly by induction of G0-G1 cell cycle arrest [468, 470, 562, 578]. In this study, miR-34b-5p induced cell cycle arrest at G0-G1 in thyroid carcinoma cells. The suppression of cell cycle was more pronounced in the cells from more aggressive type of thyroid

carcinomas. These results imply the potential regulatory roles of miR-34b-5p in cancer cell growth and proliferation by targeting various checkpoints in the cell cycle.

Notch1 is a downstream target for miR-34b[579]. In addition, Notch signalling directly or indirectly implicated in the regulation of genes involved in angiogenesis such as VEGF-A [598]. Geers et al. found that papillary thyroid carcinoma had a higher number of Notch1- positive cells in comparison to normal or nodular hyperplastic thyroid[599]. Dysregulation of Notch1 prevents differentiation and results in inhibition of apoptosis, suggesting a potential oncogenic role of Notch1 [599-601]. Similarly, this study has noted a significant downregulation of Notch1 protein expression in response to miR-34b-5p overexpression in thyroid carcinoma cells. Thus, Notch1 could be a potential mediator of miR-34b-5p's cellular effects in thyroid carcinoma.

In different cancers, enhancement of Bcl-2 expression increases the VEGF expression[602-604]. VEGF could also act as a survival factor for tumour cells by inducing the Bcl-2 expression and inhibiting apoptosis [605]. In addition, a study on prostate carcinoma revealed that Bcl-2 could interact with other factor (s) to regulate VEGF expression[602]. Notably, there was no involvement of these factors in the modulatory role of Bcl-2 in angiogenesis. Indeed, they found an exact similar expression level of transforming growth factor β-1 (TGFβ-1) and hypoxia-induced basic fibroblast growth factor (bFGF) in Bcl-2 transfected and untransfected groups[602, 606, 607]. This observation suggests that the effects of Bcl-2 on the VEGF expression level can be relatively specific.

In concurring with these observations, our results showed similar regulatory effects of miR-34b-5p on Bcl-2 and VEGF-A proteins, suggesting its potential role in the pathogenesis of thyroid carcinoma cells by modulating these genetic pathways. The current results indicated that miR-34b-5p induces cell death in thyroid carcinoma cells.

This could be a related to miR-34b-5p induced G0-G1 cell cycle arrest directly or due to suppression of Bcl-2 protein as a pro-survival factor[480, 489] or VEGF-A [582]. Regulation of Notch1 signalling pathways, as well as downstream effects on Bcl-2 and VEGF-A further, attributes to the mechanism behind miR-34b-5p induced cell cycle, apoptotic and angiogenic changes in thyroid carcinoma cells. As miR-34b-5p increases cell death in different carcinomas, miR-34b-5p induced apoptotic changes might be a common event in the pathogenesis of cancer [538, 608, 609]. Thus, our results further confirm the tumour suppressor role for miR-34b-5p in thyroid carcinomas.

In conclusion, this study indicates that miR-34b-5p downregulation may play a key role in the pathogenesis of the growth and proliferation of thyroid carcinoma. In addition, restoration of miR-34b-5p in thyroid carcinoma cells inhibited the expression of Bcl-2, VEGF-A and Notch1 proteins suggesting its target affinity towards these key regulators in molecular carcinogenesis. Furthermore, miR-34b-5p potentially functions through modulation of its downstream targets - Bcl-2, VEGF-A and Notch1. Thus, miR-34b-5p could be a target in the development of novel molecular therapeutic targets for metastatic thyroid carcinoma.

Chapter 5: Introduction of the miR-34b (PEGlyated-miR-34b) displayed an antiproliferative effect on thyroid cancer and reduced VEGF-A expression *in vitro* and *in vivo*

Antiproliferative Activity and VEGF Expression Reduction in thyroid cancer via Intravenous Liposomal Delivery of miRNA-34b-5p

This section includes a co-authored published paper. The bibliographic details of co-authored paper, including all authors, is:

Hamidreza Maroof, Vinod Gopalan, Alfred King-Yin Lam.

First author contribution to the submitted paper involved: Literature review, Experimental design, laboratory performance, data collection, data analysis, categorisation of the data into a usable format and providing direction on the scope and structure of the analysis, drafting the manuscript and revision.

Other authors contribution to the submitted paper: Critical review and revisions were conducted by King-Yin Lam and Vinod Gopalan.

(Signed) _____ (Date) 19-09-2017

Name of Student: Hamidreza Maroof

(Countersigned) _____ (Date) 19-09-2017

Principle Supervisor and
Corresponding author of paper: Prof. Alfred K-Y Lam

Paper 05: Antiproliferative Activity and VEGF Expression Reduction in thyroid cancer via Intravenous Liposomal Delivery of miRNA-34b-5p

Hamidreza Maroof, Vinod Gopalan, LanFeng Dong, Alfred K. Lam

(Manuscript is under preparation)

5.1 Abstract

The aim of this study is to determine the functional role of microRNA-34b-5p (miR-34b) in thyroid cancer progression. We used a hydration-of-freeze-dried-matrix (HFDM) formulated liposomes (PEGlyated-miR-34b) for systemic delivery of this microRNA to the thyroid cancer in vitro and in vivo. miR-34b expression was low and significantly ($P<0.05$) overexpressed following transfection with PEGlyated-miR-34b in thyroid cancer cell lines. miR-34b expression level was quantified and confirmand by real time-PCR. Immunocytochemistry, western blot and ELISA were carried out to determine the effect of this manipulation on VEGF-A expression. Protein level of VEGF-A were remarkably reduced in transfected cell lines when compared to controls. Furthermore, miR-34b overexpression significantly ($P<0.05$) reduced proliferation, wound healing potential, cell cycle progression and increased apoptosis in thyroid cancer cell lines. in vivo xenotransplantation mouse model was used to investigate the functional role of miR-34b in thyroid cancer cell biology in response to its overexpression. Xenotransplantation model showed that smaller and low-vascularized tumours were formed upon intravenous (IV) liposomal administration. Taken together, these data indicated that miR-34b may acts as a tumour suppressor via modulation of angiogenesis in thyroid cancer, and delivery of miR-34b using this cationic liposome may provide an effective therapeutic delivery strategy for thyroid cancer treatment.

5.2 Introduction

Thyroid cancer is the most rampant endocrine malignancy, and its occurrence has been steadily increasing and accounting for >80% of endocrine malignancy and 1.8% of all recently distinguished cancer reports [8]. Papillary thyroid carcinoma has an invasive behaviour and may spread to another organ such as lung as a primarily metastasis site. Life expectancy of patient with papillary thyroid cancer is age depended and increased to eighth position of cancer female's cancer. It shows an increase of 4% annually in the world [610]. Several genetic and epigenetic mutations are involved with thyroid cancer initiation and progression, which lead in activation several cellular pathways such as proliferation and angiogenesis [611].

Angiogenesis is an essential process for tumour growth, metastasis, and tumours, which have lost growth regulatory function and therefore proliferate aberrantly. Controlling of tumour-associated angiogenesis is a tactic for inhibition of cancer progression [612]. Angiogenesis is primarily activated when growing tumour creates a low oxygen microenvironment and an essential process for tumour growth and metastasis. The cancer cell undergoes an angiogenic switch directly leading to the secretion of angiogenic factors such as Vascular endothelial growth factor (VEGF) [205]. Inhibition studies strongly show the causative role of VEGF-A in tumour angiogenesis. It has been demonstrated that introducing nude mice harbouring sarcoma and glioblastoma cell tumours with anti-VEGF antibody through intraperitoneal administration, significantly decreases tumour vessel density and declines tumour cell growth [215]. Due to the large vascular supply of thyroid gland, the large range of lesions and the spectrum of aggressiveness, so far have made the thyroid cancer a suitable pattern for study on cancer mechanism and angiogenesis [8].

Recent evidence suggests that a defined class of non-coding 21–25 nucleotides RNA (miRNA) plays key roles in the regulation of gene expression, especially function at the post-transcriptional level through interaction with the 3' untranslated region (3’ UTR) of target mRNA [613]. In addition, studies have highlighted the role of miR-34b as a tumour suppressor in a different type of tumours including non-small cell lung cancer (NSCLC), small cell lung cancer cell SCLC, prostate cancer, lung cancer, colorectal cancer and thyroid cancer [579]. However, very little is known about the role and expression state of miR-34b in thyroid cancer progression.

Various miRNA-based delivery strategies have been developed to enhance the uptake of miRNA to target cell after systematic administration. Most of these methods harbors its own limitation. The cellular uptake of miRNA is often poor due to rapid uptake by reticuloendothelial (RES) organs and their high instability due to rapid degradation in serum and renal clearance [614]. Therefore, many studies have been devoted to developing an effective *in vitro* and *in vivo* RNA delivery system. Of these, the polyethylene glycol (PEG) ylated lepidic system showed effectiveness in several cancer types [615-617]. The formulation in those studies had own limitations and required special equipment and skills. Considering these limitations, our laboratory developed an efficient hydration-of-freeze-dried-matrix (HFDM) method to formulate a carrier which is able to protect RNA from degradation of RES as well as nuclease and accumulate in tumour cells *in vitro* and *in vivo* [618]. The aim of this study was therefor to investigate the biological effect of miR-34b on proliferation and angiogenesis using this carrier. Importantly, PEGlyated-miR-34b showed that our designed particle can effectively deliver the miR-34b in tumour cells, resulting in downregulation of VEGF-A expression and inhibition of proliferation and angiogenesis process. Therefore, our results indicated

the potential of PEGlyated liposomes in systematic delivery of miRNA, particularly miR-34b in modulation of thyroid cancer angiogenesis.

5.3 Materials and Methods

5.3.1 In vitro study

5.3.1.1 Cell culture.

The thyroid cancer cell lines used in this study, 8505C (undifferentiated carcinoma) and BHT-101 (undifferentiated thyroid carcinoma from the lymph node metastasis) were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmBH-German Collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany). 8505C cell line was cultured in Roswell Park Memorial Institute medium (RPMI 1640) (Invitrogen Carlsbad, CA, USA), 2 mM l-glutamine (Invitrogen) supplemented with 10% (v/v) heat & inactivated fetal bovine serum (Invitrogen). BHT-101 cells were cultured in 80% RPMI 1640 (Invitrogen), 10% heat & inactivated fetal bovine serum (Invitrogen) and 2 mM l-glutamine (Invitrogen). The cell lines were authenticated in the standard protocol (using multiplex polymerase chain reaction of mini-satellite markers for DNA fingerprinting and identification of short tandem repeats of cell lines and cytogenetics) and the passage number of these cell lines was less than nine.

5.3.1.2 HFDM Formulated miRNA-entrapped PEGlyated Lipid particle.

DOTAP, DOPE, cholesterol, and PEG2000-C16 Ceramide were purchased from Avanti Polar Lipids (Alabaster, AL). Hydration of a freeze-dried matrix (HFDM) method was used for preparation of Liposomes as described previously [618]. Briefly, DOTAP, cholesterol, DOPE and PEG_{2000}-C16 Ceramide at a molar ratio of 50:35:5:10, were mixed with mature miR-34b mimic sequence (guide strand) 5'

UAGGCAGUGUCAUUAGCUGAUUG-3′ (Qiagen, Venlo, Limburg, Netherlands) at an nitrogen: phosphate (N:P) ratio of 4 in sucrose-containing water/*tert*-butanol (1:1 v/v) co-solvent system. The resultant formulation was then snap frozen and freeze-dried overnight (ALPHA 1–2 LDplus, Martin Christ, Germany) at −80 °C and <0.1 mbar. Sterile water was used for hydration of Freeze-dried matrix immediately before use. The final product contained 40 μg miR-34b in 300 μl of isotonic sucrose solution.

5.3.1.3 Nanoparticle characterization.

Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) was used for measurement of size, polydispersity index, miR-34b entrapment efficiently and zeta potential of the resultant liposomes following appropriate dilution in distilled water. Measurements were carried out at room temperature. Two size measurements were performed with 10 runs per measurement undertaken.

5.3.1.4 PEGlyated-miR-34b Transfection.

Cell lines were transfected with PEGlyated-miR-34b and with PEGlyated-miR-1, a non-targeting control, positive control (miR-1) (Qiagen) and empty liposome, immediately after being seeded at a density of 20×10^{4} cells / well in 6 well-plate. A measurement of 1 ml of 5nM liposome-entrapped miR-34b suspended in antibiotic-free complete media was then added to each well. Cells were maintained at 37 °C and 5% CO2, and monitored for 48 hours afterward. The same protocol was performed for PEGlyated-miR-1 and empty liposome transfection.

5.3.1.5 Quantification of miR-34b and VEGF-A expression.

The miR-34b expression level was quantified by real-time quantitative polymerase chain reaction (qRT-PCR) using Hs_miR-34b*_2 miScript Primer Assay

(Qiagen, Venlo, Limburg, Netherlands) following the suggested protocol. Samples were normalised using the housekeeping gene RNU6B RNA (Hs_ RNU6B_2 miScript Primer Assay, Qiagen). Total RNA from cells was extracted using NucleoSpin® miRNA Kit (MACHEREY-NAGEL, Duren, Germany) with a DNase supplementary step. Then, cDNA was synthesised using miScript II RT Kit (Qiagen) according to the manufacturer instructions. Amplification, detection and analysis were performed with an IQ5 multicolour Real-Time PCR detection system (BIO-RAD, Hercules, CA, USA). Real-time PCR amplifications were performed in a 20 µl reaction volume consisting of 10 µl QuantiTect SYBR Green PCR Master Mix (Qiagen), 1 µl miScript Primer Assay (Qiagen), 1 µl of miScript Universal Primer (Qiagen), and 5 µl of cDNA template at 2 ng/µl stock and 3 µl RNase- free water. All qRT-PCR reactions were carried out in triplicates with non-template controls as previously published protocol [585]. The PCR primers for VEGF-A and GAPDH were reported previously [619]. PCR was performed using cDNA iQ SYBR green supermix (Bio-Rad). Thermal cycling conditions included initial denaturation in one cycle of 3 min at 95 ˚C, followed by 40 cycles of 10 second at 95 ˚C, 30 second at 60 ˚C and 30 second at 72 ˚C. Melt curve analysis was also performed using eighty-one cycles of 30 second increasing from 55 ˚C. Expressions was presented as the ratio between miR-34b/RNU6B and VEGF-A/ GAPDH. The $2^{-\Delta\Delta ct}$ method was used to calculate the fold changes in each sample group. Less than 0.5-fold changes were considered as low expression. Fold changes between 0.5 and 2 were considered as normal expression whereas fold changes of more than 2 were considered as high expression.

5.3.1.6 Western blot analysis.

Cells were lysed in Cell Lysis Buffer NP40 (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1% Nonidet P40, 0.02% NaN3) (Invitrogen) supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA),

phenylmethanesulfonyl fluoride solution (PMSF) (Sigma) and phosphatase inhibitor cocktail (Cell Signaling, Danvers, MA, USA). Then, whole protein lysates were quantified using the Macherey-Nagel protein assay kit (MACHEREY-NAGEL). Equal quantities of 25 µg protein samples were run on a 4–15% precast polyacrylamide gel (Mini-PROTEAN® TGX ™ Precast Gel, Bio-Rad). Blocking was performed with 5% non-fat milk w/v in TBST (Tris buffered saline Tween 20: 120 mmol/l Tris–HCl, pH 7.4, 150 mmol/l NaCl, and 0.05% Tween 20) for 2 hours at room temperature.

After blocking, the membrane was incubated with anti-VEGF-A (Sc-152), 1:300 dilution and anti-ß-actin (Sc-4778), 1:5000 dilution (Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4˚C. According to the manufacturer's protocol, blots were washed five times with TBST. Then, incubated for 2 hours with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000 dilution) (Santa Cruz Biotechnology) at room temperature. Blots were then developed using Clarity™ Western ECL Blotting Substrate kit (BIO-RAD). Blots were visualized by using VersaDoc-MP Imaging System (BIO-RAD) and analysed with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

5.3.1.7 Cell proliferation assay.

Cell viability was evaluated by Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich) reagent. Cell density of 1×10^4 cells/well was seeded in a 96-well tissue culture plate (Becton Dickson and Company, Franklin Lakes, NJ). Cell proliferation with PEGlyated-miR-1 Transfection and empty liposome, were set as controls. On days 0 to 3 after the initial seeding, Cell proliferation was determined with CCK-8 (cell counting kit-8) following manufacturer instructions.

5.3.1.8 FACS analysis for cell cycle distribution.

Cells were transfected with PEGlyated-miR-34b, PEGlyated-miR-1 and empty liposome for 48 hours, then fixed with cold 70% ethanol for one hour as reported recently [620]. Briefly, after washing with cold Phosphate-buffered saline (PBS), 5ml of RNase A (10 mg/ml) was added to the cells and incubated for one hour at 37 °C. Finally, 10 ml of propidium iodide solution (1 mg/ml) was added to the cell suspension. Cell cycle distribution was then analysed by flow cytometry using FACS analysis (BD FACSCalibur, BD Biosciences). Finally, percentage of cells in different phases of cell cycle was determined by FlowJo single-cell analysis software (FLOWJO, LLC, Ashland, OR, USA).

5.3.1.9 Quantitative apoptosis assay.

Apoptosis assay was performed to measure the percentage of apoptotic cells using a Membrane permeability/Dead Cell Apoptosis Kit (Invitrogen). After 48 hours of transfection, cells were harvested and washed twice with ice-cold PBS and resuspended at the 25×10^{4} cells/ml in PBS. For staining, 1 µl of YO-PRO®-1 and 1 µl of PI were added and kept in the dark for 20 minutes at room temperature. After staining, flow cytometry was performed for the quantification of apoptotic cells using FlowJo single-cell analysis software (FLOWJO, LLC, Ashland, OR, USA).

5.3.1.10 Enzyme-linked immunosorbent (ELISA) assay.

Cells were incubated in low-serum media (DMEM: F12 with 1% FBS to preserve VEGF stability) and conditioned media collected after two days for the analysis of VEGF secretion levels as previously described [621]. Cells with PEGlyated-miR-1 Transfection and empty liposome, were set as controls. The concentration of secreted VEGF was

measured using a Novex Human VEGF ELISA kit (Life Technologies Carlsbad, CA, USA) following manufacturer instructions.

5.3.1.11 Wound Healing Assay.

To determine the migration capacity of thyroid cancer cells after transfection with PEGlyated-miR-34b, a wound healing assay was performed. Cells were cultured in six-well plates. Transfection was performed at a final concentration of 5nM when cells grew up to 70% confluency as a monolayer. 48 hours later, scratches were made in PEGlyated-miR-34b, PEGlyated-miR-1 treated cells and empty liposome treated cells with a 200-ml pipette tip across the centre of culture plates. Media was added to the culture after the cells were washed three times with PBS. Images were then taken under inverted microscope at 0,24, 48 and 72 hours after the wounding. The mobility of cells in different days were measured and compared with ImageJ 1.48 software.

5.3.2 In vivo Study

Subcutaneous BHT-101 tumours were established in female NU/NU nude mouse of 6-8 weeks old (Animal Resource Centre). Two million cells suspended in 100 µl of sterile PBS were subcutaneously injected into each mouse on the right abdominal side. The study was designed for 56 days. Tumour establishment and progression were monitored at days 7, 14, 21, and 28 using callipers during the course of the experiment. After 4 weeks, mice were injected intravenously with PEGlyated-miR-34b and with PEGlyated-miR-1 (2mg/kg of microRNAs - 40 µg per dose per mouse) and empty liposome on days, 29, 33, 37, 41, 45, 49 and 53. All formulation were prepared at an N:P ratio of 4 . On day 56, mice were killed, and tumours were collected. Tumour volume was assessed by measurement with callipers using the following formula: tumour volume =

(length × width× height)/0.5236 and 6 mice were used per treatment group (n=6) [622-625].

5.3.2.1 Histological analysis.

Formalin-fixed, Paraffin-embedded (FFPE) tissue samples were used to investigate the expression level of Ki-67 proteins. Immunostaining was performed by the peroxidase-indirect-polymer method. Tumour tissue sections were deparaffinized, rehydrated and subjected to epitope antigen retrieval 2 (ER2) (20 minutes, 94˚C) (Leica Biosystems, AR9640, Biosystems, Wetzlar, Germany) with target retrieval solution high pH 50x Dako EnvisionTM Flex (Dako, Glostrup, Hovedstaden, Denmark) in a pre-treatment module PTlink (Dako, Model PT 10130). Primary monoclonal mouse antibody anti-human KI67 (Clone MIB-1, M7240; Dako) at 1:50 was used. Immunohistochemistry was performed using an automated stainer (BOND-III, Leica, Biosystems, Wetzlar, Germany) by the peroxidase-indirect- polymer method (K8000, Dako) for Ki-67. Tonsil was used as positive control. For negative controls, the primary antibody was omitted during the staining. Proliferation was evaluated in tumour section images at 400 x, only considering areas with dense tumour cell mass, displaying similar cell density between PEGlyated-miR-34b, PEGlyated-miR-1 and empty liposome groups of xenografts.

A grading scale ranging from 0 to 3 was used for this assessment, where 0 represented a negative staining, 1 represented weak staining (1-30%), 2 represented moderate staining (31 to 70%) and 3 represented strong staining (>70%). Assessment of the slides according to the extent of positive Ki-67 staining was also taken in consideration. Pale yellow indicating weak-positive staining (1); brown yellow indicating moderate-positive staining (2) and nut-brown yellow indicating strong-positive staining (3). Image J software was used for quantitation of Ki-67 positive cells. Proliferation was

expressed as percentage of Ki-67-positive cells. Hematoxylin and eosin (HE) staining of the specimen was performed for the section taken from the paraffin block according to as previously published protocol [626]. The grade and differentiation of tumours from the block were analysed and confirmed by a pathologist.

5.3.3 Data analysis.

Results were analysed using GraphPad Prism 7.0 (Graph Pad Software, SanDiego, CA, USA) and were expressed as means ± SD (standard deviation). All the *in vitro* experiments were performed at least three times. Tumour volume from the mice xenograft model was analyzed using a two-tailed paired t-test. Statistical comparisons between groups were conducted using one-way ANOVA. Levels of significance are shown as follow; * $P < 0.05$** $P < 0.01$, *** $P < 0.001$ using Student's t-test, when compared to the control groups and individual p-values was shown in the figures.

5.4 Results

5.4.1 Characterization of lipid nanoparticles entrapped miR-34b.

Jana and *et al* previously described a novel liposome formulation method, the hydration of a freeze-dried matrix (HFDM). They displayed that PEGlyated siRNA-loaded lipid particles containing 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), polyethylene glycol (PEG) 2000-C_{16}Ceramide, Cholesterol, and N-[1-(2,3-dioleoyloxy)propyl]-N,N,N trimethylammonium methyl-sulfate (DOTAP), at the molar ratio of 5: 10: 35: 50 as well as DiR, a lipidic dye, can significantly accumulate in spleen, lung and liver [618]. This observation led us to investigate further whether this method could provide an effective delivery of miR-34b in thyroid cancer cells. Therefore, we prepared PEGlyated-miR-34b containing particles via HFDM method. After rehydration, the size, zeta potential, polydispersity index of the resulting lipid particles and the

entrapment efficiency of miR-34b were measured (Table 12). The results indicated that the prepared PEGlyated-miR-34b liposomes have favourable characteristics for effective delivery of miR-34b such as high entrapment efficiency and a size below 200 nm.

Size (nm)[a]	135.3 ± 10.8
Polydispersity index	0.311 ± 0.06
Zeta potential (mV)	39.16 ± 0.451
miR-34b-5p entrapment efficiency (%)	96.9 ± 2.18

Table 14. characterization of miR-34b-5p-loaded PEGlyated lipid particles. The results indicated that the prepared PEGlyated-miR-34b liposomes have favourable characteristics for effective delivery of miR-34b such as high entrapment efficiency and a size below 200 nm. Each sample contained 40 µg miR-34b in 300 µl isotonic sucrose solution. Three batches HFDM liposomes were analysed (n = 3). [a] Size represents Z_{ave}± SD as measured by Malvern Nano Zetasizer.

5.4.2 PEGlyated-miR-34b overexpression cause downregulation in VEGF-A at protein and RNA expression level as a hallmark of angiogenesis in vitro and in vivo.

VEGF-A play important role in thyroid cancer progression through modulation of angiogenesis and proliferation pathways and it is regulated by miR-34 family in some cancers [578, 579]. qRT-PCR was used to confirm the increased expression of miR-34b in vitro and in vivo (Figure 12A and D). Cells treated with the PEGlyated-miR-34b showed significant overexpression of miR-34b levels (shown as a ratio of expression) when compared to the PEGlyated-miR-1 and empty liposome transfected cells ($P < 0.05$). miR-34b was significantly overexpressed to investigate its specific regulatory function on VEGF-A expression level. Therefore, thyroid cancer cell lines and tissue were

transfected with PEGlyated-miR-34b, PEGlyated-miR-1 and empty liposome in a concentration of 5 nM for 48 hours and intravenously for 28 days. The expression level of VEGF-A in thyroid cancer cell lines and tissue were analysed by western blot and RT-PCR while the secretion of VEGF-A protein level in serum was analysed by ELISA. As shown in Figure 1B, western blot analysis showed that VEGF-A was significantly decreased in 8505C (undifferentiated carcinoma) (Figure 12B-I) ($P < 0.05$), more evidently in BHT-101 (undifferentiated thyroid carcinoma from the lymph node metastasis) (Figure 12B-II) ($P < 0.01$), and tissue ($P < 0.05$) at RNA level (Figure 12E) when thyroid cancer cell lines and tissues were treated with PEGlyated-miR-34b whereas the PEGlyated-miR-1 and empty liposome had no changes (Figure 12B-III). Similar trend was also noted in protein expression of VEGF-A in the serum (Figure 12C). ELISA analyses indicated that, compared with the PEGlyated-miR-1 and empty liposome control, the serum VEGF-A protein expression in transfected group was remarkably ($P < 0.01$) decreased more in BHT-101 than 8505C ($P < 0.05$). In agreement with the overexpression of miR-34b through induction with PEGlyated-miR-34b, the aforementioned finding showed that miR-34b could be involved in the thyroid cancer progression, particularly in the development of angiogenesis via modulation of VEGF-A expression.

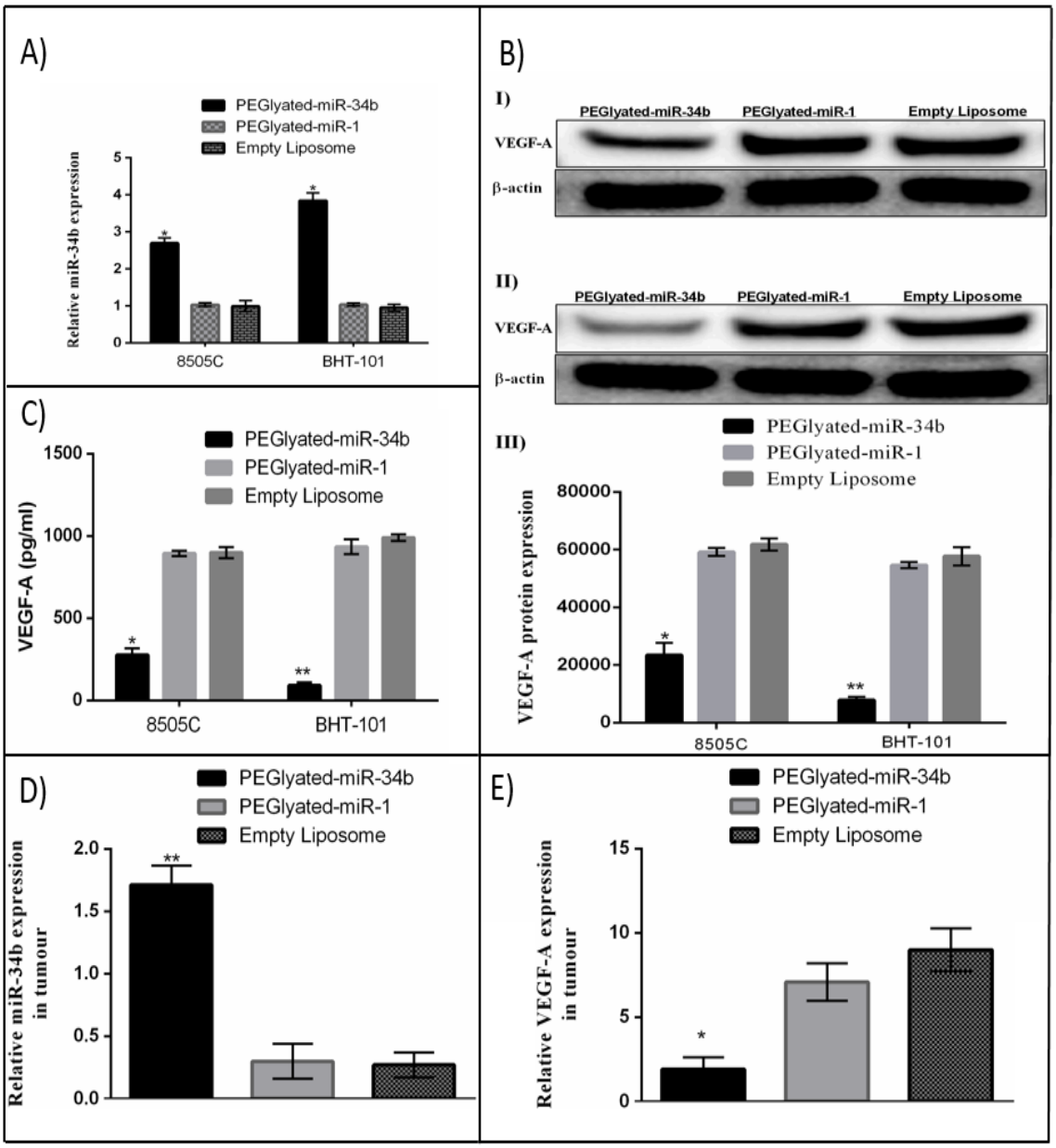

Figure 12. The effects of miR-34b overexpression on VEGF-A expression in 8505C and BHT-101 cell lines: (A) PEGlyated-miR-34b transfection led to overexpression of miR-34b in thyroid cancer cell lines (8505c and BHT-101) and (D) in tumour. Relative miR-34b expression after transfection with PEGlyated-miR-34b, PEGlyated-miR-1 and empty liposome, detected by qRT-PCR. miR-34b expression level was significantly increased after transfection with PEGlyated-miR-34b when compared to PEGlyated-miR-1 and empty liposome transfected cells and tissue.

(B) miR-34b alters VEGF-A expression in thyroid cancer cell lines. Western blot analysis showed the decreased expression levels of angiogenesis related protein VEGF-A, after overexpression of miR-34b. VEGF-A expression were changed upon PEGlyated-miR-34b transfection in the 8505C and BHT-101 cell

lines. VEGF-A protein expression was downregulated in 8505C and BHT-101 cell lines when compared to PEGlyated-miR-1 and empty liposome transfected cells.

C) Overexpression of miR-34b inhibits VEGF expression in thyroid cancer cell lines supernatant. Protein levels of VEGF-A of conditioned media measured by ELISA, showing significant reduction of secreted VEGF-A after PEGlyated-miR-34b, PEGlyated-miR-1 and empty liposome transfection in 8505C and BHT-101 cell lines when compared to the control groups. (E) PEGlyated-miR-34b transfection led to downregulation of VEGF-A in thyroid cancer tumour. Relative VEGF-A expression after transfection with PEGlyated-miR-34b, PEGlyated-miR-1 and empty liposome, detected by qRT-PCR. VEGF-A expression level was significantly decreased after transfection with PEGlyated-miR-34b when compared to PEGlyated-miR-1 and empty liposome transfected cells. Data are presented as mean ± SD from three independent tests. Level of significance, * $P < 0.05$, ** $P < 0.01$ when compared to the control groups.

5.4.3 Tumour Suppressor Properties of PEGlyated-miR-34b In Vitro.

Transfected thyroid cancer cell lines with PEGlyated-miR-34b demonstrated a significant decrease in cell proliferation when compared to PEGlyated-miR-1 and empty liposome-transfected cells (Figure 13). 8505C showed a notable ($P <0.001$) decreased proliferation in day 3 of transfection (Figure 13A). Similarly, the decreased proliferation of BHT-101 cells was observed in all 3 days of transfection, particularly in day 3 ($P < 0.01$) (Figure 13B). Likewise, would healing assay showed that PEGlyated-miR-34b transfected cells had a lower migration potential than PEGlyated-miR-1 and empty liposome-transfected control. As shown in Figure 14, the created wound healed faster in PEGlyated-miR-1 and empty liposome-transfected control whereas wound in transfected cell lines healed slightly ($P<0.05$).

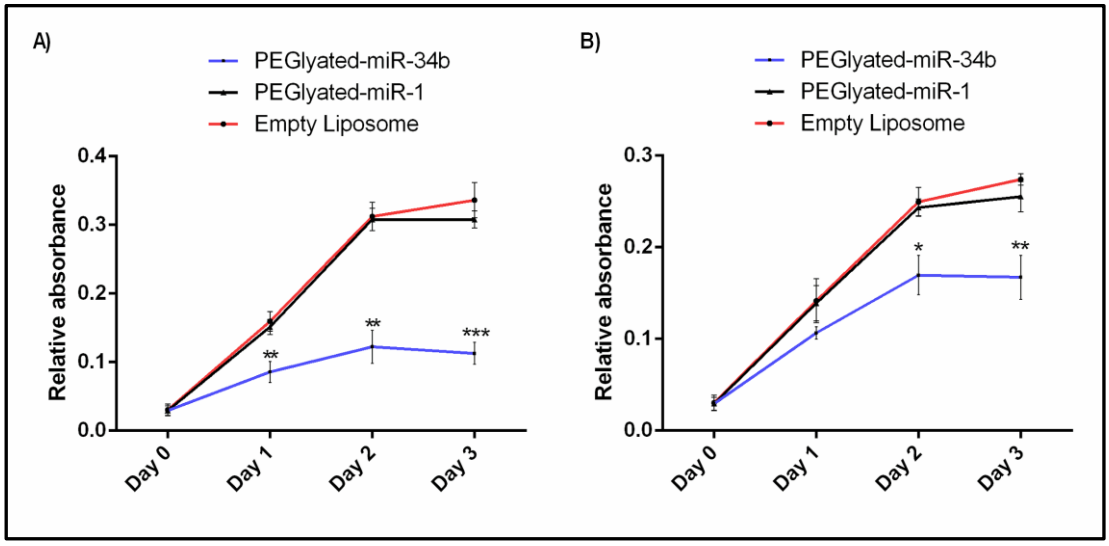

Figure 13. The effect of miR-34b upregulation on proliferation of thyroid cancer cell lines: Overexpression of miR-34b significantly inhibited cell proliferation in 8505C (A) and BHT-101 (B) cell lines. Both thyroid cancer cell lines treated with PEGlyated-miR-34b showed notable reduced cell proliferation when compared to PEGlyated-miR-1 and empty liposome transfected groups on different days after transfection. The results are shown as mean ± SD from three independent tests. Level of significance, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared to the control groups.

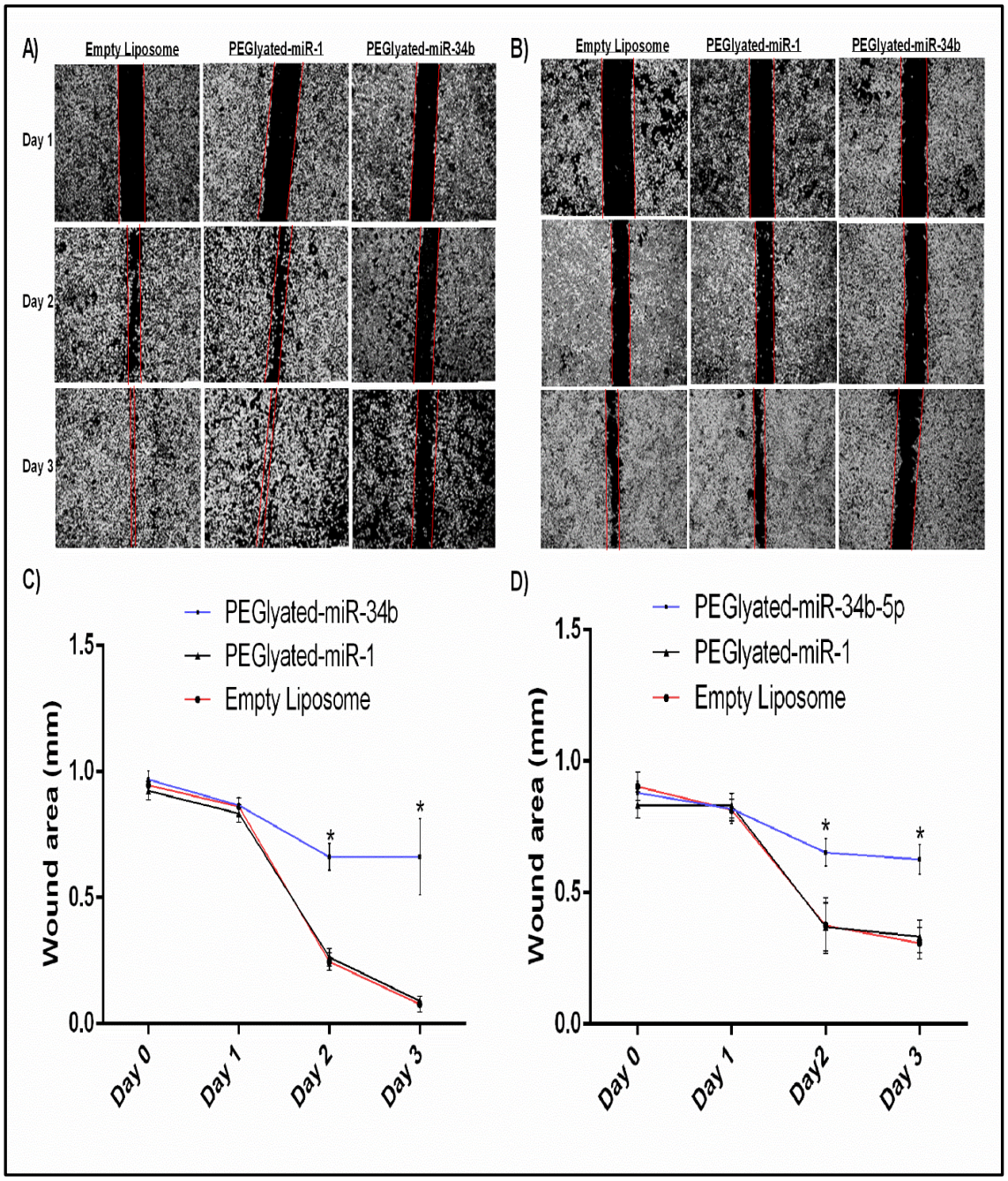

Figure 14. The influence of miR-34b overexpression in wound healing potential of thyroid cancer cells: The migration capacity of 8505C cell line significantly decreased after day 3 of transfection with miR-34b (A), and wound healed faster in PEGlyated-miR-1 and empty liposome transfected control groups in comparison to PEGlyated-miR-34b transfected group (C). Similar trend was observed in BHT-101 cell line (B). The wound healed slower in PEGlyated-miR-34b treated compared to PEGlyated-miR-1 and empty liposome transfected control group (D). Wound areas of all experimental cell types on different days

(day 0 to day 3) were recorded from three independent measurements and shown as mean ± SD. Level of significance, * P <0.05, when compared to the control groups.

5.4.4 The suppressive role of miR-34b in apoptosis and cell cycle distribution.

Following noticeable reduction in cell proliferation of thyroid cancer cell lines, apoptosis and cell cycle assays were performed to further examine the suppressive role of miR-34b in proliferation of 8505C and BHT-101 cell lines in this study (Figure 15). Apoptosis was induced after treatment with PEGlyated-miR-34b when compared to PEGlyated-miR-1 and empty liposome-transfected control. The percentage of early and late apoptosis event were significantly increased in 8505C cell line (Figure 15A) after two days of transfection with PEGlyated-miR-34b, compared with PEGlyated-miR-1 (5 nM) and the empty liposome-transfected control group (11.6±3.72) (P <0.05). At the same time, introduction of PEGlyated-miR-34b into the 8505C cell line (Figure 15C), exhibited a significant accommodation of cells in the G0-G1 phase (18.3±0.60) and a significant drop in S and G2-M phase after 48 hours (P <0.05). In BHT-101 cell line (Figure 15B), the early and late apoptotic features were significantly increased after day two of transfection with PEGlyated-miR-34b when compared to PEGlyated-miR-1 and the empty liposome-transfected control group (12.1±1.30) (P <0.05). A significant increased accommodation of cells in G0-G1 phase of cell cycle were also noted up to 15.33±0.23 in BHT-101 cell line (Figure 15D) after two days of transfection compared to PEGlyated-miR-1 and the empty liposome-transfected control group (P <0.05).

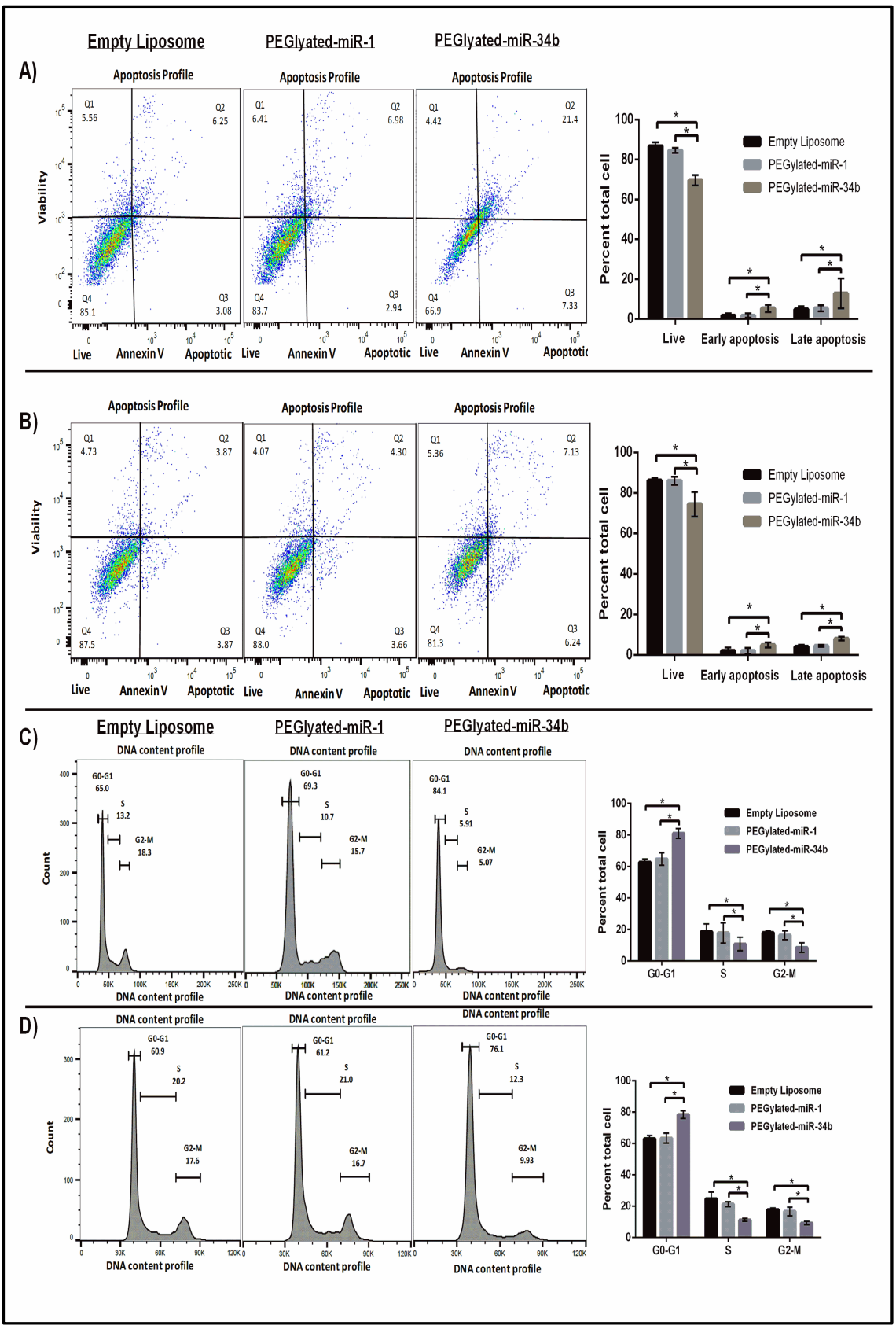
Empty Liposome
PEGlyated-miR-1
PEGlyated-miR-34b
A)
Apoptosis Profile
Q1 5.56
Q2 6.25
Q4 85.1
Q3 3.08
Q1 6.41
Q2 6.98
Q4 83.7
Q3 2.94
Q1 4.42
Q2 21.4
Q4 66.9
Q3 7.33
Viability
Live
Annexin V
Apoptotic
Percent total cell
Empty Liposome
PEGylated-miR-1
PEGylated-miR-34b
Live
Early apoptosis
Late apoptosis
B)
Q1 4.73
Q2 3.87
Q4 87.5
Q3 3.87
Q1 4.07
Q2 4.30
Q4 88.0
Q3 3.66
Q1 5.36
Q2 7.13
Q4 81.3
Q3 6.24
C)
DNA content profile
G0-G1 65.0
S 13.2
G2-M 18.3
G0-G1 69.3
S 10.7
G2-M 15.7
G0-G1 84.1
S 5.91
G2-M 5.07
Count
G0-G1
S
G2-M
D)
G0-G1 60.9
S 20.2
G2-M 17.6
G0-G1 61.2
S 21.0
G2-M 16.7
G0-G1 76.1
S 12.3
G2-M 9.93

Figure 15. Effect of miR-34b overexpression on apoptosis and cell cycle kinetics of thyroid cancer cells: Thyroid cancer cell lines transfected with PEGlyated-miR-34b PEGlyated-miR-1 and empty liposome for 48 hours. Apoptosis level of 8505C (A) and BHT-101 (B) were determined using Annexin V/propidium iodide (PI) staining and flow cytometry analysis. In both cell lines, miR-34b overexpression decreased the total number of apoptotic cells in early and late apoptosis phase. Data were shown as mean ± SD of three independent experiments and represent the percent of AnnexinV-positive cells with miR-34b-5p related to PEGlyated-miR-1 and empty liposome treatment groups. The percentage of dead cells (Q1; upper left quadrant), live cells (Q4; lower left quadrant), late apoptosis cells (Q2; PI+/Annexin V+; upper right quadrant) and early apoptosis cells (Q3; PI-/Annexin V+; lower right quadrant) were indicated.

Cell cycle analysis revelaled that miR-34b overexpression caused a significant accumodation of 8505C (C) and BHT-101 (D) cells in G0-G1 phase after 48 hours of transfection with PEGlyated-miR-34b when compared to PEGlyated-miR-1 and empty liposome transfected control group. Nuclei of the cells were stained with propidium iodide (PI) solution and analysed for DNA content by flow cytometry. Data were shown as mean ± SD of three independent experiments and represent the percentage of cells in different phases of the cell cycle with PEGlyated-miR-34b related to PEGlyated-miR-1 and empty liposome treatment. Flow cytometry results indicated the cell number increased in G0-G1 phase and decreased in S and G2-M phases when compared with PEGlyated-miR-1 and empty liposome treatment groups. Asterisks indicate statistically significant differences (* $P < 0.05$, Student's t-test) when compared to control cells.

5.4.5 In-vivo Confirmation of miR-34b Tumour Suppressor Properties.

To examine the functional role of miR-34b in thyroid carcinoma pathogenesis in vivo, we used a xenograft nude mouse model. Tumour cell were subcutaneously injected into mice. 28 days was considered to tumour establishment in all experimental animals (24/24). However, the tumours were detectable by palpation after one week of cancer cell implantation. Mice treated with PEGlyated-miR-34b, PEGlyated-miR-1 and the empty liposome every 4 days for another 28 days. At day 56, mice received PEGlyated-miR-34b, produced significantly smaller tumours in comparison with those in PEGlyated-miR-

1 and the empty liposome group (Figure 16A and B). The tumour volume was significantly decreased in the PEGlyated-miR-34b group (7 ± 1.2 mm3) compared with those of PEGlyated-miR-1 (16 ± 0.9 mm3) and the empty liposome (18 ± 0.6 mm3) group at the end of this study ($P<0.05$) (Figure 16C). At the beginning of the *in vivo* study, the weight of mice in PEGlyated-miR-34b, PEGlyated-miR-1 and empty liposome treated group were 20.8 ± 0.04, 21.9 ± 0.1, 22.0 ± 0.09 gram respectively. After 56 days of observation, the weight of mice in PEGlyated-miR-34b treated group was 23 ± 2.0 gram whereas in PEGlyated-miR-1 and empty liposome treated group were 24.8 ± 0.6 and 23.8 ± 0.4 gram, respectively. There was no statistical difference among treated and non-treated group and it indicates that there is no toxicity in the treatment of PEGlyated-miR-34b, PEGlyated-miR-1, empty liposome.

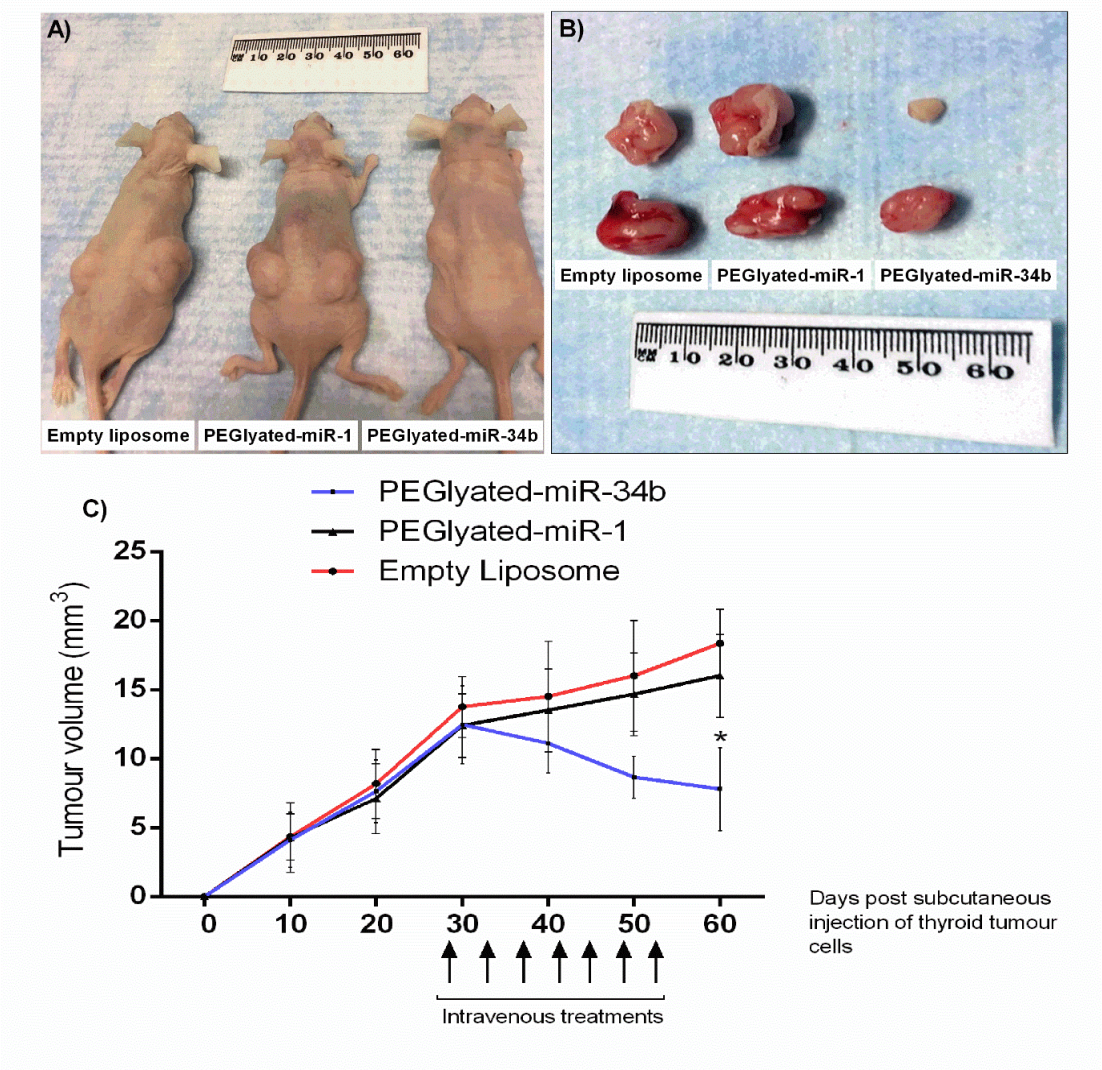

Figure 16. Inhibition of subcutaneous thyroid tumour growth in xenograft nude mouse by PEGlyated-miR-34b: All treatments were administrated on days 29, 33, 37, 41, 45, 49 and 53, after tumour cell inoculation and 40 µg miRNA was used per dose. The subcutaneous injection of thyroid cancer cell line (BHT-101) in mice, formed tumour (A). Mice that intravenously injected with PEGlyated-miR-1 and empty liposome, generated larger tumour size, whereas smaller tumour observed in mice that received PEGlyated-miR-34b (B). Mice treated with PEGlyated-miR-34b had significant reduced tumour volume (mm^3) when compared to mice treated with PEGlyated-miR-1 and empty liposome (n=6) (C). Results are shown as mean ± SD. Level of significance, * $P < 0.05$, when compared to the control groups.

In vitro apoptosis data and decrease of tumour volume in mice received PEGlyated-miR-34b reinforce the view that induction of apoptosis is part of the in vivo mechanism of action of miR-34b in this xenograft nude mouse model. Notably, immunohistochemistry for Ki-67 in xenograft tissue sections indicated that the fraction of proliferating cells is significantly decreased (62.08 ± 1.4) in PEGlyated-miR-34b when compared to PEGlyated-miR-1 and empty liposome treated group ($P<0.05$) (Figure 17). Therefore, PEGlyated-miR-34b reduces proliferation of thyroid carcinoma cells xenografted in mice.

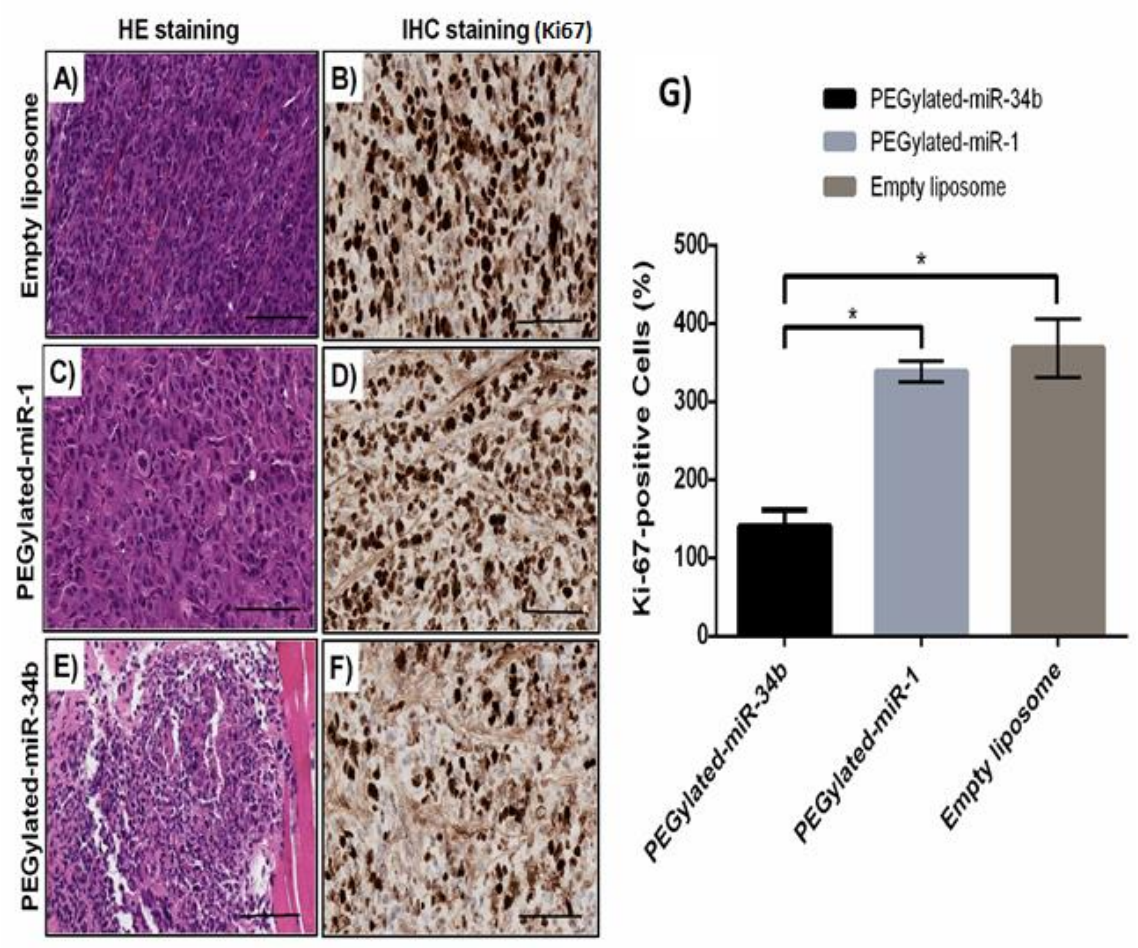

Figure 17. PEGlyated-miR-34b decreases proliferation in xenograft nude mouse tumour: Tumour xenografts obtained by subcutaneous injection of BHT-101 were formalin fixed and paraffin embedded. 3μm-thick sections were cut and used for histopathological and Ki-67 immunohistochemistry analysis. photographs of the hematoxylin and eosin-stained tissue section of thyroid tumour on day 56 after seven intravenous injections of PEGlyated-miR-34b, PEGlyated-miR-1 and empty liposome. 40 μg miRNA was used per dose (A, C, E).

Analysis of cell proliferation by Ki-67 immunostaining in tumour sections of thyroid carcinoma. Representative images of Ki-67 immunostaining (40 x) (B, D, F), and percentage of Ki-67-positive cells (G) in thyroid tumour. High-grade carcinomas were formed in mice receiving PEGlyated-miR-1 and empty liposome (B, D). Low-grade carcinomas were generated in mice treated with PEGlyated-miR-34b (F). Results are expressed as mean ± SD. Level of significance, * $P < 0.05$, when compared to the control groups. Bar represents 250 μm (A-F).

5.5 Discussion

microRNA-based therapeutics have recently been established as a novel approach in treatment of many disease including cancer. Based on the ability of microRNA in silencing the specific cancer-related genes or regulating the selective pathways that are involved in cancer progression, miRNA therapy is considered as a highly effective therapy method.

miRNA, has been identified as a potential therapeutic agent that regulates several cellular pathways through specific gene targeting and downregulating mRNAs by nearly perfect base pairing [627]. Specific miRNAs have been recognised as tumour suppressor and can potentially inhibit tumourigenesis [628]. many studies have shown that variations in miRNA expression level can cause the characteristics of cancer cells [629-633]. The deregulation of proliferation, differentiation and apoptosis has been identified as the major consequences of miRNAs level alteration in malignancies [634]. There is multiple evidence that have shown the pivotal roles of miR-34b in thyroid cancer initiation and progression and conceivably is one critical part of the p53 tumour suppressor network [33, 34].

In addition, one of the most important and renown mediator of angiogenesis is VEGF, a highly conserved glycoprotein with high levels of expression in many organs during normal angiogenesis as well as in human cancers including thyroid during tumour angiogenesis[399]. Our previous finding demonstrated that miR-34b is significantly downregulated and at the same time, the VEGF-A expression level is significantly high in different type of thyroid cancer cell line (unpublished data). Therefore, we aimed to further disclosed modulatory role of miR-34b in VEGF-A function as a hallmark of

angiogenesis process in thyroid cancer using PEGlyated liposome as our new delivery method.

Here, it showed that our delivery system provides an excellent platform for miR-34b to deliver into the tumour. We generated this liposome based on our published HFDM method [618]. This simple method was used to formulate a PEGlyated miR-34b-loaded lipid particle with desirable features for *in vitro* and *in vivo* delivery. In agreement with our previous findings, the polydispersity index was higher than that of extruded particles [618]. In addition, previous studies showed that this liposome do not activate any inflammatory cytokine responses [624] and liver toxicity [623, 625]. Considering that the characterization of miR-34b-5p-loaded PEGlyated lipid particles in our study, was more favorable than previous studies, it is possible to assume that this liposome also do not stimulate any inflammatory cytokine and liver toxicity, however, further investigations require to confirm the effectiveness of this liposome in thyroid cancer.

In this study, miR-34b remarkably overexpressed in cell lines and tissue. Consequently, VEGF-A expression significantly decreased at RNA and protein level following PEGlyated-miR-34b transfection in cell lines and tissue. By restoring the miR-34b expression in thyroid cancer, cellular viability was notably reduced, cellular proliferation and apoptosis were promoted *in vitro,* tumourigenesis suppressed *in vivo*. These finding demonstrates that miR-34b is a novel component in the p53 tumour suppressor pathway and negatively regulates VEGF-A signalling pathway in thyroid cancer. In addition, the ability of the PEGlyated lipid particle to target tumour site further enhances the therapeutic value of the miR-34b therapeutics. To our knowledge, this is the first report of systemic delivery of miR-34b for thyroid cancer therapy using a targeted RNA delivery system.

Chapter 6: Conclusion and Future perspective

6.1 Conclusion

Cancer is a disease characterized by a population of cells that proliferate and divide uncontrollably. Cells proliferation requires an effective delivery of oxygen and nutrients which is supplied via vasculature network. New blood vessel form using processes termed angiogenesis. It plays critical role in normal and cancer cell proliferation. Vast variety of genes are involved with angiogenesis process. Many studies have been performed to modulate the expression of these genes in different cancers.

However, the major obstacle in recognition and inhibition of angiogenesis progression in cancers, is the cross-talk between these genes. As a clear instance, the cross talk between VEGF-A/ Notch1/ Bcl-2 can be mentioned which regulates by miRNAs. The vascular endothelial growth factor (VEGF) is a critical regulator and best-characterized angiogenic factor in cancer. Upregulation of VEGF, especially VEGF-A, has also been observed in many cancers.

The Notch1 transmembrane receptor is a critical regulator of cell fate determination. Beside its role in multiple signalling pathways, Notch1 is also directly or indirectly implicated in regulation of genes involved in angiogenesis such as VEGF-A and vice versa. Bcl-2, well known as an anti-apoptotic gene, has been shown to protect cells against apoptosis and a direct correlation between Bcl-2 and VEGF-A expression has been noted in different cancers.

The potential of miRNAs as a therapeutic component and as an oncogenes expression regulator, have achieved great attention because miRNAs play an important role in the modulating of expression of myriad genes such as Bcl2, Notch1 and VEGF-A by its dysregulation. To date, there are much evidence that have shown the pivotal roles of mir-34b in cancer development as a regulator. miR-34b is now considered by many researchers as one critical part of the p53 tumour suppressor pathway. In addition,

accumulating evidence suggests that miR-34b is involved in the angiogenesis process, modulating new vessel formation through their upregulation or downregulation. The aims of this investigation were therefore to evaluate the effect of miR-34b, known as a tumour suppressor, on expression of these genes and the consequences of these manipulation in angiogenesis and proliferation process. To address our research aims, thyroid carcinoma was chosen because it is highly vascular and therefore is a suitable model for study of angiogenesis and proliferation process.

The first aim of this study was to evaluate the expression of miR-34b in different thyroid cancer tissues and cell lines. miR-34b was significantly downregulated in both thyroid tissue and cell lines. Our study demonstrates that the expression levels of miR-34b are inversely correlated with the pathological nature and morphological features of thyroid cancer tissue and cell lines. We also investigate the protein expression of major angiogenesis regulatory genes (VEGF-A, Notch1, Bcl-2) in thyroid cancer cell lines. VEGF-A, Notch1 and Bcl-2 protein expressions were upregulated in thyroid cancer cell lines in absent of miR-34b expression. Therefore, it seems that VEGF-A/Notch1/Bcl-2 and miR-34b might contribute in thyroid carcinoma progression.

Our second aim was to determine the interplay of thyroid cancer cell lines with VEGF-A, Notch1 and Bcl-2 expression level following miR-34b overexpression. Our results indicated that miR-34b restoration caused a significant reduction of the VEGF-A expression, accompanied by a significant inhibition of Notch1 expression levels in thyroid cancer cell lines. Additionally, the VEGF-A secretion in cell media was also decreased following miR-34b overexpression. Suggesting that VEGF-A-induced angiogenesis is partly due to the activation of the Notch1 pathway and provides the first evidence that miR-34b is involved in thyroid cancer angiogenesis, as a tumour suppressor, potentially via the direct modulation of downstream targets VEGF-A and Notch1. Bcl-2

expression level was also downregulated after miR-34b overexpression. As a result, we additionally hypothesised that, the miR-34b-mediated reduction of the VEGF-A was associated with the potent and simultaneous inhibition of its downstream target genes Notch1 and Bcl-2, both of which are involved in cell angiogenesis and proliferation. Therefore, we further investigate the modulatory effect of miR-34b on cell cycle progression and apoptosis process. We observed that miR-34b overexpression induced cell cycle arrest and apoptosis in thyroid cancer cell lines.

Further investigation the role of miR-34b using polyethylene glycol (PEG) ylated lepidic system, as an efficient delivery system *in vitro* and *in vivo,* and biological effect of miR-34b on proliferation, migration and angiogenesis potential of thyroid carcinoma as a result, was the third aim of this study. Our investigation indicated that inhibition of VEGF-A expression using PEGlyated-miR-34b delivery system, significantly suppressed cellular viability and migration capacity of thyroid cancer cell lines. Regulation of VEGF-A signalling pathways attributes to the mechanism behind miR-34b induced cell cycle, apoptotic and angiogenic changes in thyroid carcinoma. Therefore, the introduction of PEGlyated-miR-34b remarkably suppressed the cell cycle distribution and induced apoptosis in thyroid cancer cell lines. As a consequence of this suppression, our *in vivo* study showed a significant tumour size reduction with lower vascular density in mouse treated with PEGlyated-miR-34b.

6.2 Future Direction

The present investigation successfully addressed the raised hypothesis and aims regarding the modulatory roles of miR-34b in thyroid carcinoma. The results reported in this study, exemplify the importance of miR-34b in thyroid cancer proliferation and angiogenesis. In addition, the results indicated for the first time, the suitability of PEGlyated liposome method for miR-34b delivery in thyroid cancer cells *in vitro* and *in vivo.* Our results clearly showed that overexpression of miR-34b inhibited VEGF-A/Notch1 feedback loop and Bcl-2, as hallmarks of *in vitro* angiogenesis and apoptosis pathways, respectively.

However, additional enquires need to be carefully addressed in the future investigation on this area. This investigation addressed the interaction between VEGF-A, Notch1, Bcl-2 genes and miR-34b dysregulation that facilitate a desirable environment for carcinogenesis when their normal expression is perturbed. Results showed that Bcl-2 is less responsible than VEGF-A/Notch1 feedback loop and Bcl-2 expression was not completely abolished following overexpression of miR-34b.

We suspect that either every Bcl-2 binding site does not interact with miR-34b or miR-34b does not exactly match its Bcl-2 binding site, which necessitate further studies in the future to identify the missing link between VEGF-A/Notch1 feedback loop and Bcl-2 in thyroid carcinoma. This study evaluated the effect of miR-34b overexpression on VEGF-A, Notch1, Bcl-2 only; an angiogenesis PCR assay need to be performed to determine expression levels of a cohorts of predominate mutated genes involved in tumour angiogenesis as an inhibitor and stimulator including ,VEGF-A, VEGF-C, Notch1, EGFR, bFGF, bFGFR , PDGF-AA, PDGF-BB, Ang-2, IL-8, MMP-2, INF-α, INF-β, INF-γ, TIMPs, Angiostatin , Endostatin, TSP-1 and TSP-2, after this manipulation

in thyroid carcinoma and other type of cancers. These results will provide a compelling rationale to implication of miR-34b in cancer angiogenesis. As the miR-34b increases cell death in different carcinomas, miR-34b induced apoptotic changes might be a common event in the pathogenesis of cancer and this investigation performed only in thyroid carcinoma; other type of cancers can be investigated to achieve clearer picture of this interaction.

The miR-34b also has potential to be used as additional or even surrogate markers for prognosis and diagnosis of human cancers. Interestingly, there are many known compounds that can upregulate miR- 34b expression level, which in turn could serve to improve cancer treatment and inhibit growth of cancer cells. Without a doubt, our current understanding of the functional role of miR-34b and its targets, is remained incomplete and deserves future investigation. This incomplete knowledge highlights the complexity of the role of miR-34b in cellular pathways. Of the questions that remain, the relationship between elevated resistance to certain chemotherapeutic drugs and radiation with loss of expression of the miR-34b is one greatly worth investigation. Thus far, there is not a clear mechanistic explanation of how this occurs. Meanwhile, no miR-34b target gene has been identified to explain this activity of the miR-34b. Therefore, this mechanism need to be thoroughly addressed in future investigation. Regardless of the answer to this question, restored expression of miR-34b in cancers where it has been lost holds great therapeutic potential for treatment of cancer. Therefore, continued investigation on the detailed characterization of miR-34b function and its target genes will be needed to clarify their role in carcinogenesis and this, will assist in improving the design of therapeutic drug targeting regimes in human cancers.

Our current investigation addressed the functional role of miR-34b in epithelial cells only. However, increased number of studies, indicate the regulatory role of miR-34b

in proliferation, migration and angiogenesis in endothelial cell as well. Therefore, it is vital to identify its potential for anti-angiogenesis therapeutics in cancer with particular reference to epithelial cell. Studies have already shown that the altered expression of miR-34b in the endothelial cells is related on VEGF-stimulation, hypoxia, or tumour signalling. Therefore, it is possible to assume that miR-34b has a similar effect on VEGF-A/Notch1 feedback loop and Bcl-2 in endothelial cell, and need to be investigated in future research. However, utilization of miRNAs in therapy has the potential side effect of off-targeting effects, which are likely due to the cross-talk between a miRNA and many target mRNAs. Based our results, our liposome delivery method could be a good candidate to reduce this off-targeting effect and 'switch off' the angiogenesis process at least in thyroid cancer model.

Evaluation of the roles of miR-34b in endothelial biology and its relation in various diseases is a relatively new field of research, with high expectations for research and therapy applications. Despite few years of study and many published researches in this field, it is still in its initial steps, and many pitfalls have to be overcome, before successful miR-34b angiogenesis therapies reach the clinic. Considering our results, a better understanding of miR-34b regulation in both epithelial and endothelial cell is essential. Moreover, a comprehensive mapped miRNA profile is necessary to identify the specific miRNAs that are involved in thyroid cancer angiogenesis. Hopefully, this new emerged research field will open prospect full horizons for development of anti-angiogenesis drugs involving miR-34b.

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Appendices

Appendix 1. Notification of Animal ethics approval

3-May-2017

Dear Prof Alfred Lam

I write further to the additional information provided in relation to the provisional approval granted to your application for animal ethics approval for your project 'MicroRNA delivered tumour regulation in human cancers' (GU Ref No: MED/01/17/AEC).

Your response to the request for additional information regarding the points below has been considered by the Committee:

The Animal Ethics Committee Executive are satisfied with the responses provided regarding the Provisional Approval of your application "MicroRNA delivered tumour regulation in human cancers." (GU Ref No: MED/01/17/AEC) however a copy of the amended protocol document and animal usage table is requested before full approval is granted. Please use the attached excel file to complete the new animal usage table for this project and provide these by return email.

The additional information provided in relation to the provisional approval granted to your application has satisfied all the conditions and you are authorised to immediately commence this research on this basis.

Please note, if the overall weight loss for an animal reaches 15% the animal must be humanely euthanised using a technique approved by the Animal Ethics Committee. This standard condition of animal ethics approval applies in all cases unless an alternative arrangement has gained prior approval from the Animal Ethics Committee.

Please also note, the Australian Code for the Care and Use of Animals for Scientific

Purposes 8th edition (2013) requires that Investigators must monitor and assess animals, and must maintain records of the care and use of animals, and make such records available for audit by the institution, the AEC and authorised external reviewers. Animal monitoring score sheets and other documentation relating to the monitoring of animal management and welfare in research are considered primary materials and are to be stored for at least seven years after the last action is taken on the research project. As part of the audit process, the Animal Ethics Committee or animal facilities manager may check records of personnel accessing animal facilities, and reconcile these with animal monitoring records/reports.

For work involving the use of Griffith Animal Facilities, the use of Genotrack software is essential. Please contact the Animal Facilities Manager to arrange for access and training.

The animal ethics approval for this protocol runs from 3-May-2017 to 15-February-2019.

Please find attached an Unexpected Adverse Event Decision Tree as well as an Animal Ethics Approval, Conditional Approval or Provisional Approval Notification Fact sheet.

The standard conditions of approval attached to our previous correspondence about this protocol continue to apply.

Regards

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Researchers are reminded that the Griffith University Code for the Responsible Conduct of Research provides guidance to researchers in areas such as conflict of interest, authorship, storage of data, & the training of research students.
You can find further information, resources and a link to the University's Code by visiting http://policies.griffith.edu.au/pdf/Code%20for%20the%20Responsible%20Conduct%20of%20Research.pdf

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