DETERMINATION OF THE ROLE OF miR-210 AND miR-629 IN REGULATING RESPONSE TOWARDS 1'S-1'-ACETOXYCHAVICOL ACETATE IN HUMAN CERVICAL CARCINOMA CELLS CaSki AND SiHa

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ABSTRACT

Cervical cancer is the fourth most frequent malignancy affecting women worldwide and third most common cancer among Malaysian women. Chemotherapy is one of the treatments used in treating cervical cancer patients but drug resistance and toxicities remains a major challenge. The use of natural compounds is promising because they are less toxic and able to target multiple signalling pathways. The 1'S-1'-acetoxychavicol acetate (ACA) is a natural compound isolated from wild ginger *Alpinia conchigera*, capable of inducing cytotoxicity in various cancer cells including cervical cancer. MicroRNAs (miRNAs) are short non-coding RNAs that regulate numerous biological processes, such as apoptosis and chemosensitivity. Past studies have reported that miR-210 and miR-629 were up-regulated in many cancers and their expressions were altered in cervical cancer cells treated with ACA and/or cisplatin. However, the functional role of miR-210 and miR-629 in regulating sensitivity towards ACA or other anti-cancer agents remains unexplored. Hence, the main aims of this study were to investigate functional role of miR-210 and miR-629 in regulating sensitivity towards ACA in cervical cancer cells, and identify the novel targets of these miRNAs. Results from reverse transcription quantitative real-time PCR (RT-qPCR) showed that ACA down-regulated the expression of miR-210 and miR-629 in both CaSki and SiHa cervical cancer cells. Data from MTT cell viability assays indicated that inhibition of miR-210 and miR-629 enhanced sensitivity towards ACA. Apoptosis assays using flow cytometric analysis for annexin V and propidium iodide dual staining and caspase 3/7 assays demonstrated that suppression of miR-210 and miR-629 induced apoptosis when the cells were treated with ACA. However, no significant changes in sensitivity towards ACA were observed when miR-210 and miR-629 were up-regulated in these cells. Bioinformatic analyses predicted mothers against decapentaplegic homolog 4 (SMAD4) and Ras suppressor-1 (RSU1) as putative novel targets of miR-210 and miR-629,

respectively. The direct interactions between miRNAs and their putative targets were subsequently confirmed with luciferase reporter assays. Western blot analyses revealed that ACA up-regulated the expression of SMAD4 and RSU1 in these cervical cancer cells. Moreover, suppression of miR-210 and miR-629 also increased SMAD4 and RSU1 protein levels in these cells, respectively. MTT cell viability assays showed that ectopic expression of SMAD4 and RSU1 augmented anti-proliferative effects of ACA. Both flow cytometric analysis for annexin V and propidium iodide dual staining and caspase 3/7 assays demonstrated that over-expression of SMAD4 and RSU1 enhanced apoptosis in ACA-treated cells. In conclusion, these results demonstrated that down-regulation of miR-210 and miR-629 in cervical cancer cells conferred sensitivity towards ACA by regulating SMAD4 and RSU1. Furthermore, over-expression of SMAD4 and RSU1 augmented anti-proliferative and apoptosis-inducing effects of ACA in cervical cancer cells. Therefore, this study suggests that combinatorial treatment involving miRNAs and natural compounds could provide new strategies in treating cervical cancer.

ABSTRAK

Kanser serviks adalah malignansi keempat paling kerap di kalangan wanita di seluruh dunia dan ketiga paling kerap di kalangan wanita di Malaysia. Kemoterapi merupakan salah satu rawatan yang digunakan untuk mengubati pesakit kanser serviks tetapi halangan utama adalah rintangan dan ketoksikan ubat kemoterapi. Penggunaan kompaun semulajadi amat menjanjikan kerana ia kurang toksik dan boleh menyasarkan pelbagai laluan isyarat. 1'S-1'-acetoxychavicol acetate (ACA) adalah satu kompaun semulajadi yang diperolehi daripada halia liar *Alpinia conchingera*, yang boleh mencetuskan sitotoksik ke atas pelbagai sel-sel kanser, termasuk kanser serviks. MicroRNA (miRNA) merupakan RNA tanpa pengekodan pendek yang boleh mengawal selia pelbagai proses biologikal seperti apoptosis dan sensitiviti terhadap ubat kemoterapi. Kajian lepas telah melaporkan ekspresi tinggi miR-210 dan miR-629 dalam pelbagai kanser dan ekspresi mereka berubah apabila sel-sel kanser serviks dirawat dengan ACA dan/atau cisplatin. Namun begitu, peranan miR-210 dan miR-629 dalam mengawal selia sensitiviti terhadap ACA atau ubat kemoterapi yang lain masih belum lagi diterokai. Justeru, tujuan utama penyelidikan ini adalah untuk menyiasat peranan miR-210 dan miR-629 dalam mengawal selia sensitiviti terhadap ACA dalam sel-sel kanser serviks, dan mengenal pasti sasaran novel miRNA ini. PCR masa sebenar kuantitatif menunjukkan bahawa rawatan ACA merencatkan ekspresi miR-210 dan miR-629 dalam kedua-dua sel kanser serviks CaSki dan SiHa. Data daripada eksperimen MTT menunjukkan bahawa perencatan ekspresi miR-210 dan miR-629 meningkatkan sensitiviti terhadap ACA. Analisis apoptosis melalui penggunaan aliran sitometer dengan annexin V dan propidium iodide dan cerakin caspase 3/7 menunjukkan perencatan ekspresi miR-210 dan miR-629 peningkatkan apoptosis apabila sel-sel kanser serviks dirawat dengan ACA. Tetapi peningkatkan ekspresi miR-210 dan miR-629 tidak memaparkan sebarang penemuan ketara dalam sensitiviti

terhadap ACA. Analisis bioinformatik meramalkan mothers against decapentaplegic homolog 4 (SMAD4) dan Ras suppressor-1 (RSU1) sebagai sasaran putatif miR-210 dan miR-629. Interaksi secara langsung antara miRNA dan sasaran putatif disahkan melalui penggunaan cerakin pemberitahuan luciferase. Analisis pemblotan western menunjukkan bahawa rawatan ACA meningkatkan ekspresi SMAD4 dan RSU1 dalam sel-sel kanser serviks. Tambahan, perencatan ekspresi miR-210 dan miR-629 juga meningkatkan ekspresi protein SMAD4 dan RSU1 dalam sel-sel ini. Eksperimen MTT menunjukkan bahawa pengekspresian ektopik SMAD4 dan RSU1 meningkatkan kesan anti-proliferatif ACA. Penggunaan analisis aliran sitometer dengan annexin V dan propidium iodide serta cerakin caspase 3/7 menunjukkan bahawa peningkatan ekspresi SMAD4 dan RSU1 menambahkan apoptosis apabila sel-sel dirawat dengan ACA. Kesimpulannya, hasil penyelidikan menunjukkan bahawa perencatan ekspresi miR-210 dan miR-629 dalam sel-sel kanser serviks meningkatkan sensitiviti terhadap ACA melalui pengawal aturan SMAD4 dan RSU1. Tambahan, pengekspresian ektopik SMAD4 dan RSU1 juga meningkatkan kesan anti-proliferatif serta induksi apoptosis ACA dalam sel-sel kanser serviks. Justeru, hasil penyelidikan mencadangkan bahawa rawatan kombinasi yang menglibatkan miRNA dengan kompaun semulajadi boleh memberi strategi baru dalam rawatan kanser serviks.

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<	:	less than
≤	:	less than or equal to
>	:	greater than
±	:	plus-minus
×	:	times / multiplication
%	:	percent
°C	:	degree Celsius
µg	:	microgram
µg/ml	:	microgram per millilitre
µl	:	microlitre
µM	:	micromolar
3'UTR	:	three prime untranslated region
4-OHT	:	4-hydroxytamoxifen
5'UTR	:	five prime untranslated region
ABC	:	ATP-binding cassette
ACA	:	1'S-1'-acetoxychavicol acetate
AIFM3	:	apoptosis-inducing factor, mitochondrion-associated 3
AKT	:	protein kinase B
APAF-1	:	apoptotic protease-activating factor 1
APS	:	ammonium persulfate
ARH1	:	aplysia ras homolog I
ARHGDIA	:	Rho GDP dissociation inhibitor (GDI) alpha
ATCC	:	American Type Cell Culture
BCL2	:	B-cell lymphoma 2

BIK	:	BCL2-interacting killer
BIRC5	:	survivin
BCRP	:	breast cancer resistance protein
BMI1	:	B lymphoma Mo-MLV insertion region 1 homolog
CASZ1	:	castor zinc finger 1
c-Met	:	tyrosine-protein kinase Met
CCL20	:	chemokine (C-C motif) ligand 20
CCND2	:	cyclin D2
CCND3	:	cyclin D3
CDC42	:	cell division control protein 42 homolog
CDH11	:	cadherin-11
CDKN	:	cyclin dependent kinase inhibitor
cDNA	:	complementary DNA
CENAR	:	Centre for Natural Product Research and Drug Discovery
CHL1	:	close homolog of L1
CIN	:	cervical intraepithelial neoplasia
CK1α	:	casein kinase 1α
cm	:	centimetre
CO_2	:	carbon dioxide gas
CUL5	:	cullin 5
CXCL	:	chemokine (C-X-C motif) ligand
CYP	:	cytochrome P450
DAVID	:	Database for Annotation, Visualization and Integrated Discovery
DMEM	:	Dulbecco's Modified Eagle Medium
DMSO	:	dimethyl sulfoxide

DNA	:	deoxyribonucleic acid
DNMT3A	:	DNA methyltransferase 3A
DGCR8	:	DiGeorge syndrome critical region 8
DKK2	:	dickkopf 2 homolog
E2F3	:	E2F transcription factor 3
ECM1	:	extracellular matrix protein 1
EEF1A2	:	eukaryotic translation elongation factor 1A2
EGCG	:	epigallocatechin-3-gallate
EGF	:	epidermal growth factor
EGFR	:	epidermal growth factor receptor
EMT	:	epithelial to mesenchymal transition
EP300	:	E1A binding protein p300
ERBB3	:	erythoblastic leukemia viral oncogene homolog 3
ERK	:	extracellular signal-regulated kinase
FADD	:	Fas-associated death domains
FAK	:	focal adhesion kinase
FBS	:	fetal bovine serum
FBW7	:	F-box and WD-40 domain protein 7
FBXW7	:	F-box and WD repeat domain-containing 7
FDA	:	Food and Drug Administration
FGFRL1	:	fibroblast growth factor receptor-like 1
FGF	:	fibroblast growth factor
FIGO	:	Fédération of Internationale de Gynécologic et d'Obstétrique
FOX	:	forkhead box
FZD	:	frizzled family receptor
g	:	gravity

GAPDH	:	glyceraldehyde 3-phosphate dehydrogenase
GRB2	:	growth factor receptor-bound protein 2
GST	:	glutathione-S-transferase
h	:	hour
HIF-1α	:	hypoxia-inducible factor 1-alpha
HOTAIR	:	HOX transcript antisense RNA
HPV	:	human papillomavirus
hTERT	:	human telomerase reverse transcriptase
IAP	:	inhibitor of apoptosis protein
IGF	:	insulin-like growth factor
IGF2BP1	:	insulin like growth factor 2 mRNA binding protein 1
IL1RAPL1	:	interleukin 1 receptor accessory protein-like 1
IKKβ	:	inhibitor of nuclear factor kappa-B kinase subunit beta
kb	:	kilobases
KITLG	:	KIT ligand
KRAS	:	Kirsten rat sarcoma viral oncogene homolog
LB	:	Luria-Bertani
LEF1	:	lymphoid enhancer-binding factor 1
mA	:	milliampere
MAPK	:	mitogen-activated protein kinase
MCM2	:	minichromosome maintenance complex component 2
MDR1	:	multi-drug resistance protein 1
min	:	minutes
miRNA	:	microRNA
ml	:	millilitre
mm	:	millimetre

MMP2	:	matrix metalloproteinase-2
mRNA	:	messenger RNA
MRP1	:	MDR-associated protein 1
MST2	:	mammalian sterile 20-like kinase 2
MUC4	:	mucin 4
MYB	:	v-myb avian myeloblastosis viral oncogene homolog
MYC	:	v-myc avian myelocytomatosis viral oncogene homolog
N4BP1	:	NEDD4 binding protein 1
NCBI	:	National Center for Biotechnology Information
NCI	:	National Cancer Institute
NFκB	:	nuclear factor-κB
ng	:	nanogram
ng/µl	:	nanogram per microlitre
nM	:	nanomolar
nm	:	nanometre
p53	:	protein p53
Pap	:	Papanicolaou
PBS	:	phosphate buffered saline
PCR	:	polymerase chain reaction
PDCD4	:	programmed cell death 4
PI	:	propidium iodide
PI3K	:	phosphoinositide 3-kinase
PIK3CD	:	phosphoinositide 3-kinase catalytic subunit delta
PIK3R5	:	phosphoinositide-3-kinase, regulatory subunit 5
PINCH1	:	particularly interesting new cysteine-histidine-rich protein 1
PLK1	:	polo-like kinase 1

PLXNB1	:	plexin B1
pRb	:	retinoblastoma protein
PRKCA	:	protein kinase C, alpha
PTEN	:	phosphatase and tensin homolog
PTK2	:	protein tyrosine kinase 2
PTTG1	:	pituitary tumor-transforming gene 1
PUMA	:	p53-upregulated-modulator of apoptosis
RAB11FIP1	:	Rab11 family-interacting protein 1
RAC1	:	Ras-related C3 botulinum toxin substrate 1
RELA	:	transcription factor p65
RHO	:	ras homolog gene family
RIP	:	receptor interaction protein kinase
RISC	:	RNA-induced silencing complex
RMND5A	:	required for meiotic nuclear division 5 homolog A
RNA	:	ribonucleic acid
ROS	:	reactive oxygen species
rpm	:	revolutions per minute
RPMI	:	Roswell Park Memorial Institute
RSU1	:	Ras suppressor-1
RT	:	reverse transcription
RT-qPCR	:	reverse transcription quantitative real-time PCR
SDS	:	sodium dodecyl sulfate
sec	:	second
sFRP1	:	secreted frizzled-related protein 1
SOX17	:	sex determining region Y-box 17
SP1	:	specificity protein 1

SRC	:	v-Src avian sarcoma viral oncogene homolog
TBE	:	Tris/Borate/EDTA
TBS	:	tris-buffered saline
TBST	:	tris-buffered saline with 0.1% (v/v) Tween-20
TCF7L2	:	transcription factor 7 like 2
TEMED	:	Tetramethylethylenediamine
TET1	:	ten-eleven translocation methylcytosine dioxygenase 1
TET3	:	ten-eleven translocation methylcytosine dioxygenase 3
TGFβ1	:	transforming growth factor beta 1
TGS	:	tris-glycine-sodium dodecyl sulfate
THBS2	:	thrombospondin 2
TNF	:	tumour necrosis factor
TNKS2	:	tyrosine kinase, nonreceptor 2
TP53INP1	:	tumour protein p53-induced nuclear protein 1
TRADD	:	tumour necrosis factor receptor type 1-associated death domain
TRIM33	:	tripartite motif containing 33
SMAD	:	mothers against decapentaplegic homolog
UV	:	ultra violet
V	:	volt
v/v	:	volume to volume
VLP	:	virus-like particles
w/v	:	weight to volume
WNT16	:	wingless-type MMTV integration site family, member 16
ZBTB	:	zinc finger and BTB domain-containing protein

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Cancer of the cervix is the fourth most common malignancy affecting women worldwide with an estimated 527,600 new cases diagnosed and 265,700 mortalities recorded in 2012 (Torre *et al.*, 2015). Although cervical cancer is potentially curable at early stage with surgery or a combination of low-dose chemotherapy with radiotherapy, clinical outcomes in advanced, recurrent or persistent cervical cancer remained dismal with chemotherapy response rates of between 20% to 36% and a median overall survival of around one year (Waggoner, 2003; Monk *et al.*, 2009). The approval for the use of bevacizumab, a humanized monoclonal antibody against vascular endothelial growth factor A (VEGF-A), in combination with chemotherapy by US Food and Drug Administration (FDA) in 2014 demonstrated the first major improvement in overall survival in advanced, recurrent or persistent cervical cancer in decades (Liu *et al.*, 2015a). This has spurred interests to develop new drug combinations or targeted therapies to improve overall response and survival outcome in cervical cancer patients.

Natural compounds have been used in cancer treatment either as stand-alones or adjuvants to improve therapeutic efficacy for many years. Studies have demonstrated that natural compounds are less toxic, besides being able to target multiple signalling pathways compared to some conventional anti-cancer agents (Millimouno *et al.*, 2014; Cragg & Pezzuto, 2016). Some examples of the plant-derived natural compounds currently used in chemotherapy are paclitaxel, topotecan, vinblastine and vincristine (Newman & Cragg, 2016). The 1'S-1'-acetoxychavicol acetate (ACA) is a natural compound isolated from the rhizomes of wild ginger, *Alpinia conchigera*. We have previously reported that it is able to induce comparable level of cytotoxicity to cisplatin when used as stand-alone, and potentiated the effects of cisplatin when used in

combination on various cancer cells including cervical cancer (Awang *et al.*, 2010; Phuah *et al.*, 2013).

MicroRNAs (miRNAs) are highly conserved short non-coding RNAs which regulate genes post-transcriptionally by suppressing translation or inducing messenger RNA (mRNA) degradation (Lagos-Quintana *et al.*, 2001). Since one miRNA can target many genes and one gene can be targeted by different miRNAs (Wu *et al.*, 2010), they are able to regulate a wide range of cellular processes such as metastasis (Chan & Wang, 2015), proliferation (Liang *et al.*, 2015), cell death (Su *et al.*, 2015) and chemosensitivity (Yu *et al.*, 2015b). Studies have shown that natural compounds such as curcumin and resveratrol can modify miRNAs expression, and alterations in the expression of these miRNAs can subsequently affect their anti-cancer activities (Phuah & Nagoor, 2014).

The present work is a follow-up study from an earlier MSc. research project (Phuah N. H., MSc. thesis, 2012). In the earlier study, it was demonstrated that both ACA and cisplatin induced dose- and time-dependent cytotoxicity in cervical cancer cells when used as a stand-alone agent, and synergistic effects were observed when they were used in combination. A total of 25 miRNAs were found to be differentially expressed in response towards ACA and/or cisplatin using miRNA microarray. Among these miRNAs, miR-629 was found to be up-regulated with highest fold-change in cells treated with ACA; while miR-210 was up-regulated in two treatment groups: cells treated with cisplatin alone and cells treated with combination of ACA and cisplatin. These results indicated the possible roles of these miRNAs in mediating response towards anti-cancer drugs.

Since miR-629 exhibited the highest fold change in ACA-treated cervical cancer cells, it was selected for further investigation in the current study, which aimed to

investigate the involvement of miRNAs in mediating ACA's anti-cancer activities. On the other hand, miR-210 was selected for further investigation in the current study based on several reasons. First, it was found to be differentially expressed in two different treatment groups. Second, positive correlation between miR-210 expression with trastuzumab resistance have been reported previously (Jung *et al.*, 2012). Third, inhibition of miR-210 expression enhanced sensitivity towards radiation in liver cancer cell lines and xenografts in nude mice (Yang *et al.*, 2012; Yang *et al.*, 2013). Fourth, various studies have revealed that miR-210 plays a pleiotropic role in regulating diverse biological processes such as cell proliferation, migration, invasion, apoptosis, cell cycle and angiogenesis (Qin *et al.*, 2014; Dang & Myers, 2015). Finally, the role of miR-210 in mediating response towards anti-cancer drugs has not been reported previously.

1.1 Study objectives

The aims for the present study were to elucidate the functional roles of miR-210 and miR-629 in regulating sensitivity towards ACA in CaSki and SiHa cervical cancer cells, as well as identification and validation of the novel targets of these miRNAs. The specific objectives for this study were as follows:

i. To determine the effects of ACA on miR-210 and miR-629 expression in cervical cancer cells.
ii. To elucidate the role of miR-210 and miR-629 in regulating sensitivity towards ACA in cervical cancer cells.
iii. To investigate the effects of miR-210 and miR-629 expression on cell proliferation and apoptosis in cervical cancer cells.
iv. To identify the putative gene targets of miR-210 and miR-629 involved in regulating response towards ACA.
v. To experimentally validate novel gene targets of miR-210 and miR-629.

vi. To examine the role of validated gene targets in regulating sensitivity towards ACA in cervical cancer cells.

CHAPTER 2: LITERATURE REVIEW

2.1 Cancer

According to National Cancer Institute (NCI), cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells, which can result in death if the spread is not halted. These abnormal cells arise from a group of cells that are no longer subjected to normal cellular growth control to form a mass of tissue called tumours. While many cancers formed solid tumours, some cancers such as leukemias (blood cancer cells) move freely through circulation.

Tumours can be benign or malignant. Although they can grow to be quite large, benign tumours are not cancerous because they do not spread to other parts of the body. Unlike benign tumours, malignant tumours are cancerous, whereby their cells are capable of invading nearby tissues. In addition, some cancer cells can also break off and travel to distant sites in the body to form new tumours in a process known as metastasis. It is estimated that metastases are responsible for approximately 90% of cancer-related deaths (Mehlen & Puisieux, 2006).

Cancer can be caused by internal factors such as inherited mutations, hormones and immune conditions or external factors such as chemical, radiation, infectious organisms and lifestyle behaviours (smoking, poor diet, physical inactivity and low parity). These factors often act together or in sequence to initiate or promote carcinogenesis and in most cases, cancer is only detected after ten or more years from the time of exposure to external factors (Anand *et al.*, 2008). The standard treatments in cancer are surgery, chemotherapy, radiation, hormone therapy, immune therapy and targeted therapy.

2.1.1 Carcinogenesis

Carcinogenesis is a prolonged multistep process whereby normal cells are transformed into cancer cells. This process is thought to arise when a single cell of origin acquired a series of stable genetic mutations, followed by sequential selection of more aggressive cells (Nowell, 1976). These mutated cells gain a selective growth advantage and undergo clonal expansion resulting from activation of proto-oncogenes and/or inactivation of tumour suppressor genes and/or altered expression of cancer-associated molecules (Knudson, 1986; Harris, 1991). Although it was easier to transform rodent cells compared to human cells, studies have shown that rodent cells required at least two introduced genetic changes to facilitate complete tumourigenesis (Bergers *et al.*, 1998; Hahn *et al.*, 1999). These findings strongly suggest that multiple genetic and epigenetic changes have to accrue for cells to develop malignant phenotype. This multistep process (Figure 2.1) can be divided into three distinct stages: initiation, promotion and progression (Hennings *et al.*, 1993).

Initiation: The first stage of carcinogenesis. It is an irreversible process involving single base mutations in the DNA caused by genotoxic carcinogens such as chemicals, radiation or virus, resulting in activation of proto-oncogene and/or inactivation of tumour suppressor genes mutations (Bishop, 1991; Marshall, 1991).

Promotion: The second stage of carcinogenesis whereby initiated cells undergo clonal expansion to form visible premalignant lesions. Compared to initiation, this stage is reversible in the early stages since it occurs over a long duration and requires the continued presence of tumour promoters. There are two categories of promoters: specific ones that interact with receptors and non-specific ones that altered gene expression without involvement of known receptors (Klaunig *et al.*, 2000).

Progression: The final and irreversible stage resulted in transformation of benign tumours into malignant neoplasms. Transformed cells are capable of invading nearby tissues and metastasizing to distant sites in the body. Progression is caused by repetitive mutations and/or epigenetic changes, which gave rise to aberrant cells with better surviving traits such as increased growth rate and invasiveness (DiGiovanni, 1992).

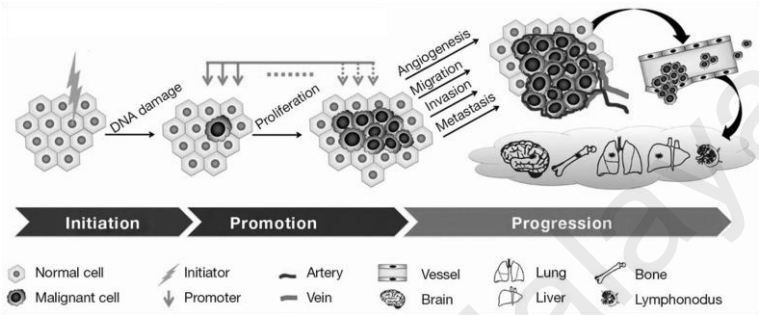

Figure 2.1: Multistep carcinogenesis.

(Figure adapted with permission from Liu et al., 2015)

2.1.2 Epidemiology in cancer

Cancer is among the leading cause of morbidity and mortality in the world, and the burden is expected to increase worldwide due to population growth and aging. Based on GLOBOCAN database, an estimated 14.1 million new cancer cases (7.4 million in males and 6.7 million in females) and 8.2 million cancer deaths (4.7 million in males and 3.5 million in females) occurred in 2012 (Torre *et al.*, 2015). Of these, the most commonly diagnosed cancers worldwide were those of the lung (1.8 million, 13.0% of the total), breast (1.7 million, 11.9%), and colorectal (1.4 million, 9.7%) while the most common causes of cancer death were cancers of the lung (1.6 million, 19.4% of the total), liver (0.8 million, 9.1%), and stomach (0.7 million, 8.8%). Based on gender, the three most common sites of cancer diagnosed in 2012 were lung (1.2 million, 16.7% of the total), prostate (1.1 million, 15.0%) and colorectal cancer (0.7 million, 10.0%) among men and breast (1.7 million, 25.2%), colorectal (0.6 million, 9.2%) and lung

cancer (0.6 million, 8.8%) among women. On the other hand, the three most common causes of cancer death in 2012 were lung (1.1 million, 23.6% of the total), liver (0.5 million, 11.2%) and stomach cancer (0.5 million, 10.1%) among men and breast (0.5 million, 14.7%), lung (13.8%) and colorectal cancer (0.3 million, 9.0%) among women.

According to the Malaysian National Cancer Registry Report 2007-2011 published by National Cancer Institute of Malaysia in 2016, a total of 103,507 new cancer cases (46,794 in males and 56,713 in females) were reported in Malaysia during 2007-2011 (Azizah, 2016). The three most common cancers diagnosed among Malaysians were breast (18,343 cases, 17.7% of total), colorectal (13,693 cases, 13.2%) and lung (10,608 cases, 10.2%) cancers. Based on gender, the three most common sites of cancer diagnosed were colorectal, lung and nasopharyngeal cancer among men and cancers of breast, colorectal and cervix among women.

2.2 Cervical cancer

According to NCI, cervical cancer is defined as the formation of cancer in the tissues of cervix. The cervix is the lower part of the uterus (womb) that connects the uterus and vagina, and is anatomically divided into endocervix (non-visible part) and ectocervix (visible part). The endocervix is the upper part of the cervix that is closer to the uterus and is lined with mucus producing columnar epithelium. On the other hand, the ectocervix is mainly lined with non-keratinizing stratified squamous epithelium and is located in the lower part of the cervix that is closer to the vagina. The region of the cervix where the columnar epithelium has been replaced and/or is being replaced by new metaplastic squamous epithelium is known as transformation zone. Most cervical cancers begin in the transformation zone, because these cells are less stable and thus more susceptible to viral infections (Richart, 1973; Crum, 2000).

2.2.1 Cervical cancer subtypes

Around 80% of cervical cancers are squamous cell carcinoma, which develop from the squamous cells that cover the ectocervix's surface. The remaining 20% constitute the adenocarcinoma, which arises from the mucus producing columnar cells that lined the endocervix. Besides these two major types, there are also other subtypes such as adenosquamous carcinoma (which shared the characteristics of both squamous cell carcinoma and adenocarcinoma), glassy cell carcinoma and small cell carcinoma (Sasieni & Adams, 2001).

2.2.2 Etiology of cervical cancer

Infection with human papillomavirus (HPV), a sexually transmitted infection, has been identified as the primary cause of cervical cancer. The association between HPV infection with development of cervical cancer was suggested by Harald zur Hausen in 1976, and this was subsequently shown in 1981 when he isolated, characterized and cloned HPV DNA from genital warts with his colleagues (zur Hausen, 1976; zur Hausen *et al.*, 1981). There are more than 100 types of HPV with over 40 of which capable of infecting genital area, which are classified by their association to cancer (Munoz *et al.*, 2003). The low-risk HPV types such as HPV type 6 and 11 can cause anogenital warts and benign or low-grade abnormalities in cervical cells (Lacey *et al.*, 2006), while the high-risk HPV types such as HPV type 16 and 18 are known to cause intraepithelial neoplasia of the anogenital region such as cervical, vulvar, penile and anal (Parkin & Bray, 2006). Almost all cervical cancers showed the presence of high-risk HPV types, with HPV type 16 and 18 detected in approximately 70% of cervical cancers worldwide (Bosch *et al.*, 2008).

While infection with high-risk HPVs is associated with development of cervical cancer, this alone is insufficient as most HPV infections are transient and asymptomatic.

More than 90% of new HPV infections including those with high-risk types become undetectable in two years with clearance usually occurring within 6 months after infection (Molano *et al.,* 2003). This was further evidenced in other epidemiological studies, which showed only a small fraction of women with HPV infection developing cervical cancer although majority of them were infected with HPV at some point in their lifetime (Ho *et al.*, 1998; Franco *et al.*, 1999).

Due to the small fraction of women developing cervical cancer despite being infected with high-risk HPV types, the possible involvement of environmental co-factors in cervical cancer was investigated. Results revealed an overwhelming number of cases showed the involvement of environmental co-factors in progression from HPV infection to cervical cancer, strongly suggesting that environmental co-factors also play an important role in cervical cancer development (Castellsague *et al.*, 2002). Among the environmental co-factors associated with cervical cancer development are high parity (Munoz *et al.*, 2002), use of oral contraceptives (Moreno *et al.*, 2002), tobacco smoking (Deacon *et al.*, 2000) and infection by other sexually transmitted agents such as *Herpes simplex virus-2* (HSV-2) and *Chlamydia trachomatis* (*C. trachomatis*) (Smith *et al.*, 2002a; Smith *et al.*, 2002b).

2.2.3 Cervical carcinogenesis

Most of the cervical cancers are thought to arise from a cervical lesion caused by the prolonged infection with high-risk HPVs. The E6 and E7 oncoproteins produced by the high-risk HPVs contribute to cervical cancer development by inactivating the tumour suppressor protein p53 (p53) and retinoblastoma protein (pRb), respectively (Dyson *et al.*, 1989; Scheffner *et al.*, 1990). Mild and moderate dysplasia lesions displayed low levels of E6 and E7 expressions, whereas high levels expressions were detected in severe dysplasia and invasive carcinoma (Klaes *et al.*, 1999). These results suggest that

persistent infection of high-risk HPVs is necessary for progression and invasion in cervical cancer. Cervical carcinogenesis consists of four major steps: HPV infection of normal cervix, persistent infection with HPV over a long period of time, progression of HPV-infected cervix to pre-cancer and cancer. However, HPV infection would not lead to cervical cancer if HPV infection is cleared or regression of pre-cancer. The HPV-infected cervix is often associated with mild cytologic and/or histologic abnormalities (Figure 2.2) (Schiffman & Kjaer, 2003).

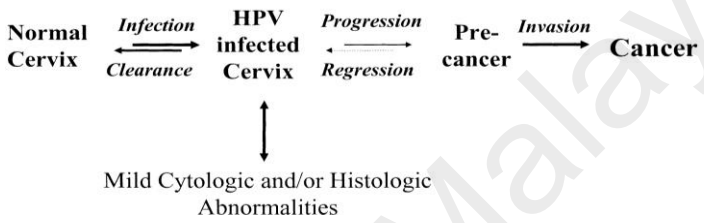

Figure 2.2: Cervical carcinogenesis.

(Figure adapted with permission from Schiffman & Kjaer, 2003)

2.2.4 Grading in cervical cancer

Cervical cancer is the result of a multistep process involving the transformation of normal cervical epithelium to a pre-neoplastic cervical intraepithelial neoplasia and subsequently to invasive cervical cancer (Richart, 1990). Dysplasia or cervical intraepithelial neoplasia (CIN) is a disturbed proliferation in the cells and precursor to invasive carcinoma. The CIN is graded based on the severity and proportion of the epithelial layer containing atypical cells. In CIN I a third, in CIN II two third and in CIN III (almost all) of the total layer of epithelium contains atypical cells. The likelihood of regression of CIN I, CIN II and CIN III is 60%, 40% and 33% respectively, while the likelihood of progression of CIN I, CIN II and CIN III to invasive carcinoma is 1%, 5% and greater than 12% respectively (Ostor, 1993).

2.2.5 Staging of cervical cancer

The different clinical stages of invasive cervical cancer are defined by the Fédération of Internationale de Gynécologic et d'Obstétrique (FIGO) based on clinical examinations using diagnostic tests such as palpation, inspection, colposcopy, endocervical curettage, hysteroscopy, cystoscopy, proctoscopy, intravenous urography, and X-ray examination of the lungs and skeleton, and cervical conization. The staging consists of four broad stages with sub-stages within each stage (Table 2.1). In Stage I, carcinoma is strictly confined to the cervix. Stage II involves carcinoma that extends beyond the cervix, but does not extend into pelvic wall (involves the vagina but not as far as the lower third of vagina). In Stage III, carcinoma is extended to pelvic wall and lower third of the vagina while Stage IV is carcinoma that extended beyond pelvis and involves the mucosa of bladder and/or rectum (Shepherd, 1996; Pecorelli *et al.*, 2009).

Table 2.1: FIGO staging of cervical cancer.

Stage I – limited to the cervix **IA** - diagnosed only by microscopy, no obvious lesion • IA1 – stromal invasion < 3 mm depth and ≤ 7 mm spread • IA2 – stromal invasion 3-5 mm depth and ≤ 7 mm spread **IB** – visible lesion with invasion > 5 mm depth or > 7 mm spread • IB1 – lesion ≤ 4 cm in dimension • IB2 – lesion > 4 cm dimension
Stage II – invades beyond cervix but does not extend into pelvic wall (involves the vagina but not as far as the lower third of vagina) **IIA** – without parametrial invasion • IIA1 – lesion ≤ 4 cm in dimension with involvement of < upper 2/3 of vagina • IIA2 – lesion > 4 cm in dimension with involvement of < upper 2/3 of vagina **IIB** – with parametrial invasion
Stage III – extends to pelvic wall/lower one-third of vagina **IIIA** – involves lower one-third of vagina **IIIB** – extends to pelvic wall and/or causes hydronephrosis or non-functioning kidney
Stage IV– extends beyond pelvis and involves the mucosa of bladder and/or rectum **IVA** – spread into adjacent pelvic organs **IVB** – spread to distant organs

2.2.6 Cervical cancer prevention

Since cervical cancer develops slowly over a long period in most cases, it can be prevented through regular screening using Papanicolaou (Pap) test that detects the pre-cancerous lesions before they progress into cancer (Papanicolaou & Traut, 1997). The introduction of this test, whereby sample of cells collected from the cervix were examined under the microscope for cytological abnormalities, has led to significance decrease in morbidity and mortality rates (Saslow *et al.*, 2002). The availability of molecular testing for detection of high-risk HPVs associated with cervical cancer has provided another screening method besides Pap test. These HPV tests are more robust and sensitive, hence they are able to predict cervical cancer risk many years in advance and detect adenocarcinoma often missed by Pap tests. The HPV tests are recommended to be used in conjunction with the Pap tests as an additional screening test or when results from Pap tests are inconclusive (Ratnam *et al.*, 2000).

Besides regular screening, the occurrence of cervical cancer can also be greatly reduced through vaccinations. There are two types of vaccines approved by US FDA in the market, which are composed of virus-like particles (VLPs) prepared from recombinant L1 capsid protein of the targeted HPV types. The quadrivalent vacccine Gardasil® from Merck & Co., Inc., which protects against HPV type 6, 11, 16 and 18, was approved for its use in 2006 while bivalent vaccine Cervarix® from GlaxoSmithKline, which protects against HPV type 16 and 18, was approved for its use 2009 (Markowitz *et al.*, 2014). In 2015, the FDA approved the use of nine-valent vaccine Gardasil® 9 from Merck & Co., Inc., which protects against HPV type 6, 11, 16, 18, 31, 33, 45, 52 and 58. This new improved vaccine is expected to prevent around 80% to 90% of cervical cancer cases (Kirby, 2015). Nevertheless, vaccinated women should still be regularly screened as the HPV vaccines do not protect against all types of HPV or provide therapeutic effect against established infections.

2.2.7 Cervical cancer treatment

Treatments for cervical cancer are planned according to the clinical stage of the disease, type of cervical cancer and patient's desire to have children in the future. Patients with stage IA1 may be treated by hysterectomy or conisation, if they wish to preserve fertility. For patients with stage IA2, IIB1 or IIA, radical hysterectomy that preserves ovarian function, is the preferred treatment among young healthy patients. However, patients with early stage cancer who wish to preserve fertility may opt for radical trachelectomy (Sonoda *et al.*, 2004). Besides surgery, radiation has been demonstrated to be equally effective for patients with early stage disease (Landoni *et al.*, 1997). On the other hand, radical surgery alone is often insufficient for patients with stage IB2 disease since majority of the patients will require adjuvant radiotherapy as well. Study has shown that chemo-radiation with hysterectomy markedly reduced the risk of recurrence and death in women compared to those receiving only radiation with hysterectomy (Keys *et al.*, 1999).

In locally advanced stage IIB, III and IVA, chemo-radiation has led to improvements in progression free survival and overall survival compared to radiation alone (Green *et al.*, 2001). Unfortunately, curative treatments are no longer possible for patients with stage IVB disease. However, patients are often given radiation and/or chemotherapy to relieve the symptoms caused by the cancer to improve their quality of life. Compared to localized cervical cancer which has a five year survival rate of 91.5%, metastatic cervical cancer has a low 16.5% five year survival rate with a median survival time of 8 to 13 months (Ferlay *et al.*, 2013; van Meir *et al.*, 2014).

2.2.8 Epidemiology in cervical cancer

Although cervical cancer is largely preventable, studies have found that cervical cancer remains one of the leading causes of cancer-related deaths among women

worldwide, especially in developing countries. Based on GLOBOCAN database, cervical cancer is the fourth most common cancer and cause of cancer-related deaths among women worldwide with an estimated 527,600 cases and 265,700 mortalities in 2012, with more than 80% of these cases recorded in developing countries (Torre *et al.*, 2015).

In the Malaysian National Cancer Registry Report 2007-2011 published by National Cancer Institute of Malaysia in 2016, cervical cancer is the seventh most common cancer among Malaysian population and third most common cancer among Malaysian women with 4,352 cases. It is the second most common cancer reported in women aged 25 to 59 years and fourth most common cancer in women aged 60 to 74 years (Azizah, 2016).

2.2.9 Human cervical cancer cell lines

The human cervical cancer cell lines, CaSki and SiHa, used in this study were obtained from American Type Culture Collection (ATCC). They are both squamous cell carcinomas with adherent growth properties and epithelial-like morphology. The CaSki cells were derived from the metastatic site in the small intestine of 40 years old Caucasian female and the SiHa cells were derived from the primary site in the cervix of 55 years old Asian female. Both cell lines are positive for the high-risk HPV type 16, although sequence related to HPV type 18 was also reported in CaSki cells (Yee *et al.*, 1985). Even though both CaSki and SiHa cells contain wild-type *p53* and *pRb* genes, their activities were greatly reduced by the expression of E6 and E7 oncoproteins produced by high-risk HPVs such as HPV type 16 (Scheffner *et al.*, 1991).

2.3 Chemotherapy

Besides surgery and radiation, chemotherapy is commonly employed to treat various cancers. Chemotherapy refers to the use of drugs known as cytostatics, which inhibit cell growth and proliferation. Compared to localized treatments such as surgery and radiation, chemotherapy is usually used as systemic treatment. There are many types of chemotherapy available, depending on its goal in cancer treatment. Curative chemotherapy aims to eliminate all cancer cells from the body to achieve a lasting cure. In adjuvant chemotherapy, the aim is to remove any remaining cancer cells in the body after surgery or radiation, while neoadjuvant chemotherapy is used to reduce the tumour size prior to commencing with other treatments such as surgery or radiation. On the other hand, palliative chemotherapy is considered when removing all tumour cells from the body are no longer possible. Instead, the goal of palliative chemotherapy used on terminal cancer patients is to slow down the progression of the disease or ease the symptoms caused by the cancer (Birk & Beger, 2001).

Due to its systemic effect and the fact that chemotherapy drugs do not discriminate between cancerous and healthy cells, there are many adverse effects associated with chemotherapy. As these chemotherapy drugs inhibit cell growth and proliferation, fast proliferating cells such as hair cells, blood-producing cells, and the cells of the mucous membranes of the mouth, throat area and digestive tract are more susceptible to the effects from these chemotherapy drugs compared to other types of normal healthy cells. Among some of the side effects associated with chemotherapy are hair loss, anaemia, fatigue, nausea, diarrhoea, vomiting, and infections in the mouth and throat (Partridge *et al.*, 2001).

2.3.1 Drug resistance

Although chemotherapy has led to improvement in the overall response and survival of cancer patients, its effectiveness is often limited due to drug resistance. While some tumours have inherent resistance (also known as intrinsic or *de novo* resistance) such as malignant melanoma, others may develop resistance during the course of treatment in a phenomenon known as acquired resistance (Selby, 1984). In intrinsic resistance, resistance-mediating factors already pre-exist in most of the tumour cells prior to chemotherapy, and treatment is doomed to fail from the beginning. In acquired resistance, tumour cells that are initially responsive to the anti-cancer drugs develop mutations or other adaptive responses to render subsequent treatment ineffective (Longley & Johnston, 2005). Moreover, studies have shown that tumour cells consist of population of cells with high degree of molecular and genetic heterogeneity. Thus, drug resistance can also arise from positive selection of drug-resistant subpopulation of cells present in the original tumour (Swanton, 2012). There are many molecular mechanisms involved in drug resistance that can act independently or in combination to reduce drug sensitivity in cancer cells (Figure 2.3).

Drug efflux: Drug efflux, whereby drug accumulation in the cells is greatly reduced by enhanced efflux, is one of the most studied mechanisms in drug resistance. Members of the ATP-binding cassette (ABC) transporter family proteins play an important role in preventing excess toxins within the cell (Sauna & Ambudkar, 2001). Besides removing toxins, these proteins can also transport other substrates such as peptides, amino acids, steroids, lipids, sugars, hydrophobic compounds and metabolites including anti-cancer drugs (Vasiliou *et al.*, 2009). The multi-drug resistance protein 1 (MDR1; also known as ABCB1), MDR-associated protein 1 (MRP1; also known as ABCC1) and breast cancer resistance protein (BCRP; also known as ABCG2) are members of this ABC

transporter family that are often over-expressed in cancer cells and have been implicated in drug resistance (Gottesman *et al.*, 2002).

Drug inactivation: Many anti-cancer drugs undergo metabolic activation by interacting with other proteins in the body to achieve clinical efficacy. The drug activating and inactivating molecules such as cytochrome P450 (CYP) system and glutathione-S-transferase (GST) superfamily are often dysregulated in cancer cells (Michael & Doherty, 2005). The molecules in CYP system are involved in drug metabolism. Hence, mutations or alterations in the CYP molecules will affect their metabolic activities, resulting in increased rate of drugs breakdown or secretion by kidneys (Shen *et al.*, 2007). The GST superfamily is a group of detoxifying enzymes that protect cellular macromolecules from electrophilic compounds. Studies have shown that up-regulation in GSTs in cancer cells enhances drugs detoxification. Besides that, GSTs can also suppressed mitogen-activated protein kinase (MAPK) pathway to reduce cell death and promote cell survival (Townsend & Tew, 2003).

Alteration of drug targets: The efficacy of the anti-cancer drugs is influenced by its molecular targets. Thus, any mutations or alterations in the expression levels of these targets can greatly affect the drug's response. For example, some anti-cancer drugs act by stabilizing the topoisomerase II, an enzyme that prevents DNA from becoming super- or under-coiled, resulting in DNA damage and inhibition of DNA synthesis. However, mutations in the topoisomerase II gene in some cancer cells rendered these cells to be resistant to anti-cancer drugs (Zwelling *et al.*, 1989).

DNA damage repair: The ability of cancer cells to repair DNA damage can affect response towards anti-cancer drugs that induced DNA damage directly (for example, cisplatin) or indirectly (for example, fluorouracil). Depending on the extent of the damage, the damage can be reversed by the DNA damage response mechanisms such as

nucleotide excision repair and homologous recombination. Hence, these DNA damage response mechanisms can greatly reduced the effectiveness of DNA damaging anti-cancer drugs (Bonanno *et al.*, 2014).

Cell death: The ultimate aim for chemotherapy is to induce cell death in the cancer cells, preferably by apoptosis. However, this is often challenging due to the dysregulated apoptotic circuitry that exists in cancer cells, which hinders effective therapies (Lowe *et al.*, 2004; Adams & Cory, 2007). Although anti-cancer drugs such as B-cell lymphoma 2 (Bcl-2) family inhibitors can effectively induced apoptosis in cancer cells, resistance can arise from prolonged use. However, studies have shown that these inhibitors can be used to restore the dysfunctional apoptotic pathways and sensitize the cancer cells, including those that are resistant, to cytotoxic agents (Samuel *et al.*, 2013; Tamaki *et al.*, 2014; de Jong *et al.*, 2016).

Epithelial to mesenchymal transition (EMT): EMT is the mechanism in which solid tumours become metastatic. Recent evidence showed that EMT not only contributes to metastasis, but also to development of drug resistance. Cells that exhibited stem-like and mesenchymal features were found to be more resistant towards anti-cancer drugs (Creighton *et al.*, 2009). Besides that, the elevated activity of drug efflux pump ABC transporter family in cancer progenitor cells (also known as cancer stem cells) that are involved in formation of metastatic cells, play a role in drug resistance (Saxena *et al.*, 2011). Another factor is the tumour microenvironment, which plays an important role in facilitating proliferative and invasive behaviour of cancer cells. The hypoxia environment promotes hypoxia-inducible factor 1-alpha (HIF-1α) activation, resulting in up-regulation of MDR1 expression (Jiao & Nan, 2012). Besides that, drug resistance can also develop during signalling processes of differentiation

involving molecules such as cytokines and growth factors, known to cause resistance towards anti-cancer drugs (Singh & Settleman, 2010; Shang *et al.*, 2013).

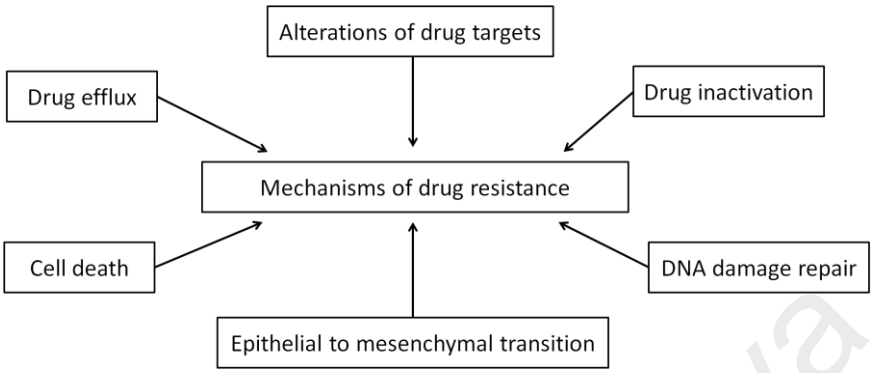

Figure 2.3: Mechanisms of drug resistance.

2.4 Cell death

In organisms, cell death is a critical and active process that eliminates potentially harmful cells and maintains homeostasis. Most cell deaths take place in a systematic manner through activation of specific signalling events. Of note, there are three major types of cell deaths distinguished by their morphological differences: apoptosis, autophagy and necrosis (Galluzzi *et al.*, 2007).

Apoptosis was first described by Kerr and his colleagues in 1972 and is characterized by morphological changes such as cell shrinkage, chromatin condensation (pyknosis), cytoplasmic blebbing as well as formation of apoptotic bodies which are engulfed by macrophages and parenchymal cells (Kerr *et al.*, 1972). Besides these morphological changes, apoptotic cells also undergo biochemical changes such as nuclear fragmentation (DNA degradation by DNAses that cut the internucleosomal regions into double-stranded DNA fragments of 180 to 200 bp), phosphatidylserine externalization and intracellular substrate cleavages by specific proteolysis (Wyllie, 1980; Martin & Green, 1995).

Autophagy is an evolutionarily conserved catabolic process characterized by the formation of autophagosomes, a double-membrane structure surrounding cytoplasmic macromolecules and organelles. The outer membrane of the autophagosome fuses with a lysosome to form autolysosome. The sequestered materials are subsequently degraded and recycled (Levine & Klionsky, 2004). Autophagy generally plays a pro-survival role in cell homeostasis and is up-regulated in response to extracellular or intracellular stress such as growth factor deprivation, starvation, endoplasmic reticulum stress or pathogen infection (He & Klionsky, 2009). Although apoptosis and autophagy have different morphological characteristics and physiological process, studies have shown that there is crosstalk between these two cell death mechanisms. In certain instances, apoptosis and autophagy could exert a synergistic effect or autophagy can be triggered when apoptosis is suppressed (Thorburn, 2008). Additionally, it was also demonstrated that both of these processes could be simultaneously triggered in tumour cells (Cheng *et al.*, 2009).

Necrosis was first described in 1972, when Kerr and his colleagues used morphological characteristics to distinguish apoptosis from necrosis (Kerr *et al.*, 1972). In contrast to apoptosis, necrosis had always been portrayed as a form of uncontrolled type of cell death that promotes local inflammation (Vakkila & Lotze, 2004). Cells undergoing necrosis exhibited chromosome clumping and organelles swelling, followed by swelling of the cell and culminates in rupture of the plasma membrane (Searle *et al.*, 1982). Necrosis is characterized by depletion of ATP, activation of calpain and cathepsin proteases, increased reactive oxygen species (ROS) and cytoplasmic calcium (Golstein & Kroemer, 2007). However, the recent discovery of key mediators of necrotic cell death indicates that necrosis could be another form of programmed cell death. Studies have shown that this form of necrosis requires the involvement of

receptor interaction protein kinase 1 and 3 (RIP1 and RIP3) and can be inhibited by necrostatins (Wu *et al.*, 2012).

2.4.1 Apoptosis

To date, there are three apoptotic pathways that have been identified: the extrinsic or death receptor pathway, the intrinsic or mitochondrial pathway and the perforin or granzyme pathway (Figure 2.4). Although these pathways are triggered differently, they converge on the activation of the caspase protease family, which ultimately leads to apoptosis.

The extrinsic pathway (also known as the death receptor pathway) is initiated by the death receptors, which are members of tumour necrosis factor (TNF) receptor gene superfamily (Locksley *et al.*, 2001). These members, which includes Fas and TNF receptors, share a similar cysteine-rich extracellular and cytoplasmic domains of about 80 amino acids known as "death domain" that plays a vital role in transmitting death signals from outside of the cell to activate the extrinsic pathway (Ashkenazi & Dixit, 1998). The death domain allows for the binding of adapter proteins such as Fas-associated death domains (FADD) and tumour necrosis factor receptor type 1-associated death domain (TRADD) (Bender *et al.*, 2005; Strasser *et al.*, 2009). The binding of FADD or TRADD form the death-inducing signalling complex (DISC) and allows for the recruitment of procaspase-8 followed by catalytic processing and activation of caspase-8. The activated caspase-8 then proceeds to activate procaspase-3 and activates the execution pathway (Taylor *et al.*, 2008).

The intrinsic pathway (also known as mitochondrial pathway) is activated in response to stimuli such as radiation, toxins, hypoxia, viral infections, free radicals or deprivation of survival factors (Elmore, 2007). These stimuli lead to changes in mitochondrial permeability transition, resulting in loss of mitochondrial membrane

potential and subsequent release of cytochrome c. This pathway is closely regulated by proteins belonging to the Bcl-2 family: the pro-apoptotic proteins (e.g. Bax, Bad, Bak, Bik, Bid, and Bim) and the anti-apoptotic proteins (e.g. Bcl-2, Bcl-xL, Bcl-W and Mcl-1). The balance between the pro- and anti-apoptotic members of the Bcl-2 family is largely responsible for apoptosis initiation (Reed, 1997). The released cytochrome c binds with apoptotic protease-activating factor 1 (APAF-1) and procaspase-9 to form apoptosome (Garrido *et al.*, 2006). The apoptosome then catalyzes the activation of procaspase-9 into caspase-9, which in turns activates the execution pathway involving caspase-3 (Kaufmann & Hengartner, 2001).

On the other hand, the perforin or granzyme pathway is utilized by cytotoxic T-cells in mediating cytotoxicity. This apoptosis pathway is activated by the transmembrane pore-forming molecule perforin, which allows the release of cytoplasmic granules containing granzyme A or B into the target cell (Trapani & Smyth, 2002). The granzyme A pathway activates caspase-independent cell death via single-stranded DNA damage (Martinvalet *et al.*, 2005), while granzyme B proceeds to activate the mitochondrial pathway through caspase-3 or -10 activation (Goping *et al.*, 2003).

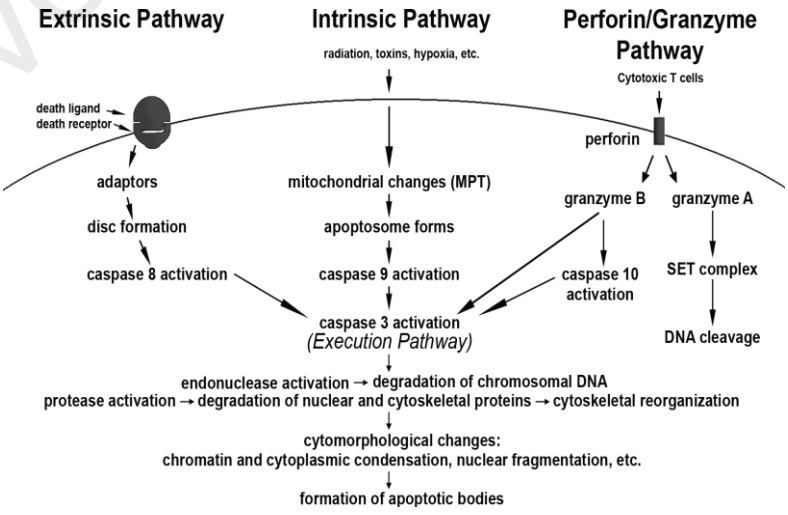

Figure 2.4: Apoptotic pathways.

(Figure adapted with permission from Elmore, 2007)

2.4.3 Apoptosis and cancer

As defined by NCI, cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. The ability of cancer cells to increase their population numbers depends not only on the rate of cell proliferation, but also on the rate of cell attrition. One of the major sources of cell attrition is apoptosis, especially the extrinsic and intrinsic apoptotic pathways. Consequently, evasion of apoptosis has been identified as a major hallmark in many types of cancers (Hanahan & Weinberg, 2011).

The possibility of apoptosis serving as a barrier to cancer development was first raised in 1972, when it was reported that widespread apoptosis occurred in fast-growing and hormone-dependent tumour cells upon hormone withdrawal (Kerr *et al.*, 1972). Over the years, other compelling functional studies conducted by different groups have further reinforced this notion (Evan & Littlewood, 1998; Lowe *et al.*, 2004).

One of the mechanisms for apoptosis evasion is disruption in the balance of pro-apoptotic and anti-apoptotic proteins. As mentioned previously, apoptosis in the cells is tightly regulated by the ratio of pro-apoptotic and anti-apoptotic proteins (Reed, 1997). Besides those proteins in the Bcl-2 family, there are also inhibitor of apoptosis proteins (IAPs) such X-linked IAP (XIAP, BIRC4) and survivin (BIRC5) (Vucic & Fairbrother, 2007). These IAPs inhibit caspase activity by promoting degradation of active caspases or by keeping the caspases away from their substrates (Wei *et al.*, 2008). Hence, disturbance in the balance of these pro-apoptotic and anti-apoptotic proteins will result in dysregulated apoptosis. Additionally, reduced caspase activity can also lead to apoptosis evasion. The caspases played a crucial role in apoptosis activation in both extrinsic and intrinsic pathways. They consist of initiator caspases which are responsible for the initiation of the apoptotic pathway such as caspase-2, -8, -9 and -10, and effector caspases which are responsible in the actual cleavage of cellular components during

apoptosis such as caspase-3, -6 and -7 (Fink & Cookson, 2005). Hence, a reduction in the levels of caspases or impairment in caspases function would consequently lead to defective apoptosis signalling. Furthermore, apoptosis evasion can also be caused by impaired death receptor signalling. This occurs when death ligands binds to decoy death receptors that do not possess a death domain, causing the latter to fail to form the requisite signalling complexes and initiate the death signalling cascade (Lavrik *et al.*, 2005). Abnormal expression of decoy receptors and reduced membrane expression of death receptors allowed the cells to evade apoptosis (Fulda, 2010).

In order for the cancer cells to grow infinitely and increase their population numbers, they must develop strategies to circumvent apoptosis. One of the ways cancer cells can acquire resistance to apoptosis is through mutation in the *p53* tumour suppressor gene. The *p53* is DNA damage sensor gene that can induce apoptosis in response to DNA breaks or chromosomal abnormalities and inactivation in *p53* activity has been found in more than 50% of human cancers (Harris, 1996). Besides this, other extracellular factors such as insulin-like growth factor 1 and 2 (IGF1 and IGF2) and interleukin 3 (IL-3), and intracellular signals from Ras, loss of phosphatase and tensin homolog (PTEN) expression and activation of phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway can also transmit anti-apoptotic signals, enabling the cancer cells to elude apoptosis (Downward, 1998; Evan & Littlewood, 1998).

While apoptosis is normally triggered in response to various physiologic stresses in normal cells, the defective apoptotic circuitry in cancer cells enable the cells to progress to high-grade malignancy and at the same time present a major barrier to effective therapies (Lowe *et al.*, 2004; Adams & Cory, 2007). The elucidation of signalling circuitry governing the apoptotic program has revealed many potentials targets for cancer treatment. The understanding of this circuitry will enable researches to develop

more effective anti-cancer therapies, either by inducing apoptosis directly in the cancer cells or restoring the defective apoptotic signalling pathways found in cancer cells.

2.5 Natural compounds

For many years, natural compounds have been widely used in the treatment of cancers and other diseases. It was found that majority of the anti-cancer drugs approved by the FDA from 1981 to 2014 were either natural compounds or their derivatives (Newman & Cragg, 2016). Hence, it is not surprising that many of the anti-cancer agents currently used in cancer treatments were developed from marine organisms (citarabine), microorganisms (dactinomycin, bleomycin and doxorubicin) and plants (vincristine, vinblastine, etoposide, paclitaxel and topotecan) (Bhanot *et al.*, 2011).

Compared to certain anti-cancer drugs such as platinum-based drugs, the use of natural compounds in cancer treatments is attractive because not only are they less toxic, they are also able to target numerous signalling pathways involved in carcinogenesis (Surh, 2003). This is useful as carcinogenesis is a multistep process caused by disruption in multiple signalling pathways during tumour initiation, promotion and progression (Hennings *et al.*, 1993). This is also one of the most plausible explanation why mono-modal therapy typically fails in cancer treatments as the specific inhibitors often targets only a single gene in a signalling pathway or only a specific signalling pathway (Li *et al.*, 2010b).

2.5.1 Plant derived natural compounds

Historically, medicinal plants have been used as folk medicines for thousands of years in many countries. Even until today, these medicinal plants continue to play an important role in primary health care system, especially among low-income populations or those staying in rural areas (Greenwell & Rahman, 2015). Plant derived natural compounds (also known as phytochemicals), constitute a set of bioactive compounds

classified according to their chemical structure such as carotenoids, alkaloids, polyphenols, and nitrogen compounds (Howes & Simmonds, 2014). These compounds, which are often responsible for the distinct plant characteristics such as smell and colour pigmentation are present naturally in plants such as vegetables, fruits and grains (Zhou *et al.*, 2016d).

Over the years, many phytochemicals have been developed as anti-cancer drugs. Among the most notable example is taxol, which is isolated from Pacific yew tree, *Taxus brevifolia* (Wani *et al.*, 1971). Taxol works by stabilizing microtubule polymer and protects it from disassembly, leading to mitotic arrest and is effective against breast, ovarian and other solid tumours (Orr *et al.*, 2003). Other noteworthy examples are the vinca alkaloids vinblastine and vincristine extracted from the Madagascar periwinkle, *Catharanthus roseus* (Noble, 1990). These vinca alkaloids bind to tubulin and de-polymerize the microtubules, leading to cell cycle arrest (Gidding *et al.*, 1999; Okouneva *et al.*, 2003). The use of these vinca alkaloids in combination chemotherapy have led to significant increase in response rates and survival for patients with advanced Hodgkin's disease (Devita *et al.*, 1970). The etoposide, derived from the mandrake plant *Podophyllum peltatum* and wild chervil *Podophyllum emodi*, is a topoisomerase II inhibitor (Stahelin, 1973; Liu, 1989) that exhibited anti-tumour activity against small-cell lung carcinoma (Harvey, 1999). Camptothecin, a topoisomerase I inhibitor, was extracted from the bark and wood of *Camptotheca accuminata*, a tree endemic to China (Wall *et al.*, 1966; Liu *et al.*, 2000). Although promising results were obtained in phase I study, phase II study showed that this natural compound is ineffective and highly toxic on patients with gastrointestinal cancer (Moertel *et al.*, 1972) and melanoma (Gottlieb & Luce, 1972). However, further investigation of this natural compound has led to the discovery of irinotecan and topotecan, which are derivatives of camptothecin. Both

irinotecan and topotecan were found to exhibit significant anti-cancer activity against colorectal and ovarian cancer, respectively (Creemers *et al.*, 1996; Fuchs *et al.*, 2006).

Among the phytochemicals are a class of dietary polyphenols such as lycopenes, curcumin, indoles, tea polyphenols, genistein and resveratrol. These dietary polyphenols isolated from fruits, vegetables or spices consumed by humans for many centuries, have been widely investigated for their anti-cancer properties. Hence, it is not surprising that high intake of fruits and vegetables reduced the risk of cancers (Block *et al.*, 1992; Slavin & Lloyd, 2012). Studies have shown that these dietary polyphenols exert their anti-cancer effects by inducing cell cycle arrest, inhibiting oncogenic signalling cascades involved in cell proliferation, angiogenesis and apoptosis, modulating ROS levels or up-regulating tumour suppressor proteins such as p53 (Anantharaju *et al.*, 2016). Therefore, these polyphenols can be used as stand-alone or adjuvant in combination chemotherapy to improve therapeutic efficacy by reducing drug-induced toxicities and/or overcoming drug resistance (Millimouno *et al.*, 2014; Cragg & Pezzuto, 2016).

2.5.2 *Alpinia conchigera*

The *Alpinia conchigera* (Family: Zingiberaceae; Section: Zingiberacea; Sub-section: Strobidia) is a herbaceous perennial plant which can grow up to 5 feet tall when fully matured and is endemic in eastern Bengal, southwards toward Peninsular Malaysia and Sumatera (Smith, 1990). This plant belongs to the *Alpinia* genus, which is the most widespread and taxonomically complex genus in Zingiberaceae family with 230 species found in tropical and sub-tropical Asia (Kress *et al.*, 2005).

In Malaysia, this plant is known locally as *lengkuas ranting*, *lengkuas kecil*, *lengkuas padang*, *lengkuas genting* or *chengkenam*. These semi wild plants are usually found in open wet fields such as edges of paddy fields, nearby streams or under the shades of

rubber and palm oil trees. In the northern part of Peninsular Malaysia, the rhizomes from this plant are used to flavour food while in the east coast of Peninsular Malaysia, it is used in traditional medicine as anti-fungal remedy (Ibrahim *et al.*, 2000). Its rhizomes are also used to relieve gastro-intestinal disorders, skin disease, bronchitis, pain, digestive stimulant, anti-inflammatory and venereal disease in Thai traditional medicine, and as condiments in food preparation (Athamaprasangsa *et al.*, 1994; Matsuda *et al.*, 2005).

The presence of four main phenylpropanoids found in *Alpinia conchigera*: nonacosane, β-sitosterol, 1'-acetoxychavicol acetate and 1'-acetoxyeugenol acetate were first reported in 1988 (Yu, 1988). Another group reported the presence of chavicol acetate, 1'-hydroxychavicol acetate, 4-acetoxycinnamyl alcohol and 4-acetoxycinnamyl acetate together with five other diarylheptanoids and two flavonoids from the n-hexane and dichloromethane extracts from the rhizomes of *Alpinia conchigera* (Athamaprasangsa *et al.*, 1994). Besides these, other natural compounds extracted from *Alpinia conchigera* that has been reported previously are essential oils, terpenoids, β-bisabolene, 1,8-cineole, β-caryophyllene, and 2',4'-dihydroxy-6'-methoxychalcone (Sirat & Nordin, 1994; Wong *et al.*, 2005; Lee *et al.*, 2006).

2.5.3 1'S-1'-acetoxychavicol acetate (ACA)

Past studies have reported that the 1'-acetoxychavicol acetate (ACA) extracted from *Alpinia galanga* (a close relative of *Alpinia conchingera*) exhibited a wide range of biological activities such as anti-tumour (Itokawa *et al.*, 1987), anti-oxidative (Kubota *et al.*, 2001), anti-inflammatory (Nakamura *et al.*, 1998), anti-allergic (Matsuda *et al.*, 2003), anti-ulcer (Mitsui *et al.*, 1976), anti-fungal (Janssen & Scheffer, 1985) and anti-xanthine oxidase (Noro *et al.*, 1988).

Besides that, it was also reported that ACA inhibited tumour promoter-induced Epstein-Barr virus activation (Kondo *et al.*, 1993) and induced apoptosis in Ehrlich ascites tumour cells and glioma cells through caspase-3 activation (Moffatt *et al.*, 2000; Williams *et al.*, 2013). Studies have also demonstrated that ACA decreased hyperphosphorylated Rb levels and increased hypophosphorylated Rb levels. Additionally, treatment with ACA caused a decrease in phosphorylated $p27^{Kip1}$ and an increase in unphosphorylated $p27^{Kip1}$ as well as an increase in the nuclear localization of $p27^{Kip1}$, although the $p27^{Kip1}$ levels were not affected by ACA (Unahara *et al.*, 2007; Xu *et al.*, 2008). It was reported that ACA induced glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1) activities, increased intracellular glutathione (GSH) level, and upregulated intranuclear nuclear factor-erythroid 2-related factor 2 (Nrf2) and cytosolic $p21^{CIP1}$ in rat intestine epithelial cells (Yaku *et al.*, 2011). In a study carried out by Pang and colleagues, it was shown that ACA suppressed proliferation and angiogenesis in prostate cancer cells by blocking the activation of VEGF-mediated Src kinase, focal adhesion kinase (FAK) and Rho GTPases (Rac1 and cdc42) (Pang *et al.*, 2011). Besides that, ACA also exerts its anti-cancer activities by inhibiting the activation of NF-κB, resulting in suppression of NF-κB-regulated proliferative, anti-apoptotic and metastatic gene products (Ichikawa *et al.*, 2005).

Furthermore, ACA also inhibited the cellular growth of myeloid leukemic cells in both *in vitro* and *in vivo* studies by inducing apoptosis through mitochondrial- and Fas-mediated dual mechanisms (Ito *et al.*, 2004). In addition, *in vivo* studies carried out by different groups demonstrated that ACA possessed strong anti-cancer effects on chemically induced tumour formation in mouse skin (Murakami *et al.*, 1996), cholangiocarcinogenesis in Syrian hamster (Miyauchi *et al.*, 2000) and cancers of colon (Tanaka *et al.*, 1997), oral (Ohnishi *et al.*, 1996) and esophageal (Kawabata *et al.*, 2000) in rats.

The ACA used in this study was isolated from the rhizomes of wild ginger, *Alpinia conchigera* Griff., collected from Jeli district of Kelantan (east coast of Peninsular Malaysia), and the voucher specimen (KL5049) was deposited in the Herbarium of Chemistry Department, Faculty of Science, University of Malaya (Awang *et al.*, 2010). The chemical structure of ACA is depicted in Figure 2.5. We have previously reported that ACA inhibited cell proliferation and migration as well as induced apoptosis when used as stand-alone, and potentiated the effects of cisplatin when used in combination on various cancer cell lines including cervical cancer and oral cancer xenografts in nude mice (Awang *et al.*, 2010; In *et al.*, 2012; Phuah *et al.*, 2013).

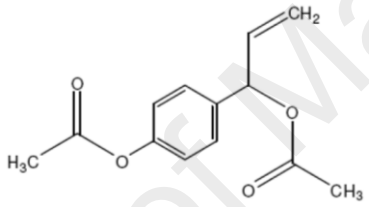

Figure 2.5: Chemical structure of 1'S-1'-acetoxychavicol acetate (ACA).

(Figure adapted with permission from Khalijah *et al*., 2011)

2.6 MicroRNAs

MicroRNA (miRNA) was first described in the early 1990s in a study that looked at the larval developmental timing in *Caenorhabditis elegans* (*C. elegans*) (Lee *et al.*, 1993; Wightman *et al.*, 1993). In this study, a 22 nucleotides RNA transcript of lin-4 was found to regulate larval developmental timing by preventing the translation of lin-14 mRNA. Further investigation revealed that lin-4 RNA has a partial sequence complementarity to a sequence repeat found in the 3'UTR of lin-14, indicating that lin-4 inhibits translation of lin-14 by interacting with its 3'UTR. Seven years later, another 21 nucleotides RNA transcript with similar function was discovered. This time, let-7 was found to repress lin-41 expression by interacting with its 3'UTR to regulate late

developmental timing in *C. elegans* (Reinhart *et al.*, 2000). These regulatory RNAs were originally thought to be unique only to nematodes. However, it was soon discovered that the sequence and temporal expression of let-7 was conserved in a wide range of animal species, including human, Drosophila, zebrafish, annelids and molluscs (Pasquinelli *et al.*, 2000). Following this, further investigations were carried out and many more RNAs resembling lin-4 and let-7 were identified from plants, *C. elegans*, Drosophila and mammals (Lagos-Quintana *et al.*, 2001; Lagos-Quintana *et al.*, 2003). However, functional analyses showed that not all of these RNA molecules play a role in regulating developmental timing, suggesting that they could be involved in regulating other signalling pathways. Subsequently, the term "microRNA or miRNA" then came into existence to refer to this class of small regulatory RNAs.

miRNAs are short non-coding RNA molecules of about 22 nucleotides long that regulate genes post-transcriptionally by facilitating mRNA degradation or inhibition of translation (Lagos-Quintana *et al.*, 2001; Bartel, 2004). However, recent evidence suggested that the predominant mechanism by which miRNAs regulate protein levels of target genes is through destabilization of target mRNAs (Guo *et al.*, 2010). While it was previously estimated that up to 1,000 unique miRNAs might be present in the human genome (Berezikov *et al.*, 2005), a recent study has identified a total of 2,469 unique miRNAs, of which 1,098 were validated by the group (Friedlander *et al.*, 2014). These miRNAs are found in abundance across different human tissues (Ludwig *et al.*, 2016) and are predicted to regulate up to one third of the human genes expression (Davis & Hata, 2009). This, undoubtedly make them one of the largest classes of gene regulators.

It had been reported that one miRNA may regulate many genes as its targets, and one gene may be targeted by multiple miRNAs (Wu *et al.*, 2010). Different groups have also demonstrated these multiple-to-multiple relationships between miRNAs and gene

targets in cancer cells (Creighton *et al.*, 2012; Hashimoto *et al.*, 2013). Meanwhile, other studies have shown that although a single miRNA may repress the protein expression in many targets, this repression is often relatively modest (Baek *et al.*, 2008; Selbach *et al.*, 2008).

2.6.1 Identification and naming conventions

All miRNAs were identified and named based on criteria and conventions that have been described previously (Ambros *et al.*, 2003). In databases and literatures, mature miRNA is designated as miR-10, whilst mir-10 refers to the miRNA gene or the predicted stem-loop of the primary transcript. It should be noted that the first three letters in the miRNA name indicate the organism. For example, hsa-miR-10 and mmu-miR-10 is used for miR-10 found in humans and mouse respectively, even though miR-10 may not be discovered in the different organisms at the same time. In addition, numbering of miRNA genes is merely sequential based on chronological discovery. Hence, closely numbered miRNAs may not be related at all. For example, miR-10 and miR-11 may not be closely related, but indicates that miR-11 is discovered after miR-10. On the other hand, miRNAs expressed from different precursor sequences and genomic loci but have identical mature sequences are designated as hsa-miR-10-1 and hsa-miR-10-2. Meanwhile, miRNAs with closely related mature sequences are denoted with lettered suffices. For example, hsa-miR-10a and hsa-miR-10b signifies that they are expressed from these two precursors, hsa-mir-10a and hsa-mir-10b.

Although one of the strands from miRNA duplex is usually degraded, miRNA cloning studies sometimes identified two sequences of miRNAs that originated from the same precursor. If the relative abundances can evidently identified the predominantly expressed miRNA, the nomenclature miR or miR* is used. The more dominant miRNA is designated as hsa-miR-10 while the less dominant product is designated as hsa-miR-

10*. On the other hand, when relative abundances could not conclusively identified the predominantly expressed miRNA, the (-5p or -3p) suffix were used. For example, hsa-miR-10-5p signified that it is derived from the 5' end of the stem-loop precursor or hsa-miR-10-3p is derived from the 3' end of the stem-loop precursor. The use of miR or miR* nomenclature has since been replaced with these suffix (-5p or -3p) to specify the origin of the mature miRNA sequence from miRBase version 21 onwards.

Although most miRNAs followed this naming convention and miRNAs that do not conform to this convention in certain cases have since been renamed, there are exceptions to these rules. For example, miRNAs found in different organisms may have slightly different naming scheme, such as miRNAs found in plants and viruses. Other than these, miRNAs such as let-7 and lin-4 are also exempted from these naming conventions with their original names retained for historical reasons.

In addition to names, each miRNA has a unique accession number, the only truly stable identifier. This is because the names for miRNA may be changed depending on the circumstances (when more information is available or when the sequences become clearer). The accession numbers allow for such changes to be tracked and at the same time provide the user with full access to history and data.

2.6.2 miRNA biogenesis

The miRNAs are first transcribed from the miRNA genes by RNA polymerase II in the nucleus as long primary miRNA molecules (pri-miRNA) with a 7-methyl guanosine cap at the 5' end and a poly (A) tail at the 3' end. These pri-miRNAs can be up to several kilobases long and contains one or more hairpin structures of about 70 nucleotides (Cai *et al.*, 2004; Lee *et al.*, 2004). Although most of the miRNA genes are present in the intergenic regions, they can also be found in the exonic and intronic regions in all human chromosomes except for Y chromosome (Rodriguez *et al.*, 2004).

The pri-miRNA is subsequently processed into short hairpin RNAs of about 70 nucleotides known as precursor miRNA (pre-miRNA) by Drosha (a RNase III enzyme) and DiGeorge syndrome critical region 8 (DGCR8) (Denli *et al.*, 2004). The resulting pre-miRNA with 5' phosphate and 2 nucleotides overhangs at 3' is then transported into the cytoplasm mediated by Exportin-5, in a RanGTP-dependent manner (Bohnsack *et al.*, 2004).

In the cytoplasm, another RNase III enzyme (Dicer) with its double-stranded RNA-binding protein, the immunodeficiency virus (HIV) transactivating response DNA binding protein (TRBP), further cleaves the pre-miRNA to release a double-stranded miRNA (around 22 nucleotides long) (Grishok *et al.*, 2001; Winter *et al.*, 2009). One of the strands from the miRNA duplex is incorporated into RNA-induced silencing complex (RISC), while the complementary miRNA strand is usually rapidly degraded. The incorporated miRNA strand then binds to their target sequences at the "seed" sequence (around 2 to 7 nucleotides) located in the 3'UTR of target mRNA (Lewis *et al.*, 2005). While miRNAs bind to the 3'UTR of target mRNAs most of the time, studies have reported that they could also bind to other regions such as in the open reading frames (Mandke *et al.*, 2012; Zhang *et al.*, 2015), coding sequences (Fang & Rajewsky, 2011; Schnall-Levin *et al.*, 2011) or 5'UTR of target mRNAs (Lytle *et al.*, 2007). The binding of miRNA to its target mRNA resulted in mRNA degradation if there is perfect complementarity, or mRNA translational repression if there is imperfect complementarity (Lagos-Quintana *et al.*, 2001; Bartel, 2004). Recent evidence suggests that miRNAs could also repress protein expression through destabilization of target mRNAs (Guo *et al.*, 2010). Although miRNAs normally suppressed protein expression, miRNAs can also up-regulate translation of target mRNAs under certain circumstances. This is by recruiting proteins complexes to the adenylate-uridylate-rich elements of

mRNA (Vasudevan *et al.*, 2007) or interfering with proteins that block the translation of target gene (Eiring *et al.*, 2010). The biogenesis of miRNAs is illustrated in Figure 2.6.

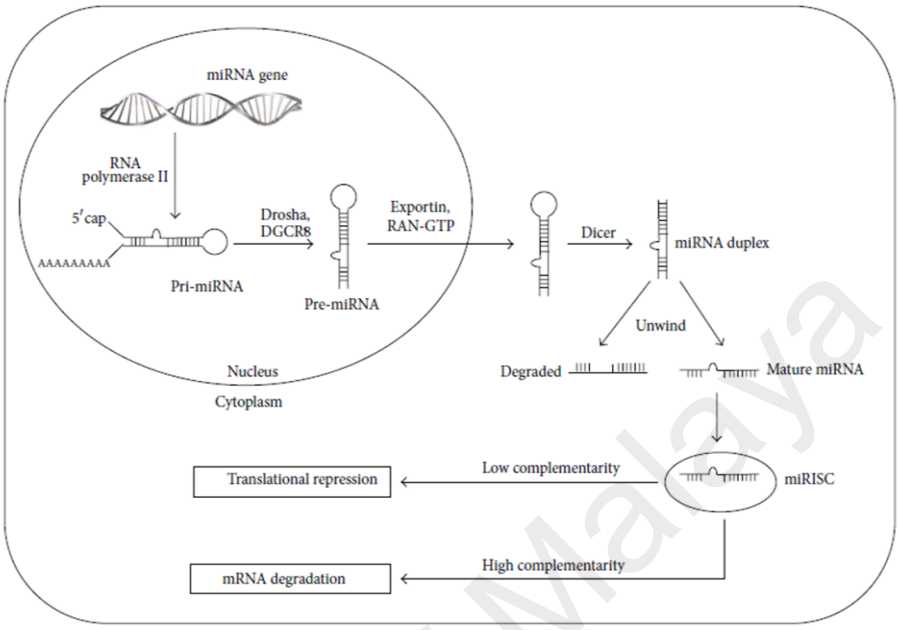

Figure 2.6: Biogenesis of miRNAs.

(Figure adapted with permission from Phuah & Nagoor, 2014)

2.6.3 miRNAs and cervical cancer

Large-scale microarray analyses have revealed aberrant miRNA expression profiles that can be used to classify different cancers (Lu *et al.*, 2005). In addition, it was demonstrated that global changes in the miRNA expression pattern promotes carcinogenesis (Kumar *et al.*, 2007). In cancer research, miRNAs that are up-regulated in cancer cells are known as oncogenic miRNAs, while miRNAs whose expression are down-regulated in cancer cells are known as tumour suppressive miRNAs (Zhang *et al.*, 2007). More often than not, oncogenic miRNAs promote carcinogenesis by targeting tumour suppressor genes, whereas tumour suppressive miRNAs repress carcinogenesis by targeting oncogenes. Over the years, many oncogenic miRNAs (Table 2.2) and tumour suppressive miRNAs (Table 2.3) have been identified in cervical cancer.

Table 2.2: miRNAs up-regulated in cervical cancer.

miRNA	miRNA function	Ref.
miR-10a	induced cell growth, invasion and metastasis; targets CHL1	(Long *et al.*, 2012)
miR-19a; miR-19b	induced cell proliferation and invasion; targets CUL5	(Xu *et al.*, 2012)
miR-20a	induced migration and invasion; targets TNKS2	(Kang *et al.*, 2012)
miR-21	induced cell proliferation; targets PDCD4 and CCL20	(Yao *et al.*, 2009; Yao & Lin, 2012)
miR-27b	induced cell proliferation, migration and invasion; targets CDH11	(Yao *et al.*, 2016)
miR-92a	induced cell proliferation and invasion; targets FBXW7	(Zhou *et al.*, 2015)
miR-93	miRNA inhibition suppressed proliferation and induced apoptosis; targets RAB11FIP1	(Zhang *et al.*, 2016c)
miR-133b	induced cell proliferation and colony formation; targets MST2, CDC42 and RHOA	(Qin *et al.*, 2012)
miR-181a	miRNA inhibition suppressed proliferation and invasion and induced apoptosis through the PTEN/AKT/FOXO1 pathway	(Xu *et al.*, 2016b)
miR-200b	induced cell proliferation and metastasis; targets FOXG1	(Zeng *et al.*, 2016)
miR-373	induced cell growth; targets YOD1	(Wang *et al.*, 2015)
miR-494	induced cell proliferation; targets PTEN	(Yang *et al.*, 2015c)
miR-519d	inhibited apoptosis; miRNA inhibition suppressed cell proliferation, migration and invasion; targets SMAD7	(Zhou *et al.*, 2016a)

Table 2.3: miRNAs down-regulated in cervical cancer.

miRNA	miRNA function	Ref.
miR-7	inhibited cell growth; induced apoptosis; targets XIAP	(Liu *et al.*, 2013b)
miR-17-5p	inhibited cell growth; induced apoptosis; targets TP53INP1	(Wei *et al.*, 2012)
miR-34a	inhibited cell viability and invasion; targets E2F3	(Wang *et al.*, 2009; Li *et al.*, 2010a; Geng *et al.*, 2015)
miR-100	inhibited cell proliferation; induced cell cycle arrest and apoptosis; targets PLK1	(Li *et al.*, 2011)
miR-125b	inhibited cell proliferation; induced apoptosis; targets PIK3CD	(Cui *et al.*, 2012; Ribeiro *et al.*, 2015)
miR-129-5p	inhibited cell proliferation and cell cycle; induced apoptosis; targets SP1	(Zhang *et al.*, 2013b)
miR-132; miR-212	inhibited cell proliferation, migration, invasion and EMT; targets SMAD2	(Zhao *et al.*, 2015)
miR-133a	inhibited cell proliferation, colony formation, migration and invasion; induced apoptosis; targets EGFR	(Song *et al.*, 2015)
miR-138	inhibited proliferation, migration and invasion; targets hTERT, RMND5A and c-Met	(Li *et al.*, 2014; Li *et al.*, 2016; Zhou *et al.*, 2016b)
miR-140-5p	inhibited cell growth and metastasis; targets IGF2BP1	(Su *et al.*, 2016)
miR-143	inhibited tumour formation; induced apoptosis; targets BCL2	(Liu *et al.*, 2012)
miR-195	inhibited cell migration and invasion; targets SMAD3, CCND2 and MYB	(Du *et al.*, 2015; Zhou *et al.*, 2016c)
miR-200b	inhibited EMT and migration; targets RhoE	(Cheng *et al.*, 2016b; Cheng *et al.*, 2016c)
miR-211	inhibited invasion and EMT; targets MUC4	(Tang *et al.*, 2013; Xu *et al.*, 2016a)
miR-214	inhibited cell proliferation, migration and invasion; targets PLXNB1 and GALNT7	(Qiang *et al.*, 2011; Peng *et al.*, 2012)
miR-328	inhibited cell proliferation and tumorigenesis; targets TCF7L2	(Wang & Xia, 2016)
miR-376c	inhibited cell proliferation and invasion; targets BMI1	(Deng *et al.*, 2016)
miR-429	inhibited cell viability and proliferation; induced apoptosis; targets IKKβ	(Fan *et al.*, 2017)
miR-486-3p	inhibited cell proliferation and metastasis; targets ECM1	(Ye *et al.*, 2016)
miR-494	inhibited metastasis; targets PTTG1	(Chen *et al.*, 2015)

2.6.4 miRNAs and natural compounds

Accumulating evidence has revealed that natural compounds exert their anti-cancer effects through epigenetic regulation (Wang *et al.*, 2013b). Given the prominent role played by miRNAs in regulating gene expression, it is not surprising to see many of the genes that can affect response towards anti-cancer agents are targeted by miRNAs. Studies have demonstrated that natural compounds can modulate miRNAs expression and alterations in these miRNAs expression can subsequently affect their anti-cancer activities (Phuah & Nagoor, 2014). Among these natural compounds, the roles of epigallocatechin-3-gallate (EGCG) (Table 2.4), curcumin (Table 2.5), genistein (Table 2.6) and resveratrol (Table 2.7) in regulating miRNAs expression have been widely investigated.

Table 2.4: miRNAs regulated by EGCG.

miRNA	Regulation	miRNA function	Cancer type	Ref.
miR-1	Up	miRNA inhibition abrogated EGCG-induced inhibition on cell growth; down-regulated c-Met expression	Osteosarcoma	(Zhu & Wang, 2016)
miR-16	Up	miRNA inhibition abrogated EGCG's activity; targets Bcl-2	Liver	(Tsang & Kwok, 2010)
miR-210	Up	inhibited cell proliferation and anchorage-independent growth; induced sensitivity to EGCG	Lung	(Wang *et al.*, 2011)
miR-221	Up	enhanced the effect of EGCG in reducing cell migration	Liver	(Arffa *et al.*, 2016)
miR-98-5p	Down	miRNA inhibition enhanced cisplatin-induced apoptosis; induced p53 expression	Lung	(Zhou *et al.*, 2014b)

Table 2.5: miRNAs regulated by curcumin.

miRNA	Regulation	miRNA Function	Cancer type	Ref.
miR-7	Up	inhibited cell proliferation and invasion; induced apoptosis; targets SET8	Pancreatic	(Ma *et al.*, 2014)
miR-9	Up	enhanced curcumin induced apoptosis; reduced phosphorylation of AKT and FOXO1	Ovarian	(Zhao *et al.*, 2014)
miR-15a; miR-16	Up	miRNA inhibition induced cell growth and up-regulated WT1 and Bcl-2 expression	Leukemia; breast	(Yang *et al.*, 2010; Gao *et al.*, 2012)
miR-22	Up	inhibited cell proliferation and migration; targets ERBB3	Retinoblastoma	(Sreenivasan *et al.*, 2012)
miR-138	Up	down-regulated SMAD4, NFκB, RELA and CCND3	Osteosarcoma	(Yu *et al.*, 2015a)
miR-181b	Up	inhibited metastasis; targets CXCL1 and CXCL2	Breast	(Kronski *et al.*, 2014)
miR-192-5p; miR-215	Up	abrogated curcumin induced apoptosis; targets XIAP	Lung	(Jin *et al.*, 2015; Ye *et al.*, 2015)
miR-203	Up	inhibited cell proliferation, migration and invasion; induced apoptosis; targets AKT2 and SRC	Bladder	(Saini *et al.*, 2011)
miR-20a; miR-27a; miR-17-5p	Down	targets ZBTB4 and ZBTB10	Colon	(Gandhy *et al.*, 2012)
miR-21	Down	abrogated curcumin induced anti-proliferative and apoptotic effects; targets PDCD4 and PTEN	Colorectal; lung	(Mudduluru *et al.*, 2011; Zhang *et al.*, 2014)
miR-125a-5p	Down	induced cell proliferation, migration and invasion; suppressed TP53 expression	Epithelial	(Gao *et al.*, 2014)
miR-186*	Down	miRNA inhibition suppressed cell proliferation and induced apoptosis; targets caspase-10	Lung	(Zhang *et al.*, 2010)
miR-208	Down	abrogated curcumin induced apoptosis; targets CDKN1A	Prostate	(Guo *et al.*, 2015)

Table 2.6: miRNAs regulated by resveratrol.

miRNA	Regulation	miRNA function	Cancer type	Ref.
miR-34a	Up	targets E2F3	Colorectal	(Kumazaki *et al.*, 2013)
miR-34c	Up	miRNA inhibition suppressed resveratrol's activity; targets KITLG	Colorectal	(Yang *et al.*, 2015a)
miR-122-5p	Up	miRNA inhibition suppressed resveratrol's activity	Breast	(Venkatadri *et al.*, 2016)
miR-328	Up	miRNA inhibition suppressed resveratrol induced inhibition of cell invasion and migration; targets MMP2	Osteosarcoma	(Yang *et al.*, 2015b)
miR-622	Up	inhibited cell proliferation and colonies formation; targets K-Ras	Lung	(Han *et al.*, 2012)
miR-663	Up	inhibited cell proliferation; targets eEF1A2, TGFβ1, JUNB and JUND	Breast; leukemia; colon	(Tili *et al.*, 2010a; Tili *et al.*, 2010b; Vislovukh *et al.*, 2013)
miR-744	Up	inhibited cell proliferation; targets eEF1A2	Breast	(Vislovukh *et al.*, 2013)
miR-21	Down	conferred resistance towards resveratrol; miRNA inhibition suppressed phospho-Akt level; targets BCL2 and PDCD4	Pancreatic; bladder; prostate	(Sheth *et al.*, 2012; Liu *et al.*, 2013a; Zhou *et al.*, 2014a)
miR-520h	Down	inhibited cell migration and invasion, reduced FOXC2 expression	Lung	(Yu *et al.*, 2013b)
miR-542-3p	Down	suppressed resveratrol's activity	Breast	(Venkatadri *et al.*, 2016)

Table 2.7: miRNAs regulated by genistein.

miRNA	Regulation	miRNA function	Cancer type	Ref.
miR-29b	Up	regulated NF-κB expression	Multiple myeloma	(Xie *et al.*, 2016)
miR-34a	Up	inhibited cell growth; induced apoptosis; targets Notch-1 and HOTAIR	Prostate; pancreatic	(Xia *et al.*, 2012; Chiyomaru *et al.*, 2013b)
miR-200c	Up	inhibited cell growth; induced apoptosis; targets DNMT3A, TET1 and TET3	Prostate	(Lynch *et al.*, 2016)
miR-574-3p	Up	inhibited cell proliferation, migration and invasion; induced apoptosis; targets RAC1, EGFR, and EP300	Prostate	(Chiyomaru *et al.*, 2013a)
miR-1296	Up	miRNA inhibition up-regulated MCM2 expression	Prostate	(Majid *et al.*, 2010)
miR-23b-3p	Down	miRNA inhibition induced apoptosis and suppressed invasion; targets PTEN	Renal	(Zaman *et al.*, 2012)
miR-27a	Down	miRNA inhibition suppressed cell growth, migration, and invasion and induced apoptosis; targets ZBTB10 and Sprouty2	Melanoma; ovarian; pancreatic	(Sun *et al.*, 2009; Xu *et al.*, 2013; Xia *et al.*, 2014)
miR-151	Down	miRNA inhibition suppressed cell migration and invasion; targets ARHGDIA, N4BP1, SOX17, CASZ1, IL1RAPL1	Prostate	(Chiyomaru *et al.*, 2012)
miR-155	Down	abrogated the anti-cancer activities of genistein; targets FOXO3, PTEN, CK1α, CDKN1B	Breast	(de la Parra *et al.*, 2016)
miR-221; miR-222	Down	targets ARH1	Prostate	(Chen *et al.*, 2011)
miR-223	Down	miRNA inhibition suppressed cell growth and induced apoptosis; targets FBW7	Pancreatic	(Ma *et al.*, 2013)
miR-1260b	Down	induced cell proliferation and invasion but suppressed apoptosis, targets sFRP1, DKK2 and SMAD4	Renal	(Hirata *et al.*, 2014)

2.7 Mothers against decapentaplegic homolog 4 (SMAD4)

The SMAD4 protein is a member of the SMAD proteins, which are a family of transcription factors found in nematodes, insects and vertebrates (Heldin *et al.*, 1997). The name SMAD is a portmanteau derived from *C. elegans* protein, SMA and *Drosophila* protein, mothers against decapentaplegic (MAD) (Derynck *et al.*, 1996). Studies have revealed that the SMAD proteins play a pivotal role in transmitting transforming growth factor-beta (TGF-β) superfamily signals from the cell surface to the nucleus, where they regulate transcription of target genes involved in regulating cell growth inhibition, senescence and apoptosis (Derynck *et al.*, 1998; Attisano & Wrana, 2000). Based on their function, the SMAD proteins can be classified into three groups: the receptor-regulated SMADs (R-SMADs), SMAD1, SMAD2, SMAD3, SMAD5 and SMAD8; the common mediator SMAD (Co-SMAD), SMAD4; and the inhibitory SMADs (I-SMADs), SMAD6 and SMAD7 (Attisano & Wrana, 1998; Massague, 1998).

TGF-β is a superfamily of cytokines that include the TGF-βs (TGF-β1, TGF-β2 and TGF-β3), activins and bone-morphogenetic proteins (BMPs) (Attisano & Wrana, 1998). The signals from the TGF-β family members are transduced across the plasma membrane by the functional receptor consisting of two TGF-β type I receptors and two TGF-β type II receptors, which are serine/threonine kinases. The initiation of the signalling requires the binding of TGF-β to the constitutively active TGF-β type II receptor, which then transphosphorylates the TGF-β type I receptor to activate a signalling cascade through phosphorylation of SMAD proteins (Wrana *et al.*, 1994). The R-SMADs are the direct substrates of the type I receptor and play an important role in maintaining specificity of the TGF-β pathway. SMAD2 and SMAD3 are activated by the TGF-β and activin receptors (Macias-Silva *et al.*, 1996; Liu *et al.*, 1997; Souchelnytskyi *et al.*, 1997), whereas SMAD1, SMAD5 and SMAD8 are targeted by the BMP receptor (Chen *et al.*, 1996; Hoodless *et al.*, 1996; Kretzschmar *et al.*, 1997).

Once phosphorylated, the R-SMADs form a complex with the Co-SMAD (SMAD4), followed by translocation to the nucleus where they regulate the expression of TGF-β target genes through interaction with DNA and DNA-binding proteins (Schmierer & Hill, 2007). On the other hand, the I-SMADs function as antagonists to the TGF-β family signalling by forming stable complexes with activated receptors and preventing the activation of R-SMADs and Co-SMAD, providing a negative feedback loop (Hayashi *et al.*, 1997; Imamura *et al.*, 1997; Nakao *et al.*, 1997; Hata *et al.*, 1998).

Although the SMAD proteins are important members in mediating TGF-β cellular responses in the canonical TGF-β/SMAD signalling pathway, numerous other signalling pathways can also be stimulated by TGF-β independently of SMAD proteins. In these non-canonical signalling pathways, the activated TGF-β receptor complex conveys signals through other pathways such as various branches of MAPK pathways, Rho GTPase signalling pathway, and PI3K/AKT pathway (Zhang, 2009; Mu *et al.*, 2012).

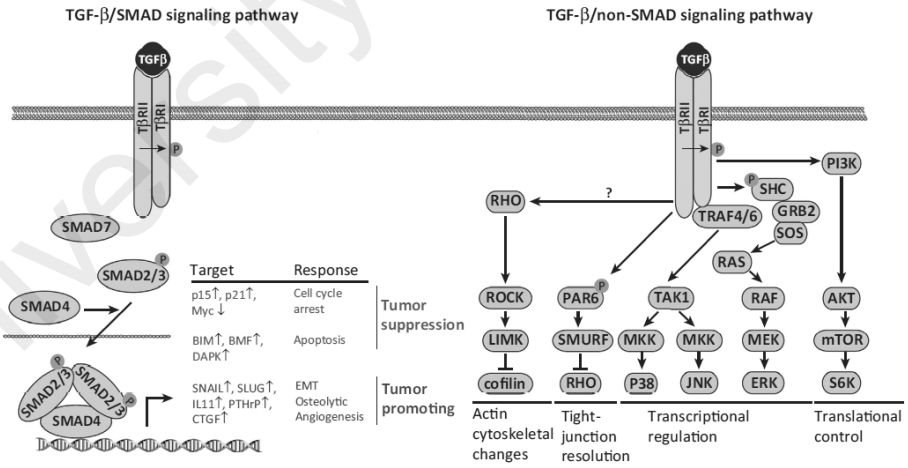

Figure 2.7: TGF-β signalling pathway.

(Figure adapted with permission from Colak & Ten Dijke, 2017)

2.7.1 Tumour suppressive functions

Various studies have demonstrated that TGF-β plays a dual role in the carcinogenesis by acting as both tumour suppressor and promoter, depending on cell context and

tumour stage (Derynck *et al.*, 2001). At the early stage of cancer, TGF-β exerts its tumour suppressive effects by inhibiting cell cycle progression and inducing apoptosis.

G1 progression in mammalian cells requires the activity cyclin dependent kinase (CDK): cyclin D-dependent kinases CDK4 and/or CDK6, and the cyclin E-dependent kinase CDK2 (Donjerkovic & Scott, 2000). TGF-β induced cell cycle arrest in G1 phase by inducing the expression of cyclin-dependent kinase inhibitors (CDKIs) $p15^{INK4B}$ and/or $p21^{CIP1}$, which binds to cyclin dependent kinase (CDK) 4 and 6, mediated by formation of complex between SMAD2, SMAD3 and SMAD4 with FOXO and SPI transcription factors (Pardali *et al.*, 2000; Ho *et al.*, 2004; Seoane *et al.*, 2004). Besides that, TGF-β also repressed the expression of oncogenic c-Myc by a transcriptional regulatory complex consisting of SMAD3, SMAD4, E2F4/5 and p107 (Chen *et al.*, 2002). Incidentally, the expressions of $p15^{INK4B}$ and/or $p21^{CIP1}$ are normally inhibited by the binding of c-Myc and zinc finger protein MIZ1, at the proximal region of their promoters (Seoane *et al.*, 2001; Seoane *et al.*, 2002). Hence, repression of c-Myc expression by TGF-β rendered these two CDKIs available for activation, further contributing to increased expressions of these two CDKIs. Besides these two CDKIs, studies have also showed that $p27^{Kip1}$ can also be induced by TGF-β (Polyak *et al.*, 1994; Reynisdottir *et al.*, 1995). The $p27^{Kip1}$ is CDKI that binds to CDK2 to induce cell cycle arrest, although its activity is normally inhibited by its binding to CDK4 and CDK6. However, the binding of $p15^{INK4B}$ to CDK4 and CDK6 would result in the release of $p27^{Kip1}$ from CDK4 and CDK6 (Reynisdottir *et al.*, 1995).

Unlike the well-documented mechanisms involved in regulating TGF-β-induced cell cycle arrest, the mechanisms underlying the pro-apoptotic effects of TGF-β remains largely uncharacterized. However, the involvements of R-SMADs together with Co-SMAD in mediating TGF-β-induced apoptosis have been demonstrated in various

studies. It was previously reported that interactions between the SMAD proteins and activator protein-1 (AP-1) played an important role in mediating TGF-β-induced apoptosis (Atfi *et al.*, 1997; Yamamura *et al.*, 2000). Studies have also shown that death-associated protein kinase (DAPK) and growth arrest and DNA-damage-inducible, beta (GADD45B), which promote cell death, are activated by TGF-β through the action of SMAD proteins (Jang *et al.*, 2002; Yoo *et al.*, 2003). Furthermore, transcriptional up-regulation of pro-apoptotic proteins such as BMF, BIM, BIK and down-regulation of BCL-xL by SMAD proteins have also been implicated in TGF-β-induced apoptosis (Ramjaun *et al.*, 2007; Yu *et al.*, 2008; Spender *et al.*, 2009).

On the other hand, it should also be noted that as tumours develop and progress, sensitivity to the tumour suppressive effects of TGF-β is often lost due to accumulation of genetic and epigenetic alterations, and TGF-β signalling switches from tumour suppressor to tumour promoter (Blobe *et al.*, 2000). The paradoxical effects of TGF-β are often seen at later stages of tumours, whereby it exerts its effects through induction of epithelial-to-mesenchymal transition, evasion of immune system and promotion of cell proliferation by modulating tumour microenvironment (Wakefield & Roberts, 2002; Derynck & Akhurst, 2007). These mechanisms resulted in enhanced tumour cell invasion and migration, leading to tumour progression and metastatic dissemination. Thus, the dual role played by TGF-β make it an attractive target for the development of novel therapies aimed at restoring the tumour suppressive arm or blocking the tumour promoting arm of its signalling pathway.

2.8 Ras suppressor 1 (RSU1)

The RSU1 was originally identified based on its ability to suppress Ras-dependant oncogenic transformation, indicative of its tumour suppressive activity (Cutler *et al.*, 1992). However, subsequent experiments revealed that RSU1 does not affect Ras

activation directly, but inhibits the activation of Rho GTPase, namely RhoA, Rac1 and cdc42. The same study also demonstrated that the activation of Rho associated kinase (ROCK), the downstream target of Rho GTPase, was also inhibited by RSU1 (Donthamsetty *et al.*, 2013). Past studies have demonstrated that the downstream targets of Ras pathway can contribute to cell transformation in response to activated Ras (Prendergast *et al.*, 1995; Khosravi-Far *et al.*, 1996). Besides that, it was also demonstrated that RSU1 expression increases ERK2 activation while inhibiting JUN kinase (Masuelli & Cutler, 1996; Vasaturo *et al.*, 2000).

2.8.1 Tumour suppressive functions

Despite its initial discovery many years ago, the mechanisms by which RSU1 functions in cancers are still not well-defined to date. However, there is generally a consensus among published studies on its role as a tumour suppressor in halting cancer cell growth. Studies have shown that ectopic expression of RSU1 exhibited tumour suppressive effects by inhibiting cell proliferation and migration in various cancer cells (Tsuda *et al.*, 1995; Dougherty *et al.*, 2008; Donthamsetty *et al.*, 2013). It was also demonstrated that over-expression of RSU1 inhibited cell growth by inducing the expression of CDKI $p21^{Cip1}$ (Masuelli *et al.*, 1999; Vasaturo *et al.*, 2000). Meanwhile, another group reported that RSU1 silencing led to increased cell proliferation in both non-invasive and highly invasive liver cancer cells, but reduced cell adhesion and invasion in highly invasive liver cancer cells only (Gkretsi & Bogdanos, 2015). Besides that, it was also reported recently that RSU1 induced apoptosis in breast cancer cells by inhibiting the pro-survival particularly interesting new cysteine-histidine-rich protein 1 (PINCH1) and activating the pro-apoptotic p53-upregulated-modulator of apoptosis (PUMA) (Giotopoulou *et al.*, 2015). PINCH1 is a cytoplasmic component of cell-extracellular matrix adhesions that contributed to apoptosis resistance by suppressing the expression of pro-apoptotic protein BIM (Chen *et al.*, 2008).

CHAPTER 3: MATERIALS AND METHODS

3.1 Cell cultures

3.1.1 Cell lines and culture conditions

The human cervical carcinoma cells CaSki and SiHa were obtained from American Type Cell Culture (ATCC, USA) and maintained in Roswell Park Memorial Institute (RPMI) 1640 (Hyclone, USA) and Dulbecco's Modified Eagle Medium (DMEM) (Hyclone, USA), respectively. Both cell lines were cultured in 25cm^2 cell culture flask containing 5 ml of complete media, grown as monolayer and maintained at 37°C and 5% CO_2 in humidified CO_2 incubator (Thermo Fisher Scientific, USA). Complete media used are basal media (RPMI-1640 or DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone, USA).

3.1.2 Cell harvesting

The spent media was decanted and cells were washed with 2 ml of 1× PBS solution to remove the remaining traces of FBS found in the cell culture media which can inhibit trypsin activity. The 1× PBS solution was subsequently discarded and 1.5 ml of 0.25% (v/v) trypsin-EDTA was added into the cell culture flask. The cell culture flask was incubated at 37°C to facilitate cells detachment, and its progress was checked every few minutes by observing under microscope (Nikon, Japan). After all cells were successfully detached, 1.5 ml of complete media was added to inactivate the trypsin activity. The cell suspension was then transferred into 15 ml centrifuge tube and centrifuged at 1,500 rpm for 5 min using Centrifuge 5702 (Eppendorf, Germany).

3.1.3 Sub-culturing cells

The cells were sub-cultured when they reached around 80% confluency. The cells were harvested as described in Section 3.1.2 and cell pellet was re-suspended in 5 ml complete media. One ml from this cell suspension was added into 25cm^2 cell culture flask containing 4 ml of complete media. The cells were then incubated at 37°C and 5% CO_2 in humidified CO_2 incubator.

3.1.4 Cell cryopreservation

To carry out cell cryopreservation, cells were grown until they have reached around 80% confluency. The cells were harvested as described in Section 3.1.2 and cell pellet was re-suspended in 4 ml of cell freezing media consisting of basal media supplemented with 20% (v/v) FBS and 5% (v/v) dimethyl sulfoxide (DMSO) (Merck, Germany) as cryoprotecting agent. A total of 1.2 ml of cell suspension was aliquoted into three 2 ml cryovials and cells were frozen gradually (4°C for 3 h followed by -20°C for 6 h) before being transferred to the liquid nitrogen storage tank (-196°C) for long term storage.

3.1.5 Cell recovery

Cryopreserved cells were removed from liquid nitrogen storage tank and immediately thawed at 37°C with gentle swirling until only a small chunk of ice remained in water bath (Memmert, Germany). The content was then transferred into a 25cm^2 cell culture flask containing 5 ml of basal media supplemented with 20% (v/v) FBS. The cells were then incubated at 37°C and 5% CO_2 in humidified CO_2 incubator. Fresh complete media was used to replace the spent media upon successful cells attachment.

3.1.6 Cell count

The cells were harvested as described in Section 3.1.2 and cell pellet was re-suspended in 5 ml of complete media. Twenty µl of the cell suspension was mixed with

20 µl of 0.4% trypan blue solution in a 0.2 ml PCR tube and incubated at room temperature. After 3 min, 10 µl of the mixture was loaded onto a clean hemacytometer and the number of viable cells was counted under the microscope at 100× magnification (dead cells stained blue while viable cells appeared as bright spheres). The concentration of the cell suspension (number of viable cells/ml) was determined using the following equation:

$$\text{Cell Concentration (cell/ml)} = \left(\frac{\text{Avg. Number of Cells Counted}}{\text{Volume Counted (ml)}}\right) \times \text{Dilution Factor}$$

From the cell concentration calculated, the cells were subsequently diluted with complete media to obtain the desired cell concentration.

3.2 Transient transfection

3.2.1 Transfection efficiencies

To determine the transfection efficiencies, cells were transfected with miRIDIAN microRNA Mimic or Inhibitor Transfection Control with Dy547 (Thermo Fisher Scientific, USA). The Dy547-labeled miRNA mimic and inhibitor, which were designed based on *C. elegans* cel-miR-67, allows for visualization of miRNA mimic and inhibitor delivery into transfected cells. The 20 µM working solutions were prepared by re-suspension in 1× siRNA buffer (Thermo Fisher Scientific, USA) and stored at -20°C until further use.

Cells were seeded at 1.0×10^5 cells per well in 6-wells plate and left overnight for cells attachment to the surface. The transfection mixture for each well was prepared as followed. In the first tube, 100 nM of miRIDIAN microRNA Mimic or Inhibitor Transfection Control with Dy547 was diluted and top up to 150 µl with basal media. In another tube, 2 µl of DharmaFECT 1 Transfection Reagent (Thermo Fisher Scientific, USA) was diluted and top up to 150 µl with basal media. Both tubes were incubated at

room temperature for 5 min. The contents from both tubes were then combined and incubated at room temperature for 20 min. After that, 1.2 ml of complete media was added to the transfection mixture and mixed well. The spent media in the 6-wells plate was decanted and cells were washed with 1 ml of 1× PBS before transfection mixture was added drop-wise into the wells. The cells were then incubated at 37°C and 5% CO_2 in humidified CO_2 incubator. After 24 h incubation, transfection efficiencies were assessed by visualizing the uptake of Dy547-labeled miRNA mimic and inhibitor using fluorescence microscopy (Nikon, Japan).

3.2.2 miRNA transfection

To up-regulate miRNA expression, cells were transfected with miRIDIAN microRNA human hsa-miR-210 mimic and miRIDIAN microRNA human hsa-miR-629 mimic (Thermo Fisher Scientific, USA). To inhibit miRNA expression, cells were transfected with miRIDIAN microRNA human hsa-miR-210 inhibitor and miRIDIAN microRNA human hsa-miR-629 inhibitor (Thermo Fisher Scientific, USA). The negative controls miRIDIAN microRNA Mimic Negative Control #1 and miRIDIAN microRNA Inhibitor Negative Control #1 (Thermo Fisher Scientific, USA) were included to ensure that the effects observed is specific. These negative controls were designed based on the mature sequence of *C. elegans* cel-miR-67 and have minimal sequence identity to miRNAs found in human. The 20 µM working solutions were prepared by re-suspension in 1× siRNA buffer (Thermo Fisher Scientific, USA) and stored at -20°C until further use.

Cells were seeded at 3.0×10^5 cells in $25cm^2$ cell culture flask and left overnight for cells attachment to the surface. The transfection mixture for each sample was prepared as followed. In the first tube, 100 nM of miRIDIAN microRNA mimic, inhibitor or negative controls was diluted and top up to 300 µl with basal media. In another tube, 6

µl of DharmaFECT 1 Transfection Reagent was diluted and top up to 300 µl with basal media. Both tubes were incubated at room temperature for 5 min. The contents from both tubes were then combined and incubated at room temperature for 20 min. After that, 2.4 ml of complete media was added to the transfection mixture and mixed well. The spent media in the flask was decanted and cells were washed with 2 ml of 1× PBS before transfection mixture was added drop-wise into the flasks. The cells were then incubated at 37°C and 5% CO_2 in humidified CO_2 incubator.

For reverse transcription quantitative real-time PCR (RT-qPCR) analysis, total RNA was extracted after 24 h incubation. For Annexin V/PI assay and western blot analysis, the transfection mixture was replaced with complete media after 24 h to reduce cytotoxicity. For other downstream experiments, cells were harvested after 24 h as described in Section 3.2.2 and re-seeded at 1.0×10^4 cells per well in 96-wells plate (cell proliferation assays) or 1.5×10^5 cells per well in 6-wells plate (caspase 3/7 assays). Cells were incubated at 37°C and 5% CO_2 in humidified CO_2 incubator overnight for cells attachment.

3.2.3 Plasmid transfection

For gene target over-expression studies, cells were transfected with mammalian expression plasmids containing the coding region of SMAD4 (pCMV6-SMAD4) or RSU1 (pCMV6-RSU1) (Origene Technologies, USA). The empty vector (pCMV6) (Origene Technologies, USA) was used as negative control to ensure that the effects observed is specific. The 100 ng/µl working solution was prepared by dissolving the plasmid in nuclease-free water and stored at -20°C until further use.

Cells were seeded at 1.5×10^4 cells per well in 96-wells plate (for cell viability assay) or 1.5×10^5 cells per well in 6-wells plate (for other downstream experiments) and left overnight for cells attachment to the surface. The transfection mixture for each

sample were prepared as followed. In the first tube, 5 ng (96-wells plate) or 50 ng (6-wells plate) of plasmid were diluted and top up to 10 µl (96-wells plate) or 150 µl (6-wells plate) with basal media. In another tube, 0.2 µl (96-wells plate) or 3 µl (6-wells plate) of DharmaFECT 1 Transfection Reagent was diluted and top up to 10 µl (96-wells plate) or 150 µl (6-wells plate) with basal media. Both tubes were then incubated at room temperature for 5 min. The contents from both tubes were combined and incubated at room temperature for 20 min. After that, 80 µl (96-wells plate) or 1.2 ml (6-wells plate) of complete media was added to the transfection mixture and mixed well. The spent media in the 96-wells and 6-wells plate was decanted and cells were washed with 1× PBS before transfection mixture was added drop-wise into the wells. The cells were then incubated at 37°C and 5% CO_2 in humidified CO_2 incubator.

For western blot analysis, total protein was extracted after 24 h incubation. However, the transfection mixture was replaced with complete media after 12 h incubation to reduce cytotoxicity. For other downstream experiments, cells were incubated with transfection mixture for 6 h (caspase 3/7 assays) or 12 h (cell proliferation and Annexin V/PI assays).

3.3 1'S-1'-acetoxychavicol acetate (ACA)

3.3.1 Re-constitution of ACA

The ACA was provided by the Centre for Natural Product Research and Drug Discovery (CENAR), Department of Chemistry, University of Malaya. The ACA stock solution was prepared by dissolving the purified ACA in DMSO and then vortexed vigorously to ensure that ACA had completely dissolved in the DMSO. The 10 mM working solution was prepared by diluting the stock solution in DMSO. Both ACA stock and working solutions were stored at -20°C until further use.

3.3.2 Treatment with ACA

To assess the effects of ACA on miRNA and miRNA target protein expressions, cells were seeded at 1.5×10^5 cells per well in 6-wells plate and left overnight for cells attachment to the surface before being treated with ACA (20 µM for CaSki cells and 30 µM for SiHa cells). To determine the effects of ACA on miRNA expression, total RNA was extracted from cells treated with ACA after 12 and 24 h incubation for analysis using RT-qPCR. To determine the effects of ACA on miRNA target protein expression, total protein was extracted from cells treated with ACA after 12 and 24 h incubation and analyzed using western blots. Untreated cells were included as controls in both experiments.

To investigate the effects of miRNA expression and gene target over-expression in regulating response towards ACA, transfected cells as described in Sections 3.2.2 and 3.2.3 were treated with ACA according to the treatment duration and concentration summarized in Table 3.1 and Table 3.2.

Table 3.1: Concentration of ACA used in treatment on miRNA transfected cells.

Assays	Treatment duration (h)	CaSki cells (µM)	SiHa cells (µM)
Cell viability	48	0 - 20	0 - 20
Annexin V/PI	48	20	30
Caspase 3/7	6	20	30

Table 3.2: Concentration of ACA used in treatment on plasmid transfected cells.

Assays	Treatment duration (h)	CaSki cells (µM)	SiHa cells (µM)
Cell viability	12	10	15
Annexin V/PI	12	20	30
Caspase 3/7	6	20	30

3.4 Total RNA extraction and quality control

3.4.1 Total RNA extraction

Total RNA from cells was extracted using miRNeasy Mini Kit (Qiagen, Germany). The cells were harvested as described in Section 3.1.2. The cell pellet was re-suspended in 1 ml of 1× PBS, transferred into a 1.5 ml microcentrifuge tube and centrifuged at 500× g for 5 min. The supernatant was then decanted and 700 µl of QIAzol lysis reagent was added to the pellet to lyse the cells by repetitive pipetting followed by vortexing for 1 min for homogenization. The homogenized samples were incubated at room temperature for 5 min to promote dissociation of nucleoprotein complexes. Next, 140 µl of chloroform was added to the homogenate and tubes were shaken vigorously by hand for 15 sec and incubated at room temperature for 3 min. This was followed by centrifugation at 12,000× *g* for 15 min at 4°C. After that, the upper aqueous phase was transferred into a new 1.5 ml microcentrifuge tube and 525 µl of 100% ethanol was added and mixed thoroughly by pipetting up and down several times. Next, 700 µl of the sample was transferred into the provided RNeasy Mini spin column and centrifuged at 8,000× *g* for 15 sec at room temperature. The flow-through was then discarded and remaining sample was transferred into the same RNeasy Mini spin column and centrifuged at 8,000× *g* for 15 sec at room temperature. The flow-through was discarded and 700 µl of Buffer RWT was added into the same RNeasy Mini spin column and centrifuged at 8,000× *g* for 15 sec at room temperature. The flow-through was again discarded and 500 µl of Buffer RPE was added into the same RNeasy Mini spin column to wash the column and followed by centrifugation at 8,000× *g* for 15 sec at room temperature. The flow-through was discarded before 500 µl of Buffer RPE was added into the same RNeasy Mini spin column and centrifuged at 8,000× *g* for 2 min at room temperature to dry the spin column membrane. The RNeasy Mini spin column was then transferred into a new collection tube and centrifuged at full speed for 1 min to

eliminate any possible carryover of Buffer RPE or residual flow-through on the spin column. The RNeasy Mini spin column was then transferred into a new 1.5 ml microcentrifuge tube and 30 µl of nuclease-free water was added directly onto the center of the spin column. Lastly, the sample was incubated at room temperature for 5 min followed by centrifugation at 8,000× *g* for 1 min at room temperature to elute the total RNA. All RNA samples were stored at -20°C until further use.

3.4.2 RNA quality control

The concentration and purity of the extracted RNA was assessed using NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), while its integrity was determined using 2200 TapeStation (Agilent Technologies, USA).

3.4.2.1 RNA analysis using NanoDrop spectrophotometer

The concentration and purity of the extracted RNA was assessed using NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). The nuclease-free water, which was used to elute the RNA, was used as blank. The purity of the extracted RNA was determined using the ratio of A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm}, and only RNA samples with a ratio of 1.8 to 2.0 were utilized for the experiments. A ratio of 1.8 to 2.0 for A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm} indicates absence or minimal amount of contaminants, which could possibly interfere and/or inhibit with the downstream experiments.

3.4.2.2 RNA analysis using 2200 TapeStation

The integrity of the extracted RNA was determined using 2200 TapeStation (Agilent Technologies, USA). The RNA ScreenTape Sample Buffer (Agilent Technologies, USA) and RNA ScreenTape (Agilent Technologies, USA) were first allowed to equilibrate to room temperature before use while the total RNA sample was allowed to thaw on ice. Five µl of RNA ScreenTape Sample Buffer was added to 1 µl of total RNA sample in a 0.2 ml PCR tube and mixed by pipetting. The samples were then heated at

72°C for 3 min followed by incubation on ice for 2 min. After a short centrifugation, the samples were then loaded into the 2200 TapeStation and analyzed using 2200 TapeStation software for Eukaryotic RNA protocol. The RNA ScreenTape and loading tips were loaded into the 2200 TapeStation prior to starting the analysis. The quality of RNA samples were then assessed based on RNA integrity number (RIN) value, whereby RIN value of 0 represents highly degraded RNA sample while RIN value of 10 represents highly intact RNA sample (Schroeder *et al*., 2006). For this study, only RNA samples with a RIN value of 7 and above were utilized for the experiments.

3.5 Reverse transcription quantitative real-time PCR (RT-qPCR)

3.5.1 Reverse transcription (RT)

Five ng of extracted total RNA samples (described in Section 3.4.1) was used for reverse transcription reactions using TaqMan® MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, USA). The volumes for each RT reaction were prepared according to Table 3.3 with the total volume of reaction mixture per tube being 10 µl. The reactions were incubated for 16°C for 30 min, 42°C for 30 min, 85°C for 5 min, and then held at 4°C using Veriti 96-Well Thermal Cycler (Thermo Fisher Scientifc, USA).

Table 3.3: RT setup.

Component	Volume per sample (µl)
Stem-loop RT primer	2.00
10× RT Buffer	1.00
dNTP (100 mM)	0.10
MultiScribe™ Reverse Transcriptase (50 U/µL)	0.67
RNase inhibitor (20 U/µL)	0.13
RNA template	x
Nuclease-free water	6.10-x
Total volume	10.00

3.5.2 Real-time PCR amplification and quantitation

The cDNA obtained in Section 3.5.1 was subsequently used for target amplification using TaqMan® MicroRNA Assays (Thermo Fisher Scientific, USA). The volumes for each PCR reaction were prepared according to Table 3.4 with the total volume of reaction mixture per tube being 10.0 µl. Reactions were incubated at 50°C for 2 min and then 95°C for 20 s followed by 40 cycles at 95°C for 3 s and 60°C for 20 s using CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, USA) and analyzed using CFX Manager v1.6 (Bio-Rad Laboratories, USA). The U6 small nuclear RNA was used as an internal control to normalize RNA input. The fold-change was calculated using the $2^{-\Delta\Delta Ct}$ method (as described by Livak and Schmittgen, 2001) and presented as fold-expression changes relative to negative controls after normalization to internal control. The TaqMan® MicroRNA Assays used were hsa-miR-210 (Assay ID: 000512); hsa-miR-629 (Assay ID: 002436) and RNU6B (Assay ID: 001093).

Table 3.4: PCR setup for real-time PCR amplification and quantitation.

Component	Volume per sample (µl)
2× TaqMan® Fast Advanced Master Mix	5.00
TaqMan® 20× Primer Probe Assay	0.50
RT product	0.67
Nuclease-free water	3.83
Total volume	10.00

3.6 Cell proliferation assay

Cell proliferation assay was carried out using 3-(4,5-dimethylthiazol-2-γl)-2,5-diphenyl-tetrazoliumbromide (MTT) reagent (Merck, Germany). At the end of incubation period, 30 µl of MTT solution (5 mg/ml) was added to each well and the plate was incubated at 37°C for 60 min. After that, media and excess MTT solution were removed and 200 µl of DMSO was added to each well to dissolve the purple formazan precipitates. Once the formazan crystals were dissolved, results were obtained

using microtiter plate reader (Tecan, Switzerland) which detects absorbance wavelength at 570 nm with reference wavelength at 650 nm and analyzed using Magellan v6.3 software (Tecan, Switzerland). Serial dilution was performed using wells containing cells at descending densities (10,000, 5,000, 2,500, 1,250, 0) to construct standard curve for quantification purposes.

3.7 Apoptosis assay

3.7.1 Annexin V/PI assay

Detection and differentiation of various apoptosis stages were determined using BD Pharmingen™ Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, USA). The cells were harvested as described in Section 3.2.2, except that the spent media was transferred to the same 15 ml centrifuge tube and not discarded. The cell pellet was re-suspended in 1 ml of 1× cold PBS, transferred into a 1.5 ml microcentrifuge tube and centrifuged at 1,000× g for 5 min. Supernatant was decanted and cell pellet was washed with 1 ml of 1× cold PBS again and centrifuged at 1,000× g for 5 min. Supernatant was decanted and cell pellet was re-suspended in 100 µl of 1× Annexin V binding buffer. Four µl of FITC Annexin V and propidium iodide staining solution were added to the samples and mixed well. The samples were then left at room temperature for 15 min in the dark. After that, 400 µl of 1× Annexin V binding buffer was added to each sample and the contents was transferred into 5 ml round bottom polystyrene tube for further analysis. Results were obtained using BD FACSCanto II flow cytometer (BD Biosciences, USA) and analyzed with BD FACSDiva software (BD Biosciences, USA). Quantification and visualization of live, early-apoptosis, late-apoptosis and dead cell populations were defined by quadrants that were created based on single signal controls of respective cancer cell lines.

3.7.2 Caspase 3/7 assay

The activation of caspase-3 and -7 activities were assessed using Caspase-Glo® 3/7 assay (Promega, USA). The cells were harvested as described in Section 3.2.2, except that the used cell culture media was transferred to the same 15 ml centrifuge tube as well and not discarded. The cell pellet was washed with 1 ml of 1× PBS and centrifuged at 1,500 rpm for 5 min using Centrifuge 5702 (Eppendorf, Germany). Next, the cell pellet was re-suspended in 300 µl of 1× PBS and 50 µl of this suspension was aliquoted into a 1.5 microcentrifuge tube. A total of 50 µl of Caspase-Glo 3/7 reagent was added to each samples and mixed well. The samples were then left at room temperature for 60 min in the dark. The caspase-3 and -7 activities were subsequently measured with GLOMAX®-Multi Jr (Promega, USA).

3.8 Luciferase reporter assay

3.8.1 miRNA target prediction

The putative miR-210 and miR-629 targets were identified using TargetScan v7.0 (Agarwal *et al.*, 2015). Gene annotation enrichment analyses of the predicted miRNA targets with total context score of less than 0 were then performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (Huang da *et al.*, 2009). The miRNA binding site on the putative target was cross-checked using miRanda (Betel *et al.*, 2010), another miRNA target prediction software, before primers were designed to isolate the gene fragment containing miRNA binding site.

3.8.2 Isolation of gene fragment containing miRNA binding site

3.8.2.1 Primer design

The forward and reverse primers flanking the region where the putative miRNA binding site is located were designed based on the FASTA sequence obtained from the National Center for Biotechnology Information (NCBI) database using Primer-BLAST

software provided by the NCBI database. The restriction site for the restriction enzyme *Nhe*I and *Xho*I together with recognition sequence were incorporated into the primer sequence. The primers designed were checked using Primer Premier for formation of hairpin and primer dimers. The finalized primers sequence (Table 3.5), were submitted to First BASE Laboratories Sdn. Bhd. for synthesis.

Table 3.5: Synthesized primers sequence.

Name	Sequence
E2F3_F	5'-TTA CTC TCG AGT AGG CAC GGC TTG AAA TGC TA-3'
E2F3_R	5'-CTT GTG CTA GCT TGC TTC GTG TGA ACT CTC CT-3'
SMAD4_F	5'-TTA CTC TCG AGT AAT TGA CAC GGT TCA AGG GAA-3'
SMAD4_R	5'-ATC ATG CTA GCT GAC AAA AGG TTA CAT AGT ATG CCC-3'
BIK_F	5'-TAT TAC TCG AGT GCC GAG GGC ATC ACA TAT CA-3'
BIK_R	5'-GTC ATG CTA GCT ATG GAC GGT TTC ACC ACA CT-3'
RSU1_F	5'-TTA CTC TCG AGT AAG CCA GAG CCC ACT ATG GA-3'
RSU1_R	5'-ATT ATG CTA GCT CTT GGC GTG TCC CAT GTT TT-3'

(Note: F denotes forward primer and R denotes reverse primer.)

3.8.2.2 Reverse transcription-PCR (RT-PCR)

First strand cDNA synthesis: One µg of RNA from untreated cells was converted into cDNA using Thermo Scientific RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). The volumes for each RT reaction were prepared according to Table 3.6 with the total volume of reaction mixture per tube being 20 µl. After adding template RNA, forward primer and nuclease-free water, the mixture was incubated at 65°C for 5 min. The mixture was then chilled on ice before other components were added. The reactions were incubated for 45°C for 60 min followed by 70°C for 5 min using Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, USA).

Table 3.6: RT setup for first strand cDNA synthesis.

Component	Volume per sample (µl)
RNA template	x
Forward primer (0.5 µM)	2
Nuclease-free water	12 - x
5× Reaction buffer	4
RiboLock RNase inhibitor (20 U/µL)	1
dNTP mix (10 mM)	2
RevertAid H Minus reverse transcriptase	1
Total volume	20

PCR amplification: The product of the first strand cDNA synthesis was used as template for subsequent PCR amplification using Thermo Scientific Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, USA). The volumes for each PCR reaction were prepared according to Table 3.7 with the total volume of reaction mixture per tube being 20 µl. The reactions were incubated for initial denaturation at 98°C for 10 sec followed by 35 cycles at 98°C for 1 sec and 62°C for 5 sec and 72°C for 15 sec followed by final extension at 72°C for 1 min and then held at 4°C using Veriti 96-Well Thermal Cycler (Thermo Fisher Scientific, USA).

Table 3.7: Setup for PCR amplification of first strand cDNA.

Component	Volume per sample (µl)
cDNA template	2
Forward primer (0.5 µM)	2
Reverse primer (0.5 µM)	2
2× Phusion Flash Master Mix	10
Nuclease-free water	4
Total volume	20

3.8.2.3 Agarose gel electrophoresis

The 1% (w/v) agarose gel was prepared by adding 0.5 g of agarose powder (Amresco, USA) to 50 ml of 1× TBE buffer (Amresco, USA) and heated with microwave oven (Panasonic, Malaysia) until the powder was totally dissolved. The

molten agarose gel was then poured into a 5.5 cm ×12 cm gel casting tray (BayGene, China) and comb was inserted to create wells for sample loading. Once the gel has solidified, the comb was removed and the gel was submerged in electrophoresis chamber (BayGene, China) containing 1× TBE buffer. The DNA sample was then mixed with 6× Orange DNA loading dye (Thermo Fisher Scientific, USA) at 1:5 ratio prior to loading into the wells. Two µl of O'GeneRuler 1kb DNA ladder (Thermo Fisher Scientific, USA) was used as the molecular weight marker. Electrophoresis was carried out at 100V for approximately 45 min or until the dye has reached the end of the gels. The gel was stained with ethidium bromide solution (0.5 µg/ml) (Promega, USA) for 5 min followed by de-staining in distilled water for 10 min and viewed under UV light using UV transilluminator (Vilber Lourmat, Germany).

3.8.2.4 DNA gel extraction

DNA gel extraction was carried out using QIAquick Gel Extraction Kit (Qiagen, Germany). The gel slice containing DNA fragment was viewed under UV light using UV transilluminator (Vilber Lourmat, Germany) and excised from the agarose gel using a clean scalpel and transferred to a 1.5 ml microcentrifuge tube. The weight of the gel slice was calculated and 3 volumes of Buffer QG were added to 1 volume of gel weight. The tube was then incubated at 50°C for 10 min and vortexed every 3 min to dissolve the gel. After the gel slice has completely dissolved, one volume of isopropanol was added to the sample and mixed well by inversion. A total of 750 µl sample was then transferred to the QIAquick column in 2 ml collection tube and centrifuged at 17,900× *g* for 1 min. The flow-through was discarded and remaining sample was loaded into the same QIAquick column in 2 ml collection tube and centrifuged at 17,900× *g* for 1 min. The flow-through was discarded and 500 µl of Buffer QG was added into the QIAquick column and centrifuged at 17,900× *g* for 1 min. The flow-through was discarded and 750 µl of Buffer PE was added into the QIAquick column. The sample was incubated at

room temperature for 5 min followed by centrifugation at 17,900× *g* for 1 min. The flow-through was discarded and 750 µl of Buffer PE was again added into the QIAquick column and centrifuged at 17,900× *g* for 1 min. The flow-through was discarded and centrifuged at 17,900× *g* for 1 min to remove the residual ethanol from Buffer PE. The QIAquick column was then transferred to a new 1.5 ml microcentrifuge tube and 30 µl of Buffer EB was added to the center of the QIAquick membrane. The sample was incubated at room temperature for 4 min followed by centrifugation at 17,900× *g* for 1 min to elute the DNA. All DNA samples were stored at -20°C until further use.

3.8.2.5 DNA quantification and quality control

The concentration and purity of the extracted DNA was assessed using NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Buffer EB, which was used to elute the DNA, was used as blank. The purity of the extracted RNA was determined using the ratio of A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm}. A ratio of 1.8 – 2.0 for A_{260nm}/A_{280nm} and A_{260nm}/A_{230nm} indicates absence or minimal amount of contaminants, which could possibly interfere and/or inhibit with the downstream experiments.

3.8.2.6 Purified PCR product sequencing

Ten µl of purified PCR product (20 ng/µl) was sent to First BASE Laboratories Sdn. Bhd. for sequencing using gene-specific primers designed in Section 3.8.2.1. Sequence alignment was then conducted using Nucleotide BLAST software provided by the NCBI database to confirm that the nucleotides sequence is correct.

3.8.3 Sub-cloning

3.8.3.1 Restriction enzyme digestion

Restriction enzyme digestion was performed to linearize and create sticky ends for pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, USA) and

insert DNA. The volumes for each reaction were prepared according to Table 3.8 with the total volume of reaction mixture per tube being 20 µl. The reactions were incubated for initial denaturation at 37°C for 2 h (vector DNA) or 3 h (insert DNA). After the incubation period, the digested products were observed using agarose gel electrophoresis as described in Section 3.8.2.3 and DNA gel extraction was carried out to excise the fragment as described in Section 3.8.2.4.

Table 3.8: Restriction enzyme digestion setup.

Component	Volume per sample (µl)
Nuclease-free water	16.8 - x
10× Buffer B	2.0
Acetylated bovine serum albumin (10 mg/ml)	0.2
DNA (50 ng/µl)	x
*Nhe*I (10 U/µl)	0.5
*Xho*I (10 U/µl)	0.5
Total volume	20.0

3.8.3.2 Ligation

Ligation reaction between vector DNA and insert DNA was carried out using insert DNA:vector DNA molar ratio of 1:1, 1:3 and 3:1. The amount of insert DNA required was calculated using the following formula:

$$\frac{ng\ of\ vector \times kb\ size\ of\ insert}{kb\ size\ of\ vector} \times molar\ ratio\ of\ \frac{insert}{vector} = ng\ of\ insert$$

The volumes for each reaction were prepared according to Table 3.9 with the total volume of reaction mixture per tube being 10 µl. The reactions were then incubated at 4°C overnight.

Table 3.9: Ligation setup.

Component	Volume per sample (µl)
T4 ligase 10× buffer	1.00
T4 DNA ligase (3 U/µl)	0.33
Vector DNA (5 ng/µl)	x
Insert DNA	y
Nuclease-free water	7.67 - x -y
Total volume	10.00

3.8.3.3 Transformation

The JM109 competent cells (Promega, USA) was removed from the -80°C freezer and thawed on ice. The tube containing the thawed competent cells was flicked gently to mix it and 100 µl of competent cells was transferred to chilled 1.5 ml microcentrifuge tube with chilled pipette tips. Ten µl of ligation reaction was added to the tube with chilled pipette tips and the tube was flicked gently to mix it before being incubated on ice for 30 min. After that, the tube was incubated at 42°C for 2 min followed by incubation on ice for 2 min. A total of 900 µl of cold LB ampicillin broth was added into the tube and incubate at 37°C for 60 min with agitation (230 rpm). Following incubation, the tube was centrifuged at 8,000× rpm for 10 min to pellet the cells. The supernatant was discarded and cell pellet was re-suspended in 100.0 µl cold LB ampicillin broth and plated on LB ampicillin plate. The plate was then incubated at 37°C overnight.

3.8.3.4 Colony PCR

The colonies found on the plate following overnight incubation were analyzed by colony PCR to screen for successful transformants. Different colonies were selected and inoculated into 0.2 ml PCR tube containing 20 µl with the end of a sterile pipette tip followed by incubation at 95°C for 5 min. Two µl of sample from the tube was used as template and PCR reactions were carried out as described in Section 3.8.2.2 and results

were analyzed using agarose gel electrophoresis as described in Section 3.8.2.3. Colonies containing positive transformants were then inoculated in 10 ml of LB ampicillin broth and incubate at 37°C overnight with agitation (230 rpm).

3.8.3.5 Plasmid extraction

Plasmid DNA was extracted from overnight bacteria culture using PureYield™ Plasmid MiniPrep System (Promega, USA). First, 600 µl bacteria culture grown in LB medium was transferred to a 1.5 ml microcentrifuge tube. Next, 100 µl of Cell Lysis Buffer was added to the tube and mixed by inversion for 6 times, whereby the solution changed from opaque to clear blue. After that, 350 µl of cold Neutralization Solution was added and mixed thoroughly by inversion, whereby the solution changed from clear blue to yellow with precipitate. The tube was then centrifuged at maximum speed for 3 min. Approximately 900 µl of the supernatant was subsequently transferred to PureYield™ Minicolumn placed in collection tube and centrifuged at maximum speed for 15 sec. The flow-through was discarded and 200 µl Endotoxin Removal Wash was added followed by centrifugation at maximum speed for 15 sec. The flow-through was again discarded and 400 µl of Column Wash Solution was added followed by centrifugation at maximum speed for 30 sec. The PureYield™ Minicolumn was transferred to a new 1.5 ml microcentrifuge tube and 30 µl of Elution Buffer was added to the center of the column membrane. The sample was incubated at room temperature for 1 min and then centrifuged at maximum speed for 15 sec to elute the plasmid DNA. The plasmid DNA was stored at -20°C until further use. The concentration and purity of the extracted DNA was assessed using NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) as described in Section 3.8.2.5. The Elution Buffer, which was used to elute the DNA, was used as blank.

3.8.3.6 Plasmid sequencing

Ten µl of plasmid DNA (100 ng/µl) was sent to First BASE Laboratories Sdn. Bhd. for sequencing using forward primer 5'-GATCGCCGTGTAATTCTAGTTGTTT-3' and reverse primer 5'-CTTCCTTTCGGGCTTTGTTAGC-3'. Sequence alignment was then conducted using Nucleotide BLAST software provided by the NCBI database to confirm successful ligation between the vector and insert DNA, and to ensure that the sequence for the nucleotides is correct.

3.8.4 Site-directed mutagenesis

3.8.4.1 Designing mutagenic primers

The mutagenic primers to incorporate point mutations in the miRNA binding site were designed using web-based QuikChange Primer Design Program provided by Agilent Technologies. The mutagenic primers sequence (Table 3.10) was submitted to First BASE Laboratories Sdn. Bhd. for synthesis. The mutagenic primer sequence for RSU1 is not shown as the site-directed mutation in RSU1 binding site was performed by First BASE Laboratories Sdn. Bhd. This was because prolonged unscheduled electrical disruption rendered the consumables unusable before site-directed mutation in RSU1 binding site could be carried out.

Table 3.10: Synthesized mutagenic primers sequence.

Name	Sequence
Control_mut	5'-CTC CCG TAT CGT AGT TAT CTA CAC GAC GGG-3'
E2F3_mut	5'-GCT GAC ATT TTA ATG AAT TTT TTA AAA AAT TAA TAA ACA AAT TGT CTA ATG CGT GTG TTG CAG GCT CCC TTG GGA AAG CCC T-3'
SMAD4_mut	5'-CTT TTG AAT TGC GTG CAC ACA CAC ACG CTG CGT GTC ACT CTG GTC AGA GTT TAT TAA GGC TTT-3'

3.8.4.2 PCR amplification of mutant strand

To obtain site-directed plasmid DNA, PCR amplification of mutant strand was performed using QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies, USA). The volumes for each PCR reaction were prepared according to Table 3.11 with the total volume of reaction mixture per tube being 25 µl. The reactions were incubated for initial denaturation at 95°C for 1 min followed by 30 cycles at 95°C for 1 min and 55°C for 1 min and 65°C for 2 min/kb of plasmid length using Veriti 96-Well Thermal Cycler (Applied Biosystems, USA). Upon completion of the PCR amplification, the reaction was placed on ice for 2 min.

Table 3.11: Setup for PCR amplification of mutant strand.

Component	Volume per sample (µl)
10× QuikChange Multi reaction buffer	2.5
QuikSolution	0.5
DNA template (4 ng/µl)	x
Mutagenic gene specific primer (0.404 µM)	1.0
Mutagenic control primer (0.404 µM)	1.0
dNTP mix	1.0
QuikChange multi enzyme blend	1.0
Nuclease-free water	18.0 - x
Total volume	25.0

3.8.4.3 *Dpn*I digestion of the amplification products

One µl of D*pn*I restriction enzyme was added to each amplification reaction and mixed by pipetting. After the mixture was spin down by short centrifugation, the reaction was incubated at 37°C for 60 min to digest the parental (non-mutated) DNA.

3.8.4.4 Transformation

The XL10-Gold ultracompetent cells (Agilent Technologies, USA) was removed from the -80°C freezer and thawed on ice. The tube containing the thawed competent cells was flicked gently to mix it and 45 µl of competent cells was transferred to a

chilled 1.5 ml microcentrifuge tube with chilled pipette tips. Two µl of XL10-Gold β-mercaptoethanol mix was added and the contents in the tube was swirled gently for mixing. Following this, 1.5 µl of *Dpn*I digested DNA was added and the contents in the tube was again swirled gently for mixing. Next, the tube was incubated on ice for 30 mins. After that, the tube was incubated at 42°C for 30 sec followed by incubation on ice for 2 min. A total of 500 µl of NZY^+ broth (which has been heated to 42°C earlier) was then added into the tube, and sample was incubated at 37°C for 60 min with agitation (230 rpm). After incubation, 10 µl of sample from the tube was mixed with 90 µl of NZY^+ broth and plated on LB ampicillin plate. In addition, 100 µl of sample taken directly from the tube was also plated onto LB ampicillin plate. The plates were then incubated at 37°C overnight. Colonies found on the plate following overnight incubation were inoculated in 10 ml of LB ampicillin broth and incubate at 37°C overnight with agitation (230 rpm). Plasmid DNA was extracted from overnight bacteria culture as described in Section 3.8.3.5 and sent to First BASE Sdn. Bhd. for sequencing as described in Section 3.8.3.6 to confirm the presence of the desired point mutations in the nucleotides sequence.

3.8.5 Luciferase assay

3.8.5.1 Co-transfection

SiHa cells were seeded at 1.5×10^5 per well in 6-wells plate and left overnight for cells attachment to the surface. The transfection mixture for each well was prepared as followed. In the first tube, 40 ng of pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, USA) containing wild-type or mutated miRNA binding site and 100 nM of miRIDIAN microRNA mimic, inhibitor or negative controls (Thermo Fisher Scientific, USA) were diluted and top up to 150 µl with basal media. In another tube, 3 µl of DharmaFECT 1 Transfection Reagent (Thermo Fisher Scientific, USA) was diluted and top up to 150 µl with basal media. Both tubes were then

incubated at room temperature for 5 min. Next, the mixtures from both tubes were combined and incubated at room temperature for 20 min. After that, 1.2 ml of complete media was added to the transfection mixture and mixed well. The spent media in the 6-wells plate was decanted and cells were washed with 1 ml of 1× PBS before transfection mixture was added drop-wise into the wells. The cells were then incubated at 37°C and 5% CO_2 in humidified CO_2 incubator for 24 h. After 24 h incubation, the transfection mixture in the 6-wells plate was decanted and cells were washed with 1 ml of 1× PBS before 1.5 ml of complete media was added drop-wise into the wells. The cells were then incubated at 37°C and 5% CO_2 in humidified CO_2 incubator for another 24 h before being assayed.

3.8.5.2 Dual-Glo luciferase assay

The interaction between miRNA and its putative gene targets was investigated using Dual-Glo® Luciferase Assay System (Promega, USA), which measures the activity of two reporter enzymes within a single system. Firefly luciferase activity was used to evaluate the interaction between miRNA and its putative gene targets while *Renilla* luciferase activity was used as normalization for transfection efficiencies. At 48 h post-transfection, the cells were harvested as described in Section 3.2.2. The cell pellet was re-suspended in 1 ml of 1× PBS and transferred into a 1.5 ml microcentrifuge tube and centrifuged at 20,000× g for 30 sec. Supernatant was decanted and 50 µl of 1× PBS was used to re-suspend the cell pellet. Fifty µl of Dual-Glo® Luciferase reagent (Promega, USA) was added into the tube and mixed well followed by incubation at room temperature for 10 min in the dark before firefly luminescence was measured with GLOMAX®-Multi Jr (Promega, USA) and recorded. Fifty µl of Dual-Glo® Stop & Glo® reagent (Promega, USA) was added into the tube and mixed well followed by incubation at room temperature for 10 min in the dark before *Renilla* luminescence was

measured with GLOMAX®-Multi Jr (Promega, USA) and recorded. The ratio of firefly luminescence to *Renilla* luminescence for each sample was then calculated.

3.9 Protein extraction and quantification

3.9.1 Protein extraction

Total protein from cells was extracted using Radioimmunoprecipitation assay (RIPA) Buffer (Thermo Fisher Scientific, USA). Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, USA) was added to RIPA buffer prior to use to prevent proteolysis and maintain phosphorylation status of proteins. The cells were harvested as described in Section 3.2.2. The cell pellet was re-suspended in 1 ml of 1× cold PBS and transferred into a 1.5 ml microcentrifuge tube and centrifuged at 1,000× g for 5 min. Supernatant was decanted and cells were washed with 1× cold PBS and centrifuged at 1,000× g for 5 min. Supernatant was decanted and 50 µl of RIPA buffer was added to lyse the cells. The sample was then incubated on ice for 15 min and vortexed every 5 min followed by centrifugation at 14,000× *g* for 15 min at 4°C. The supernatant was then transferred into a new labelled 1.5 ml microcentrifuge tube and samples were stored at -20°C for further analysis.

3.9.2 Protein quantification

The concentration of total protein extracted was determined using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA). A working reagent for all samples was prepared by mixing reagent A to reagent B in a ratio of 50:1. Pre-diluted BSA protein assay standards (25 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml, 750 µg/ml, 1000 µg/ml, 1500 µg/ml and 2000 µg/ml) were used to generate a standard curve to determine the protein concentration from unknown samples. Sample was prepared by mixing 2.5 µl of total protein sample to 22.5 µl of nuclease-free water while the blank consisted of 25 µl of nuclease-free water. A total of 200 µl working reagent was then

added to 25 µl of assay standard / sample / blank and mixed well by pipetting. The mixture was then incubated at 37°C for 30 min. After a short centrifugation, the samples were measured using NanoDrop spectrophotometer.

3.10 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and western blot

3.10.1 SDS-PAGE and sample preparation

The spacer and short plates with 1 mm thickness were first sandwiched together and placed in the casting frame and casting stand on Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories, USA). The 4% stacking and 12% resolving gels were prepared according to Table 3.12, whereby the APS was added to the mixture just prior to loading. The resolving gel solution was loaded between the plates until a marked line and 0.1% SDS solution was added immediately until the top of the plates to remove air bubbles and even the surface level. The resolving gel was then allowed to set for 30 min. After the resolving gel has polymerized, the 0.1% SDS solution was decanted and excess water was removed using kimwipes. The stacking gel solution was then added to the top of the plates and a 1 mm comb was immediately inserted into the stacking gel solution to create wells for sample loading. The stacking gel was then allowed to set for 30 min. After the stacking gel has polymerized, the sandwich cassette was assembled into gel electrophoresis tank, and filled with 1× TGS solution. Each well was then gently flushed with 1× TGS solution. To prepare the samples for loading, 40 µg of protein sample in 20 µl was mixed with 5 µl of 5× Lane Marker Reducing Sample Buffer (Thermo Fisher Scientific, USA) and heated at 95°C for 5 min. After a short spin, 20 µl of the sample mixture was loaded into the wells and 5 µl of Biotinylated Protein Ladder (Cell Signaling Technology, USA) was also added as molecular weight marker. Electrophoresis was conducted at 100V for 20 min or until the dye has reached the end

of stacking gel using Power Supply-PowerPac (Bio-Rad, USA), followed by 150V for 50 min or until the dye has reached the end of the resolving gel.

Table 3.12: Reagents for gel preparation for SDS-PAGE.

Reagents	**4% Stacking gel (µl)**	**12% Resolving gel (µl)**
40.0% (w/v) Acrylamide (Promega, USA)	500	4500
0.5M Tris HCl (pH6.8)	1260	-
1.5M Tris HCl (pH 8.8)	-	3750
10.0% (w/v) SDS	50	150
Distilled H_2O	3150	6520
Tetramethylethylenediamine (TEMED) (Acros, USA)	5	7.5
10.0% (w/v) ammonium persulphate	25	75
Bromophenol Blue (Fisher Scientific, USA)	10	-
Total Volume	**5000**	**15000**

3.10.2 Membrane transfer

The Extra Thick Blot Paper (Bio-Rad Laboratories, USA) and 2 µM nitrocellulose membrane (Bio-Rad Laboratories, USA) were used for transferring. After the completion of SDS-PAGE, the sandwich cassette was removed from the electrophoresis tank and glass plates were separated carefully. The top part stacking gel was removed and discarded. The resolving gel, extra thick blot paper and nitrocellulose membrane were then soaked in 1× TGS buffer with 20% (v/v) methanol for 10 min. The transfer sandwich was then assembled in the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories, USA) from cathode to anode in the following order: cathode, extra thick blot paper, resolving gel, nitrocellulose membrane, extra thick blot paper and anode. The air bubbles from the transfer sandwich were gently removed using a roller and membrane transfer was performed at 50mA, 25V for 90 min with MP-2AP Power Supply (Major Science, Taiwan). After membrane transfer was completed, the membrane was stained with Ponceau S (Sigma, USA) to check for the transfer

efficiency. Upon confirmation of the presence of protein bands on the membrane, the membrane was washed twice with distilled water with slight agitation for 5 min each.

3.10.3 Western blot

The membrane was blocked with 5.0% (w/v) non-fat dry milk powder (Merck, Germany) in 1× TBST and incubated for 60 min at room temperature with agitation to prevent non-specific binding of the antibodies to the membrane. After that, the membrane was washed 3 times with 1× TBST for 5 min at room temperature with agitation.

Next, the membrane was soaked in primary antibodies diluted with 5% (w/v) bovine serum albumin (Amresco, USA) in 1×TBS and incubated for 30 min at room temperature with agitation followed by incubation at 4°C overnight. The primary antibodies used were: 1:1000 rabbit polyclonal anti-SMAD4 (Cell Signaling Technology, USA), 1:1000 rabbit monoclonal anti-RSU1 (Abcam, USA) and 1:10,000 rabbit monoclonal anti-GAPDH (Cell Signaling Technology, USA). The next day, the membrane was incubated for 60 min at room temperature with agitation. After that, the membrane was washed 3 times with 1× TBST for 5 min at room temperature with agitation.

The membrane was subsequently soaked in anti-rabbit IgG HRP-linked antibody and anti-biotin HRP-linked antibody (Cell Signaling Technology, USA) diluted at 1:1000 in 5% (w/v) bovine serum albumin in 1×TBS, and incubated for 60 min at room temperature with agitation. After that, the membrane was washed 3 times with 1× TBST for 5 min at room temperature with agitation, and then incubated in 1× TBS until visualization of the bands were carried out.

The bands were visualized using WesternBright™ Quantum™ HRP Substrate (Advansta, USA), a high sensitivity substrate that reacts with horseradish peroxidase (HRP) conjugated to the secondary antibodies on the membrane by releasing chemiluminescence signal. This kit contained two solutions: WesternBright™ Quantum consisting luminol enhancer solution and WesternBright™ Peroxide consisting stabilized peroxide solution. The working solution was prepared by mixing equal parts of both solutions and kept in the dark. The membrane was first blotted dry with kimwipes and then incubated in the working solution for 2 min. Following that, the membrane was again blotted dry with kimwipes and the bands were visualized using Fusion FX7 system (Vilber Lourmat, France). Intensities of the bands were then quantified using the ImageJ Analyst software (NIH, USA), and band intensities were normalized to GAPDH, which was used as loading control.

3.11 Statistical analysis

The data presented are representative of the experiments performed in triplicates, and presented as mean values ± standard error mean. Student's *t*-test was used to evaluate the statistical significance of the results, whereby p values < 0.05 was considered as statistically significant.

CHAPTER 4: RESULTS

4.1 Effects of ACA on miR-210 and miR-629 expression in cervical cancer cells

Throughout history, natural compounds have played a prominent role in treating various human ailments, including cancers. The use of natural compounds in cancer treatments is particularly attractive because not only do they exhibited lower toxicity profiles to humans, but they are also able to target multiple different signalling pathways involved in carcinogenesis (Millimouno *et al.*, 2014). Many groups have demonstrated the involvement of miRNAs in cancer initiation and progression by regulating various signalling pathways (Ryan *et al.*, 2010; Nana-Sinkam & Croce, 2014). Since cancer is a multi-stage process caused by gene alterations in more than one signalling pathway (Sugimura *et al.*, 1992), the use of combination therapy involving natural compounds and miRNAs targeting could possibly help to improve therapeutic efficacy due to their pleiotropic effects. In line with this, different studies have reported that plant-derived natural compounds can modulate specific miRNAs expression, and changes in the expression of these miRNAs can subsequently affect their anti-cancer activities (Phuah & Nagoor, 2014). In an earlier study, following treatment with ACA and/or cisplatin in cervical cancer cells for 2 h, miR-629 was found to be up-regulated in cells treated with ACA, while miR-210 was up-regulated in two treatment groups: cells treated with cisplatin alone and cells treated with combination of ACA and cisplatin.

Hence, to investigate the effects of ACA on miR-210 and miR-629 expression at different time points, both CaSki and SiHa cervical cancer cells were treated with ACA for 12 and 24 h and changes in the miRNAs expression were determined using RT-qPCR. Compared to the untreated cells, miR-210 was down-regulated following treatment with ACA in both CaSki (0.55 ± 0.11 fold at 12 h) and SiHa (0.46 ± 0.07 fold

at 12 h and 0.76 ± 0.05 fold at 24 h) cells. Similarly, miR-629 was also down-regulated in CaSki (0.61 ± 0.05 fold at 12 h and 0.77 ± 0.02 fold at 24 h) and SiHa (0.52 ± 0.05 fold at 12 h and 0.76 ± 0.04 fold at 24 h) cells in comparison to untreated cells (Figure 4.1). Notably, the expression levels for both miRNAs increased slightly at 24 h post-treatment when compared to 12 h post-treatment in both CaSki and SiHa cells.

In summary, miR-210 and miR-629 were down-regulated in cervical cancer cells treated with ACA compared to untreated cells. The down-regulation in the miRNAs expression pattern observed here differs with the earlier study, whereby both of these miRNAs were up-regulated following treatment with anti-cancer drugs. These contradictory results are discussed in Section 5.1.

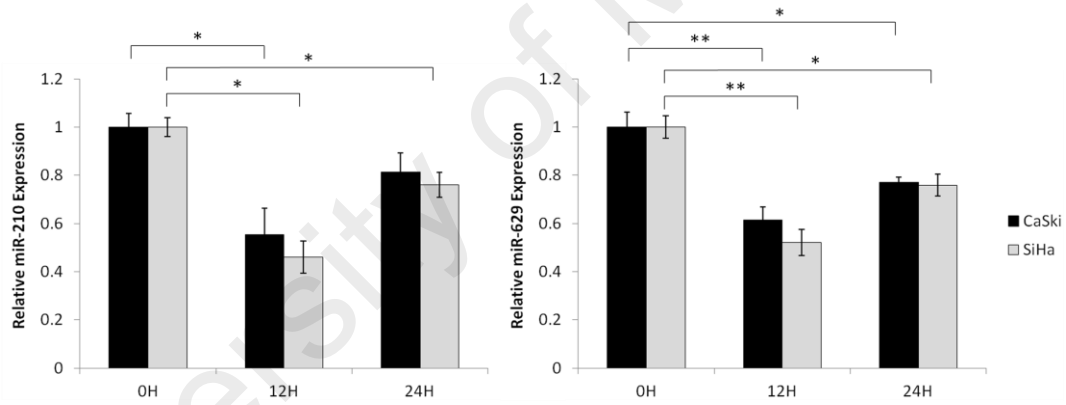

Figure 4.1: ACA down-regulated the expression of miR-210 and miR-629 in cervical cancer cells.

The changes in the miR-210 and miR-629 expression were determined using RT-qPCR following treatment with ACA in CaSki (20 µM) and SiHa (30 µM) cells for 12 and 24 h. The miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method and presented as changes relative to untreated controls after normalization to RNU6B internal control. The data presented are representive of three independent experiments. ** denotes $p<0.01$; * denotes $p<0.05$.

4.2 Confirmation of miRNAs transfection

Given that aberrant miRNA expression is implicated in cancers, normalizing miRNA expression or activity may provide new strategies in cancer therapies. Hence, several methods have been developed to up-regulate or inhibit deregulated miRNA expression. In the current study, functional studies on the role of miR-210 and miR-629 were performed using miRNA mimics and inhibitors to up-regulate and suppress the expression of the miRNAs, respectively. The miRNA mimics are double-stranded RNA oligonucleotides designed to mimic the function of endogenous mature miRNAs, and have been chemically enhanced to preferentially program RNA-induced silencing complex with active miRNA strand to exclude the passenger miRNA strand. The miRNA inhibitors are RNA oligonucleotides designed to bind irreversibly to the complementary mature miRNA, and have been chemical modified with stem-loop structures to enhance potency and extend inhibitory activity. To ensure that the effects observed is specific, both miRNA mimic and inhibitor negative controls were included in each experiments, and results obtained were subsequently normalized or compared to its respective negative controls. These negative controls were designed based on the mature sequence of cel-miR-67 found in *C. elegans*, and have minimal sequence identity to miRNAs found in humans (Sharma *et al.*, 2009).

In this study, the cervical cancer cells were transiently transfected with miRNA mimics or inhibitors using the lipofection method with transfection reagent. Lipofection (also known as liposome transfection) involves the use of cationic (positively charged) lipid to form an aggregate with the anionic (negatively charged) nucleic acids (Felgner *et al.*, 1994). The nucleic acids are then introduced into the cells using liposomes, vesicles that can easily merge with the cell membrane as both are made up of phospholipid bilayer (Felgner *et al.*, 1987). Studies have demonstrated that this method is fast, easy, has low toxicity to the cells and able to transfect a wide range of cell types

with high efficiency (Salimzadeh *et al.*, 2013). Since the nucleic acids are not integrated into the genome using this method, the nucleic acids are only transiently expressed and will be diluted through cell division or degradation, although its expression are generally stable up to 72 h post transfection (Kim & Eberwine, 2010).

4.2.1 Determination of transfection efficiencies

Transfection efficiencies were first determined using miRNA mimic and inhibitor transfection controls, before miRNA functional assays were carried out. These transfection controls were designed based on the mature sequence of cel-miR-67 found in *C. elegans*, and labeled with Dy547 dye for monitoring delivery into the cells. Both CaSki and SiHa cells were transfected with 100 nM of miRNA mimic or inhibitor transfection control with Dy547 for 24 h.Visualization in the uptake of Dy547-labeled miRNA mimic and inhibitor transfection controls using fluorescence microscopy revealed transfection efficiencies of more than 90% for miRNA mimic and inhibitor transfection control in both cell lines, above the recommended 80% transfection efficiency. Transfection efficiencies of 98.90 ± 0.64% and 93.21 ± 0.33% were observed in CaSki cells transfected with miRNA mimic and inhibitor transfection control, respectively (Figure 4.2). On the other hand, transfection efficiencies of 98.25 ± 0.65% and 94.25 ± 0.30% were observed in SiHa cells transfected with miRNA mimic and inhibitor transfection control, respectively (Figure 4.3).

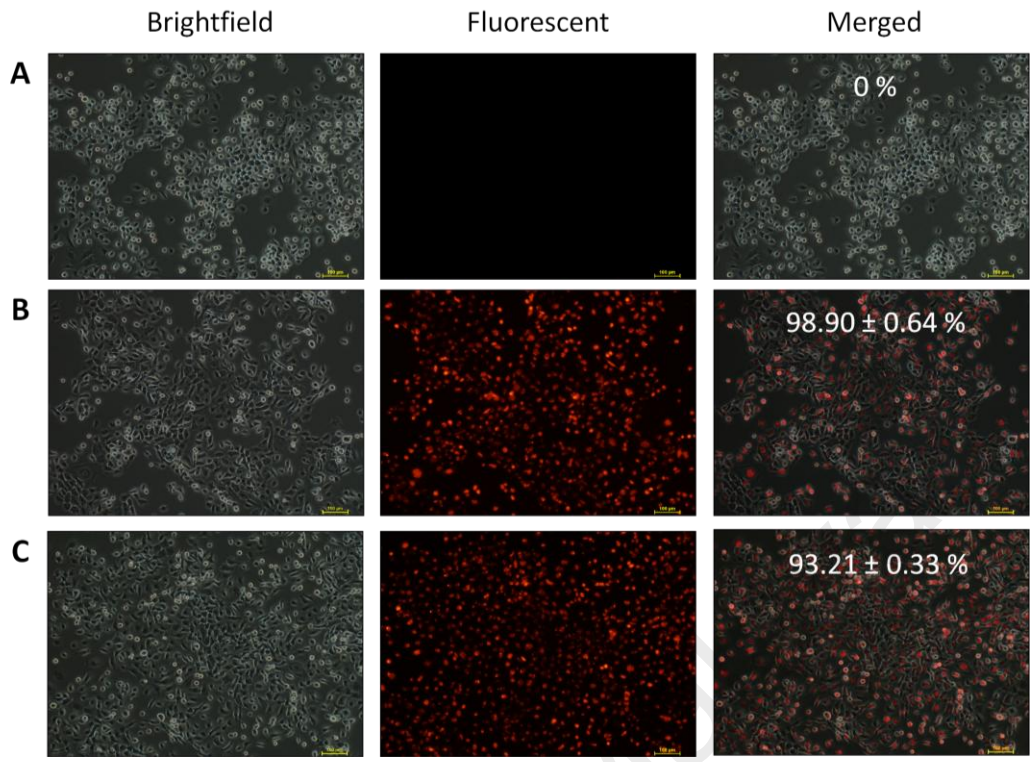

Figure 4.2: Representative photomicrographs of brightfield, fluorescent and merged images of CaSki cells.

(A) Non-transfected cells. (B) Cells transfected with miRNA mimic transfection control with Dy547. (C) Cells transfected with miRNA inhibitor transfection control with Dy547. CaSki cells were transfected with 100 nM of miRNA mimic or inhibitor transfection control with Dy547 for 24 h. The uptake of Dy547-labeled miRNA mimic and inhibitor transfection controls from four random fields was then visualized using fluorescence microscopy and analyzed. The data presented are representive of two independent experiments.

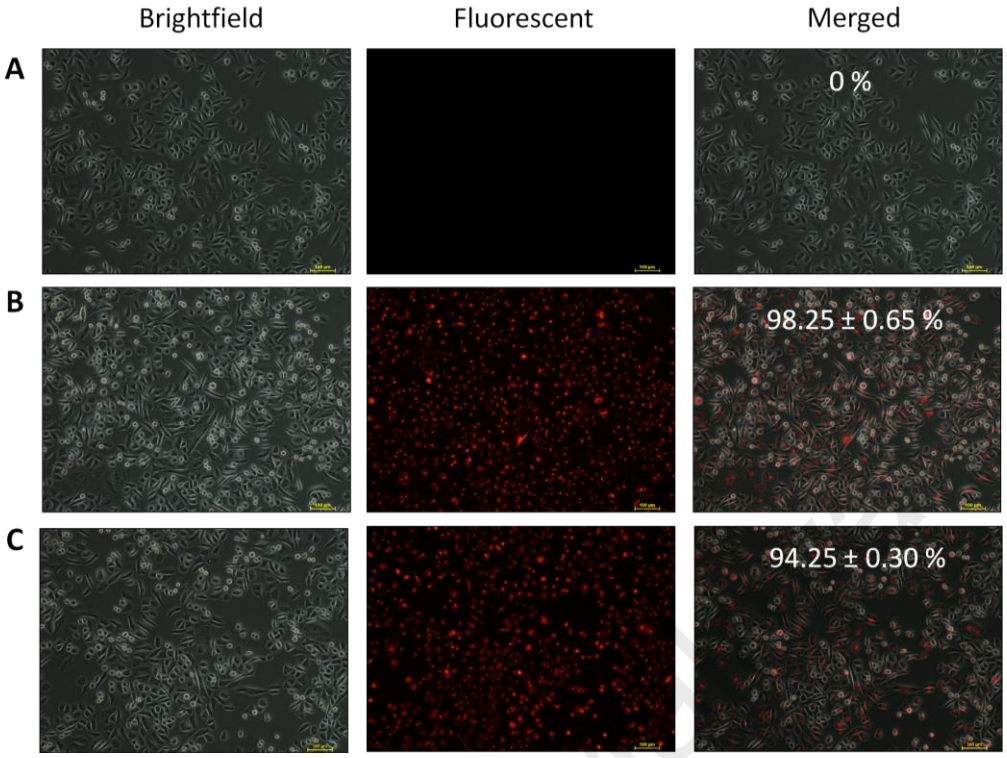

Figure 4.3: Representative photomicrographs of brightfield, fluorescent and merged images of SiHa cells.

(A) Non-transfected cells. (B) Cells transfected with miRNA mimic transfection control with Dy547. (C) Cells transfected with miRNA inhibitor transfection control with Dy547. SiHa cells were transfected with 100 nM of miRNA mimic or inhibitor transfection control with Dy547 for 24 h. The uptake of Dy547-labeled miRNA mimic and inhibitor transfection controls from four random fields was then visualized using fluorescence microscopy and analyzed. The data presented are representive of two independent experiments.

4.2.2 Validation by RT-qPCR

To confirm that the miR-210 and miR-629 expression were successfully altered in transfected cells, changes in the expression level of miR-210 and miR-629 in both cervical cancer cells were determined using RT-qPCR following transfection with 100 nM of miR-210 or miR-629 mimic, inhibitor or negative controls for 24 h. The results showed that the expression of miR-210 was significantly up-regulated following transfection with miR-210 mimic (9.00 ± 0.66 fold in CaSki and 11.26 ± 0.88 fold in SiHa), and significantly down-regulated following transfection with miR-210 inhibitor (0.28 ± 0.07 fold in CaSki and 0.11 ± 0.02 fold in SiHa) (Figure 4.4). Likewise, a

significant up-regulation in miR-629 expression were found in CaSki (17.85 ± 0.31 fold) and SiHa (14.60 ± 0.37 fold) cells following transfection with miR-629 mimic, while transfection with miR-629 inhibitor significantly suppressed its expression in both cell lines (0.52 ± 0.10 fold in CaSki and 0.43 ± 0.11 fold in SiHa) (Figure 4.5).

In summary, these findings confirmed that the expression levels of miR-210 and miR-629 were successfully altered in cervical cancer cells, following transfection with miR-210 or miR-629 mimic, inhibitor or negative controls.

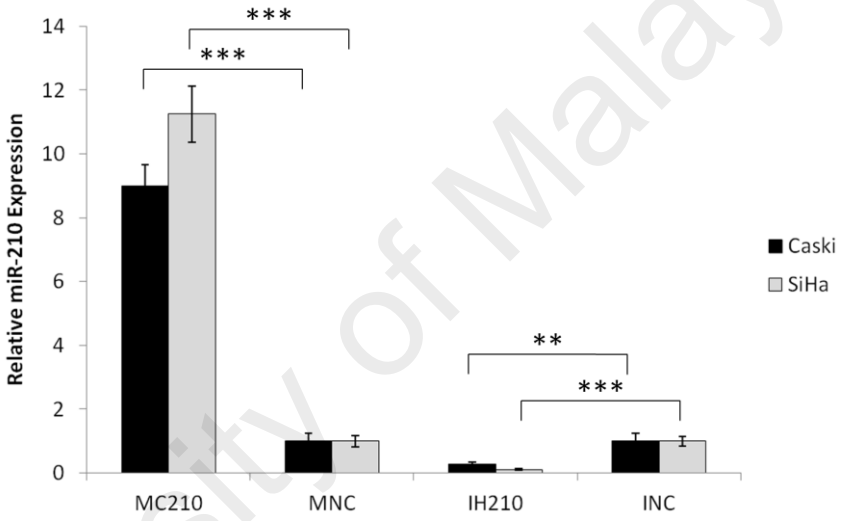

Figure 4.4: Over-expression of miR-210 increased its expression level while inhibition of miR-210 reduced its expression level in cervical cancer cells.

The changes in the miR-210 expression were determined using RT-qPCR following transfection with 100 nM of miR-210 mimic, inhibitor or negative controls for 24 h. The miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method and presented as changes relative to untreated controls after normalization to RNU6B internal control. The data presented are representive of three independent experiments. MC210: cells transfected with miR-210 mimic; MNC: cells transfected with mimic negative control; IH210: cells transfected with miR-210 inhibitor; INC: cells transfected with inhibitor negative control; *** denotes $p<0.001$; ** denotes $p<0.01$.

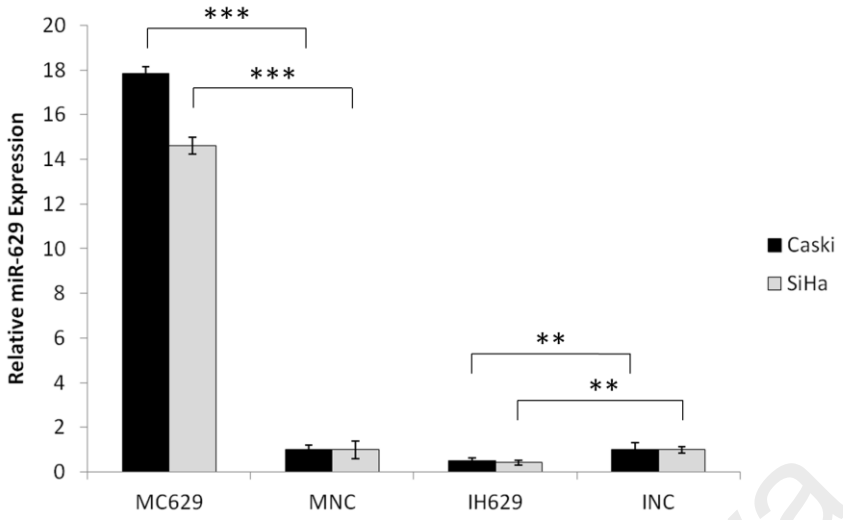

Figure 4.5: Over-expression of miR-629 increased its expression level while inhibition of miR-629 reduced its expression level in cervical cancer cells.

The changes in the miR-629 expression were determined using RT-qPCR following transfection with 100 nM of miR-629 mimic, inhibitor or negative controls for 24 h. The miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method and presented as changes relative to untreated controls after normalization to RNU6B internal control. The data presented are representive of three independent experiments. MC629: cells transfected with miR-629 mimic; MNC: cells transfected with mimic negative control; IH629: cells transfected with miR-629 inhibitor; INC: cells transfected with inhibitor negative control; *** denotes $p<0.001$; ** denotes $p<0.01$.

4.3 Effects of miR-210 and miR-629 expression on sensitivity towards ACA in cervical cancer cells

A large body of evidence has shown that the anti-cancer properties in many natural compounds are due to epigenetic regulation (Wang *et al.*, 2013b). Since it has been previously estimated that miRNAs are able to regulate up to 60% of protein-coding genes in human genome (Friedman *et al.*, 2009), natural compounds are thought to exert their anti-cancer effects through miRNAs regulation. Among these natural compounds, the relationship between curcumin and miRNAs has been widely studied. Curcumin (diferuloylmethane) is a natural compound extracted from the rhizomes of turmeric (*Curcuma longa*). Various groups have demonstrated that curcumin promotes cell cycle arrest and apoptosis as well as inhibits cell proliferation, migration, invasion and

inflammation on different types of cancers, such as breast, prostate, pancreatic, gastric and cervical cancer (Aggarwal & Shishodia, 2006; Gupta *et al.*, 2010). It was previously reported that miR-192-5p expression was up-regulated by curcumin in lung cancer cells. Subsequent investigations showed that over-expression of miR-192-5p enhanced the anti-proliferative and apoptosis-inducing effects of curcumin, which were reversed when this miRNA was inhibited in these cells (Jin *et al.*, 2015). The up-regulation of miR-192-5p expression by curcumin in lung cancer cells was also reported in another study along with miR-215, and inhibition of these miRNAs abrogated the curcumin induced apoptosis (Ye *et al.*, 2015). Besides these, miR-21 was found to be down-regulated in lung cancer cells treated with curcumin and over-expression of miR-21 reversed the anti-proliferative and apoptotic effects of curcumin (Zhang *et al.*, 2014). Given that miR-210 and miR-629 expression were down-regulated by ACA in these cervical cancer cells, it was investigated if changes in the expression of these miRNAs can similarly affect the anti-proliferative and apoptosis-inducing effects of ACA.

To evaluate whether changes in miR-210 and miR-629 expression can affect sensitivity towards ACA in cervical cancer cells, cell proliferation and apoptosis were examined following transfection with miR-210 or miR-629 mimic, inhibitor and negative controls. Cell proliferation was examined using MTT reagent, a colorimetric assay that measures the activity of mitochondrial reductase enzyme in reducing MTT to purple formazan precipitates in living cells (Mosmann, 1983). Two apoptosis assays were used in this study: Annexin V/PI and caspase 3/7 assays. The Annexin V/PI assay detects the externalization of phosphatidylserine, while the caspase 3/7 assay measures caspase-3 and -7 activities, both of which are hallmarks of apoptosis (Elmore, 2007).

4.3.1 Effects of miR-210 and miR-629 expression on cell proliferation in ACA treated cervical cancer cells

The cervical cancer cells were transfected with 100 nM of miR-210 or miR-629 mimic, inhibitor or negative controls for 48 h followed by treatment with varying doses of ACA (0, 5, 10, 15 and 20 µM) for another 48 h. Results from MTT assays showed reduced cell proliferation when miR-210 was suppressed in both CaSki and SiHa cells (Figure 4.6). Similar results were also observed in both cell lines following inhibition of miR-629 expression (Figure 4.7). On the other hand, over-expression of miR-210 or miR-629 did not resulted in any significant effect on cell proliferation in both cells lines. Taken together, these findings indicated that inhibition of miR-210 and miR-629 expression augmented the anti-proliferative effect of ACA in cervical cancer cells.

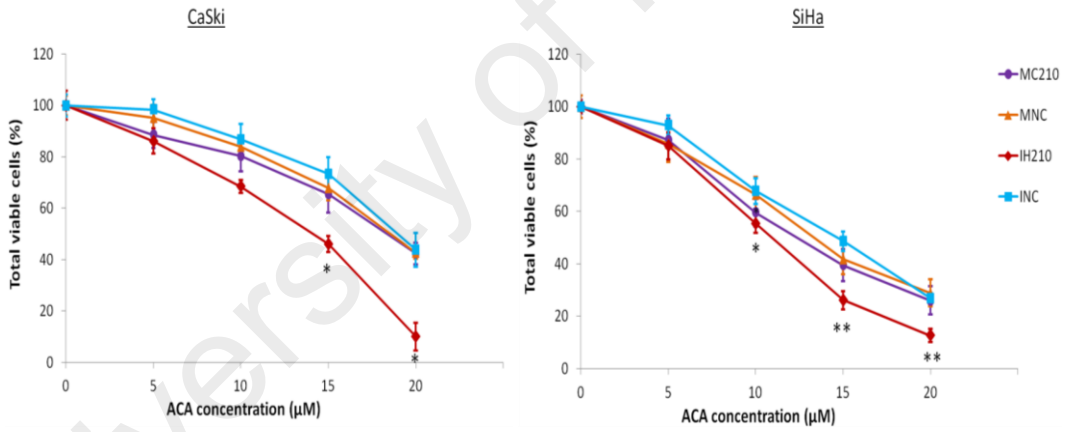

Figure 4.6: Inhibition of miR-210 augmented anti-proliferative effect of ACA in cervical cancer cells.

Cells were transfected with 100 nM of miR-210 mimic, inhibitor or negative controls for 48 h followed by treatment with ACA (0 - 20 µM) for 48 h. The data presented are representive of three independent experiments. MC210: cells transfected with miR-210 mimic; MNC: cells transfected with mimic negative control; IH210: cells transfected with miR-210 inhibitor; INC: cells transfected with inhibitor negative control; ** denotes $p<0.01$; * denotes $p<0.05$.

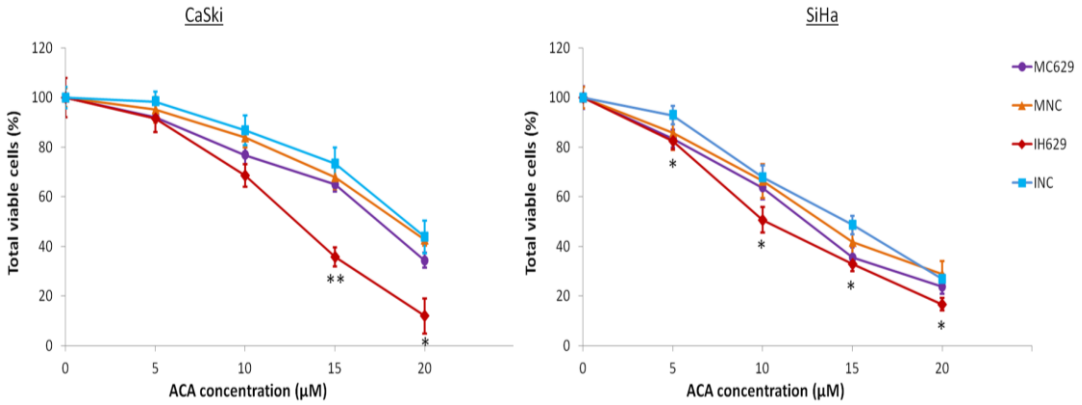

Figure 4.7: Inhibition of miR-629 augmented anti-proliferative effect of ACA in cervical cancer cells.

Cells were transfected with 100 nM of miR-210 mimic, inhibitor or negative controls for 48 h followed by treatment with ACA (0 - 20 μM) for 48 h. The data presented are representive of three independent experiments. MC629: cells transfected with miR-629 mimic; MNC: cells transfected with mimic negative control; IH629: cells transfected with miR-629 inhibitor; INC: cells transfected with inhibitor negative control; ** denotes $p<0.01$; * denotes $p<0.05$.

4.3.2 Effects of miR-210 and miR-629 expression on apoptosis in ACA treated cervical cancer cells

Given that ACA is able to induce apoptosis in different cancer cells (Moffatt *et al.*, 2000; Ito *et al.*, 2004; Awang *et al.*, 2010), it was next investigated if inhibition of miR-210 and miR-629 expression can affect apoptotic induction by ACA in cervical cancer cells. Both cervical cancer cells were transfected with 100 nM of miR-210 or miR-629 mimic, inhibitor or negative controls for 48 h followed by treatment with ACA (20 μM in CaSki cells and 30 μM in SiHa cells) for another 48 h, before being assayed with Annexin V/PI assay. Following treatment with ACA, the percentage of apoptotic cells increased by 1.72 fold and 1.62 fold in CaSki and SiHa cells transfected with miR-210 inhibitor, compared to cells transfected with inhibitor negative control. Results showed that the percentage of apoptotic cells increased by 1.31 fold and 1.47 fold in CaSki and SiHa cells transfected with miR-629 inhibitor compared to cells transfected with inhibitor negative control, upon exposure to ACA. On the other hand, no significant

differences in the percentage of apoptotic cells were observed in both CaSki and SiHa cells treated with ACA, when cells were transfected with miR-210 or miR-629 mimic in comparison to cells transfected with mimic negative control. Notably, transfection with both mimic and inhibitor negative controls did not resulted in significant effects towards ACA compared to non-transfected cells treated with ACA (Figure 4.8).

To confirm apoptotic induction in cervical cancer cells transfected with miR-210 or miR-629 inhibitor and treated with ACA, caspase 3/7 was employed. Both cervical cancer cells were transfected with 100 nM of miR-210 or miR-629 mimic, inhibitor or negative controls for 48 h followed by treatment with ACA (20 µM in CaSki cells and 30 µM in SiHa cells) for another 6 h. The caspase-3 and -7 activities were then measured using caspase 3/7 assay. Compared to the inhibitor negative control, inhibition of miR-210 and miR-629 resulted in higher caspase-3 and -7 activities upon ACA treatment in CaSki and SiHa cells. Inhibition of miR-210 increased caspase-3 and -7 activities by 1.52 fold in CaSki and 1.70 fold in SiHa cells, while inhibition of miR-629 led to an increase of 1.26 fold in CaSki and 1.64 fold fold in SiHa cells. No significant changes were found upon treatment with ACA when miR-210 and miR-629 were up-regulated in these cells, compared to cells transfected with mimic negative control. Also, no significant differences were observed in cells transfected with mimic or inhibitor negative controls compared to non-transfected cells, following treatment with ACA (Figure 4.9).

In summary, the findings shown in this section demonstrated that inhibition of miR-210 and miR-629 expression enhanced the anti-proliferative and apoptosis-inducing effects of ACA in cervical cancer cells.

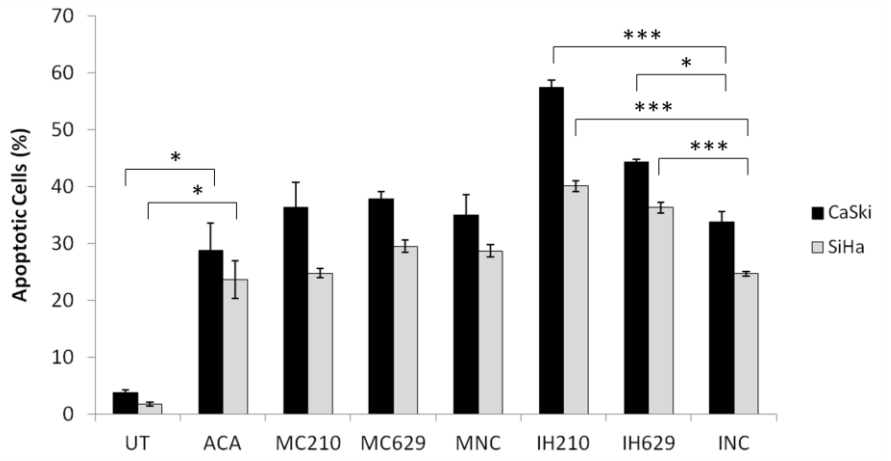

Figure 4.8: Inhibition of miR-210 and miR-629 expression increased percentage of apoptotic cells in cervical cancer cells treated with ACA.

Cells were transfected with 100 nM of miR-210 or miR-629 mimic, inhibitor or negative controls for 48 h followed by ACA treatment (20 µM in CaSki cells and 30 µM in SiHa cells) for another 48 h. The data presented are representative of two independent experiments. MC210: cells transfected with miR-210 mimic; MC629: cells transfected with miR-629 mimic; MNC: cells transfected with mimic negative control; IH210: cells transfected with miR-210 inhibitor; IH629: cells transfected with miR-629 inhibitor; INC: cells transfected with inhibitor negative control; *** denotes $p<0.01$, * denotes $p<0.05$.

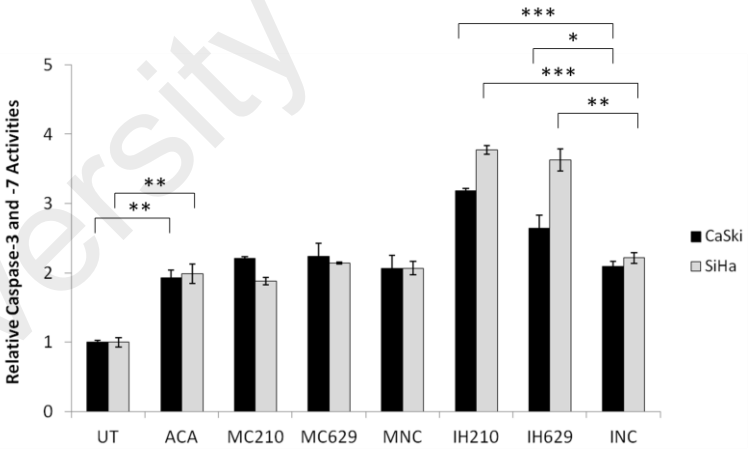

Figure 4.9: Inhibition of miR-210 and miR-629 expression increased caspase-3 and -7 activities in cervical cancer cells treated with ACA.

Cells were transfected with 100 nM of miR-210 or miR-629 mimic, inhibitor or negative controls for 48 h followed by ACA treatment (20 µM in CaSki cells and 30 µM in SiHa cells) for another 6 h. The data are presented as changes relative to untreated controls. The data presented are representative of three independent experiments. MC210: cells transfected with miR-210 mimic; MC629: cells transfected with miR-629 mimic; MNC: cells transfected with mimic negative control; IH210: cells transfected with miR-210 inhibitor; IH629: cells transfected with miR-629 inhibitor; INC: cells transfected with inhibitor negative control; *** denotes $p<0.001$, ** denotes $p<0.01$; * denotes $p<0.05$.

4.4 Effects of miR-210 and miR-629 expression on cell proliferation and apoptosis in cervical cancer cells

Different studies have shown that changes in the expression of single miRNA was able to induce significant effects on biological processes such as cell proliferation, migration, invasion and apoptosis in cancer cells. For example, inhibition of miR-210 expression, which is up-regulated in glioma cells, reduced cell proliferation and induced apoptosis in glioma cells (Shang *et al.*, 2014). On the contrary, miR-210 was found to down-regulated in esophageal squamous cell carcinoma, and over-expression of this miRNA led to reduced cell proliferation and induced cell death and cell cycle arrest in these cells (Tsuchiya *et al.*, 2011). Besides that, suppression of miR-629 inhibited cell migration and invasion in renal cell carcinoma (Jingushi *et al.*, 2015). Hence, it was also of interest to investigate if changes in the miR-210 and miR-629 expression were able to induce any significant effects on cell proliferation and apoptosis in CaSki and SiHa cells.

4.4.1 Effects of miR-210 and miR-629 expression on cell proliferation in cervical cancer cells

To evaluate if miR-210 and miR-629 can induce any significant effects on cell proliferation in cervical cancer cells, cell proliferation assays using MTT reagent were carried out on cells transfected with 100 nM of miR-210 or miR-629 mimic, inhibitor or negative controls for 24 and 48 h. Compared to cells transfected with negative controls, neither over-expression or suppression of miR-210 or miR-629 induced any significant effects on cell proliferation in both CaSki and SiHa cells at 24 h post-transfection. No significant effects on cell proliferation were also observed in both cervical cancer cells following over-expression of miR-210 or miR-629 at 48 h post-transfection, compared to cells transfected with mimic negative control. On the other hand, inhibition of miR-

210 expression reduced cell proliferation in SiHa cells by 31.07% at 48 h post-transfection, compared to cells transfected with inhibitor negative control, while no significant effects were observed in CaSki cells when miR-210 was suppressed (Figure 4.10).

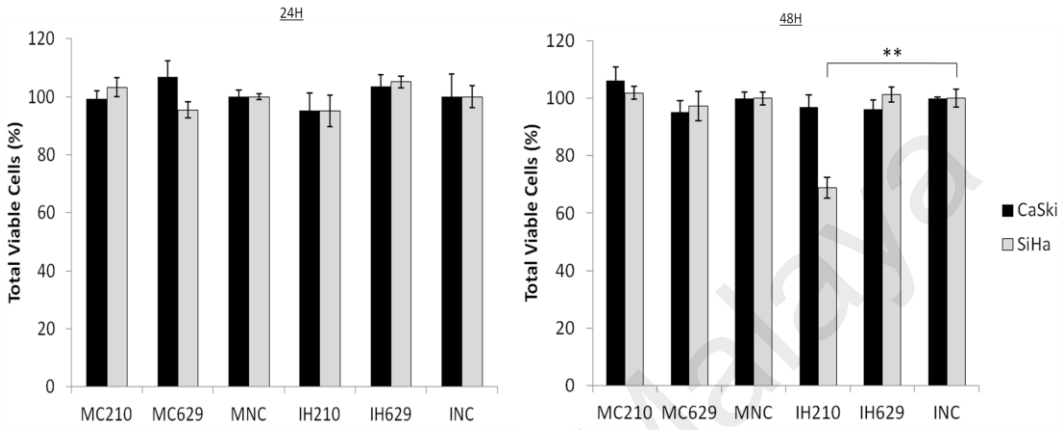

Figure 4.10: Inhibition of miR-210 reduced cell proliferation in SiHa cells, but not in CaSki cells.

Cells were transfected with 100 nM of miR-210 or miR-629 mimic, inhibitor or negative controls for 24 and 48 h. The data presented are representive of three independent experiments. MC210: cells transfected with miR-210 mimic; MC629: cells transfected with miR-629 mimic; MNC: cells transfected with mimic negative control; IH210: cells transfected with miR-210 inhibitor; IH629: cells transfected with miR-629 inhibitor; INC: cells transfected with inhibitor negative control; ** denotes $p<0.01$.

4.4.2 Effects of miR-210 and miR-629 expression on apoptosis in cervical cancer cells

To determine the effects of miR-210 and miR-629 expression on apoptosis in cervical cancer cells, Annexin V/PI assay was performed on cells transfected with 100 nM of miR-210 or miR-629 mimic, inhibitor or negative controls for 48 h. Although no significant differences were found in both CaSki and SiHa cells transfected with miR-210 or miR-629 mimic compared to cells transfected with mimic negative control, inhibition of miR-210 expression resulted in increased percentage of apoptotic cells in

SiHa cells (by 1.65 fold) compared to cells transfected with inhibitor negative control, but not in CaSki cells (Figure 4.11).

To confirm apoptotic induction, the activities of caspase-3 and -7 were measured in cells were transfected with 100 nM of miR-210 or miR-629 mimic, inhibitor or negative controls for 24 h using caspase 3/7 assays. The caspase-3 and -7 activities increased by 45.21% in SiHa cells transfected with miR-210 inhibitor compared to cells transfected with inhibitor negative control. As expected, no significant differences in caspase -3 and -7 activities were observed in CaSki cells transfected with miR-210 inhibitor compared to cells transfected with inhibitor negative control. Compared to cells transfected with mimic negative control, over-expression of miR-210 or miR-629 in both cell lines did not resulted in any significant changes in caspase-3 and -7 activities (Figure 4.12).

In summary, the results from this section demonstrated that inhibition of miR-210 expression reduced cell proliferation and induced apoptosis in SiHa cells, but not in CaSki cells. Changes in the miR-629 expression did not resulted in any significant effects on cell proliferation and apoptosis in both CaSki and SiHa cells. As both CaSki and SiHa cells are of cervical origin, the results for miR-210 were unexpected because it was thought that miRNAs would induce similar effects on similar cancer type. These contradictory results are discussed in Section 5.3.

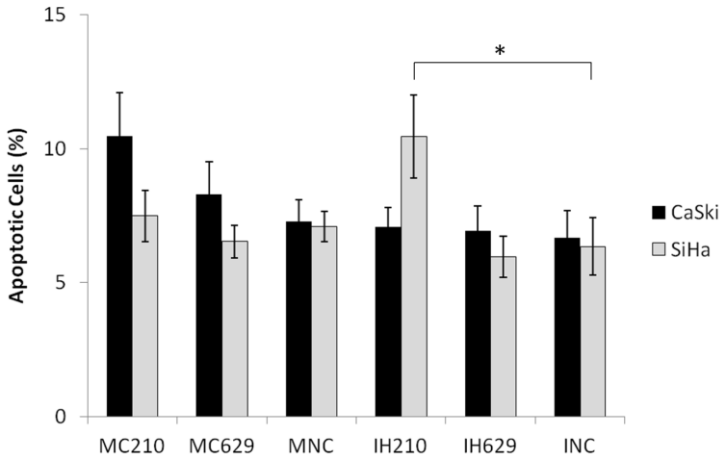

Figure 4.11: Inhibition of miR-210 induced apoptosis in SiHa cells, but not in CaSki cells

Cells were transfected with 100 nM of miR-210 or miR-629 mimic, inhibitor or negative controls for 48 h. The data presented are representative of three independent experiments. MC210: cells transfected with miR-210 mimic; MC629: cells transfected with miR-629 mimic; MNC: cells transfected with mimic negative control; IH210: cells transfected with miR-210 inhibitor; IH629: cells transfected with miR-629 inhibitor; INC: cells transfected with inhibitor negative control; * denotes $p<0.05$.

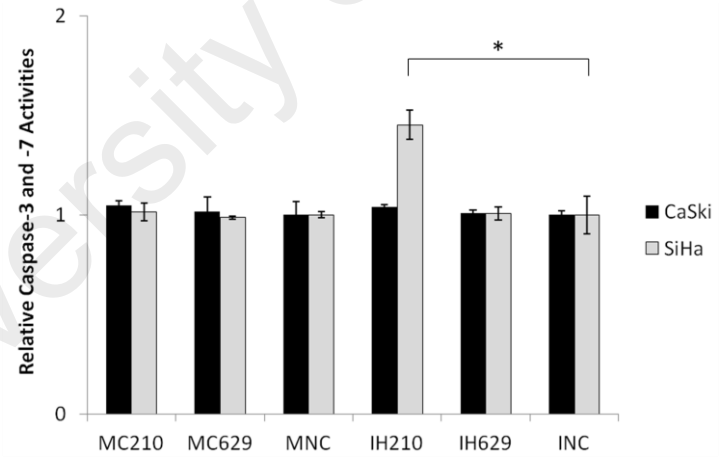

Figure 4.12: Inhibition of miR-210 increased caspase-3 and -7 activities in SiHa cells, but not in CaSki cells.

Cells were transfected with 100 nM of miR-210 or miR-629 mimic, inhibitor or negative controls for 24 h. The data presented are representative of three independent experiments. MC210: cells transfected with miR-210 mimic; MC629: cells transfected with miR-629 mimic; MNC: cells transfected with mimic negative control; IH210: cells transfected with miR-210 inhibitor; IH629: cells transfected with miR-629 inhibitor; INC: cells transfected with inhibitor negative control; * denotes $p<0.05$.

4.5 Identification and confirmation of miR-210 and miR-629 targets

Since each miRNA has multiple mRNA targets and vice versa, this poses a major challenge in identifying accurate miRNA:mRNA interactions. Furthermore, the imperfect complementarity that produces secondary structures in miRNA:mRNA duplexes, which increased the number of possible bindings because of the loops formed from unpaired nucleotides, also added to the complexity in predicting miRNA targets in animals (Rehmsmeier *et al.*, 2004). Over the years, numerous miRNA target prediction tools have been developed to predict the most probable miRNA:mRNA interactions with ease and high degree of accuracy such as TargetScan (Agarwal *et al.*, 2015), PicTar (Krek *et al.*, 2005), RNAHybrid (Kruger & Rehmsmeier, 2006), PITA (Kertesz *et al.*, 2007), miRanda (Betel *et al.*, 2010) and DIANA-microT (Paraskevopoulou *et al.*, 2013). Among these tools, TargetScan is considered the most robust because its prediction has a higher probability of it being biologically validated compared to other tools. Nevertheless, other prediction tools that utilized different algorithms such as miRanda and DIANA micro-T, which are also popular among researchers, should be used to complement results obtained from TargetScan, as they may consider other parameters that may not be considered by TargetScan (Riffo-Campos *et al.*, 2016).

4.5.1 Bioinformatic analyses of miR-210 and miR-629 targets

In this study, the putative targets of miR-210 and miR-629 were identified using TargetScan v7.0 (Agarwal *et al.*, 2015), which generated 814 and 3,726 predicted targets for miR-210 and miR-629, respectively. Since it would be difficult, if not impossible, to analyze and interpret the massive data generated manually, gene annotation enrichment was carried out, whereby the over or under-representation of gene ontology (GO) terms in a set of genes is determined statistically. Given that lower context score indicates higher probability of the predicted targets being biologically validated, list of predicted miRNA targets with context score of less than 0 (710 and

3,194 predicted targets for miR-210 and miR-629, respectively) was subjected to gene annotation enrichment analyses using DAVID v6.7 with default parameters (Huang da *et al.*, 2009).

A total of 35 predicted targets for miR-210 (Table 4.1) and 62 predicted targets for miR-629 (Table 4.2) were obtained following gene annotation enrichment analyses. Among the predicted targets of miR-210, several of them have been confirmed to be directly targeted by miR-210 previously: ACVR1B (Mizuno *et al.*, 2009), E2F3 (Giannakakis *et al.*, 2008; Gou *et al.*, 2012) and IGF2 (Tang *et al.*, 2016). None of the predicted targets for miR-629 has been identified to be directly targeted by miR-629 previously. This could be due to the limited number of studies that examine the roles and functions of miR-629.

Table 4.1: Predicted targets of miR-210.

Official Gene Symbol	Gene Name	Total context score
ACVR1B	activin A receptor, type IB	-0.51
ACVR1C	activin A receptor, type IC	-0.17
ADCY7	adenylate cyclase 7	-0.35
ATP2B3	ATPase, Ca++ transporting, plasma membrane 3	-0.46
BDKRB2	bradykinin receptor B2	-0.21
BMP6	bone morphogenetic protein 6	-0.10
CACNA1A	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	-0.13
CACNA1C	hypothetical protein LOC100131098; calcium channel, voltage-dependent, L type, alpha 1C subunit	-0.15
CCKBR	cholecystokinin B receptor	-0.30
CD38	CD38 molecule	-0.20
CHRM3	cholinergic receptor, muscarinic 3	-0.55
DRD5	dopamine receptor D5	-0.45
E2F3	E2F transcription factor 3	-0.32
GDF7	growth differentiation factor 7	-0.23
GNA15	guanine nucleotide binding protein (G protein), alpha 15 (Gq class)	-0.18
GRIN2A	glutamate receptor, ionotropic, N-methyl D-aspartate 2A	-0.05
HTR5A	5-hydroxytryptamine (serotonin) receptor 5A	-0.23

Table 4.1, continued

Official Gene Symbol	Gene Name	Total context score
IGF2	insulin-like growth factor 2 (somatomedin A); insulin; INS-IGF2 readthrough transcript	-3.03
INHBB	inhibin, beta B	-0.52
INPP5D	inositol polyphosphate-5-phosphatase, 145kDa	-0.28
MAPK1	mitogen-activated protein kinase 1	-0.25
OXTR	oxytocin receptor	-0.24
PDE3A	phosphodiesterase 3A, cGMP-inhibited	-0.21
PDPK1	3-phosphoinositide dependent protein kinase-1	-0.15
PIK3R5	phosphoinositide-3-kinase, regulatory subunit 5	-0.51
PPP1CB	protein phosphatase 1, catalytic subunit, beta isoform; speedy homolog A (Xenopus laevis)	-0.36
PPP1R3B	protein phosphatase 1, regulatory (inhibitor) subunit 3B	-0.26
PRKCA	protein kinase C, alpha	-0.23
RHOQ	ras homolog gene family, member Q; similar to small GTP binding protein TC10	-0.31
SLC8A1	solute carrier family 8 (sodium/calcium exchanger), member 1	-0.03
SMAD4	SMAD family member 4	-0.14
SORBS1	sorbin and SH3 domain containing 1	-0.17
STAT1	signal transducer and activator of transcription 1, 91kDa	-0.24
THBS2	thrombospondin 2	-0.07
ZFYVE9	zinc finger, FYVE domain containing 9	-0.14

Predicted miRNA targets with context score of <0 by TargetScan v7.0 were subjected to gene annotation enrichment analysis using DAVID v.67 with default parameters.

Table 4.2: Predicted targets of miR-629.

Official Gene Symbol	Gene Name	Total context score
AKT3	v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)	-0.36
APPL1	adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1	-0.18
BCL2	B-cell CLL/lymphoma 2	-0.13
BIK	BCL2-interacting killer (apoptosis-inducing)	-0.30
BMP6	bone morphogenetic protein 6	-0.22
BMP8A	bone morphogenetic protein 8a	-0.17
BMP8B	bone morphogenetic protein 8b	-0.10
CAMK2A	calcium/calmodulin-dependent protein kinase II alpha	-0.08
CASP8	caspase 8, apoptosis-related cysteine peptidase	-0.20

Table 4.2, continued

Official Gene Symbol	Gene Name	Total context score
CDKN2B	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	-0.22
CHP2	calcineurin B homologous protein 2	-0.18
COL4A1	collagen, type IV, alpha 1	-0.12
COL4A4	collagen, type IV, alpha 4	-0.20
CRK	v-crk sarcoma virus CT10 oncogene homolog (avian)	-0.09
CSNK1A1	casein kinase 1, alpha 1	-0.40
CSNK1E	casein kinase 1, epsilon	-0.17
CSNK1G1	casein kinase 1, gamma 1	-0.54
CTBP2	C-terminal binding protein 2	-0.17
EGF	epidermal growth factor (beta-urogastrone)	-0.14
FGF1	fibroblast growth factor 1 (acidic)	-0.96
FGF5	fibroblast growth factor 5	-0.28
FGF10	fibroblast growth factor 10	-0.35
FOXO1	forkhead box O1	-0.08
FZD3	frizzled homolog 3 (Drosophila)	-0.26
FZD6	frizzled homolog 6 (Drosophila)	-0.17
FZD9	frizzled homolog 9 (Drosophila)	-0.16
GAS1	growth arrest-specific 1	-0.16
GRB2	growth factor receptor-bound protein 2	-0.45
LEF1	lymphoid enhancer-binding factor 1	-0.27
LRP6	low density lipoprotein receptor-related protein 6	-0.55
MAPK10	mitogen-activated protein kinase 10	-0.37
MET	met proto-oncogene (hepatocyte growth factor receptor)	-0.12
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	-0.30
NFATC2	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2	-0.21
PIAS3	protein inhibitor of activated STAT, 3	-0.13
PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	-0.14
PLCB1	phospholipase C, beta 1 (phosphoinositide-specific)	-0.10
PLCB4	phospholipase C, beta 4	-0.17
PPP2R5B	protein phosphatase 2, regulatory subunit B', beta isoform	-0.23
PPP3CB	protein phosphatase 3 (formerly 2B), catalytic subunit, beta isoform	-0.09
PTK2	PTK2 protein tyrosine kinase 2	-0.10
RAB23	RAB23, member RAS oncogene family	-0.40
RAC2	ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	-0.24
RALBP1	hypothetical LOC100129773; ralA binding protein 1	-0.16
RASSF5	Ras association (RalGDS/AF-6) domain family member 5	-0.15

Table 4.2, continued

Official Gene Symbol	Gene Name	Total context score
RSU1	Ras suppressor protein 1	-0.56
RXRB	retinoid X receptor, beta	-0.23
SENP2	SUMO1/sentrin/SMT3 specific peptidase 2	-0.20
SFRP2	secreted frizzled-related protein 2	-0.49
SKP1	S-phase kinase-associated protein 1	-0.21
SKP2	S-phase kinase-associated protein 2 (p45)	-0.14
SMAD3	SMAD family member 3	-0.08
SMO	smoothened homolog (Drosophila)	-0.10
STAT1	signal transducer and activator of transcription 1, 91kDa	-0.17
STK4	serine/threonine kinase 4	-0.16
TCF7	transcription factor 7 (T-cell specific, HMG-box)	-0.17
TCF7L2	transcription factor 7-like 2 (T-cell specific, HMG-box)	-0.23
TGFB2	transforming growth factor, beta 2	-0.26
TRAF1	TNF receptor-associated factor 1	-0.18
TRAF6	TNF receptor-associated factor 6	-0.17
WNT16	wingless-type MMTV integration site family, member 16	-0.27
ZIC2	Zic family member 2 (odd-paired homolog, Drosophila)	-0.19

Predicted miRNA targets with context score of <0 by TargetScan v7.0 were subjected to gene annotation enrichment analysis using DAVID v.67 with default parameters.

4.5.2 Confirmation of miR-210 and miR-629 direct targets

Luciferase reporter assays are commonly used to confirm the direct interaction between miRNA and its predicted target. In this assay, a fragment of the 3'UTR containing the wild-type binding site for miRNA is cloned and inserted into the vector. The vector is then co-transfected into the cells together with miRNA mimic or mimic negative control, whereby reduced luciferase activity indicates the binding of miRNA to the cloned miRNA target sequence (positive interaction) (Doench *et al.*, 2003; Stark *et al.*, 2003). To confirm that the reduction in luciferase activity is mediated by the specific binding of miRNA to its predicted binding site and not due to other secondary effects, vector containing a fragment of the 3'UTR with mutated binding site is often included as control (Sotillo & Thomas-Tikhonenko, 2011).

In this study, a dual-luciferase vector that contains firely luciferase as primary reporter and *Renilla* luciferase as control reporter was used to confirm the interaction between miRNA and its predicted target. Reduced firefly luciferase activity indicated the binding of miRNA to the cloned miRNA target sequence (positive interaction), while *Renilla* luciferase activity was used for normalization. Since it was demonstrated that inhibition of miR-210 and miR-629 expression resulted in enhanced sensitivity towards ACA in cervical cancer cells and miRNAs are negative gene regulators, it was hypothesized that these miRNAs mediate their effects by targeting tumour suppressors.

4.5.2.1 Confirmation of miR-210 direct targets

From the list of predicted targets for miR-210 (Table 4.1), SMAD4 was selected as a potential target of miR-210. Studies have demonstrated that SMAD4 over-expression promoted apoptosis in cervical cancer cells (Lee *et al.*, 2001b) and suppressed cervical cancer xenografts in nude mice (Klein-Scory *et al.*, 2007), suggesting that SMAD4 plays a tumour suppressive role in cervical cancer. SMAD4 together with other SMAD proteins play a key role in mediating TGF-β induced cell cycle arrest and apoptosis through up-regulation of CDKIs such as $p15^{INK4B}$, $p21^{CIP1}$ and $p27^{Kip1}$ (Reynisdottir *et al.*, 1995; Pardali *et al.*, 2000; Ho *et al.*, 2004; Seoane *et al.*, 2004) and pro-apoptotic proteins such as BMF, BIM, BIK and down-regulation of BCL-xL (Ramjaun *et al.*, 2007; Yu *et al.*, 2008; Spender *et al.*, 2009). Incidentally, past studies have reported that ACA up-regulated the expression of $p21^{CIP1}$ (Yaku *et al.*, 2011) and suppressed the expression of anti-apoptotic proteins such as survivin, IAP1, IAP2, XIAP, Bcl-2, Bcl-xL, and FLIP (Ichikawa *et al.*, 2005). Although treatment with ACA did not affect the levels of $p27^{Kip1}$, it caused a decrease in phosphorylated $p27^{Kip1}$ and an increase in unphosphorylated $p27^{Kip1}$ as well as an increase in the nuclear localization of $p27^{Kip1}$ (Unahara *et al.*, 2007).Taken together, these findings suggest a possible link between ACA's anti-cancer activities and SMAD4. Furthermore, the direct interaction between

SMAD4 and miR-210 has not been reported previously. Meanwhile, E2F3 was included in this experiment as a positive control since its direct interaction with miR-210 has been confirmed previously (Giannakakis *et al.*, 2008; Gou *et al.*, 2012).

Luciferase reporter assays were carried out by co-transfecting 40 ng of vector containing cloned miR-210 target sequence (wild-type or mutated site) in E2F3 with 100 nM of miR-210 mimic, inhibitor or negative controls for 48 h in SiHa cells. Results showed a substantial reduction in relative luciferase activity (by 63.44%) by miR-210 mimic compared to mimic negative control when co-transfected with vector containing the wild-type binding site for miR-210. In contrast, no significant changes in relative luciferase activity were observed when the miR-210 binding site was mutated. No significant effects were also observed following co-transfection of wild-type or mutant construct with miR-210 inhibitor or inhibitor negative control (Figure 4.13). These results confirmed E2F3 as a direct target of miR-210, as reported previously.

Next, the interaction between SMAD4 and miR-210 was investigated using luciferase reporter assay. The SMAD4 gene fragment containing wild-type or mutated miR-210 binding site were cloned into the luciferase vector to generate the wild-type and mutated construct. SiHa cells were then co-transfected with 40 ng of wild-type or mutated construct with 100 nM of miR-210 mimic, inhibitor or negative controls for 48 h. Following co-transfection of wild-type construct and miR-210 mimic, relative luciferase activity reduced by 31.17%, compared to cells co-transfected with wild-type construct and mimic negative control. No obvious differences in relative luciferase activity were observed when the miR-210 binding site on SMAD4 was mutated, indicating specificity of the binding. Co-transfection with miR-210 inhibitor and inhibitor negative control also did not resulted in any significant differences in relative

luciferase activity in both wild-type construct and mutated construct (Figure 4.14). Taken together, these results confirmed SMAD4 as a direct target of miR-210.

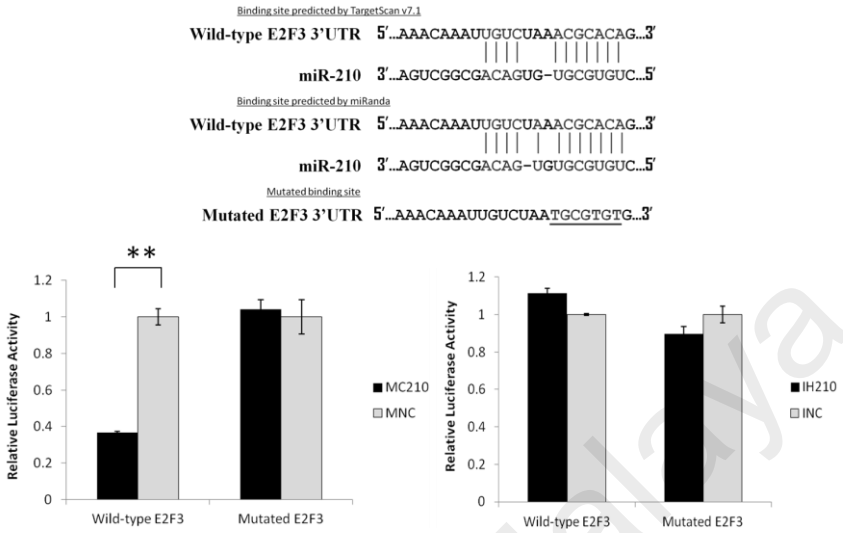

Figure 4.13: E2F3 is a direct target of miR-210.

Wild-type miR-210 binding site in E2F3 as predicted by TargetScan v7.0 and miRanda. Site-directed mutation was carried out on miR-210 binding site in E2F3 as underlined (top). SiHa cells were co-transfected with 40 ng of vector containing wild-type or mutated miR-210 binding site with 100 nM of miR-210 mimic, inhibitor or negative controls at 48 h post-transfection. Data are presented as changes relative to negative controls after normalization to *Renilla* luciferase activity. The data presented are representative of three independent experiments (bottom). MC210: cells transfected with miR-210 mimic; MNC: cells transfected with mimic negative control; IH210: cells transfected with miR-210 inhibitor; INC: cells transfected with inhibitor negative control; ** denotes $p<0.01$.

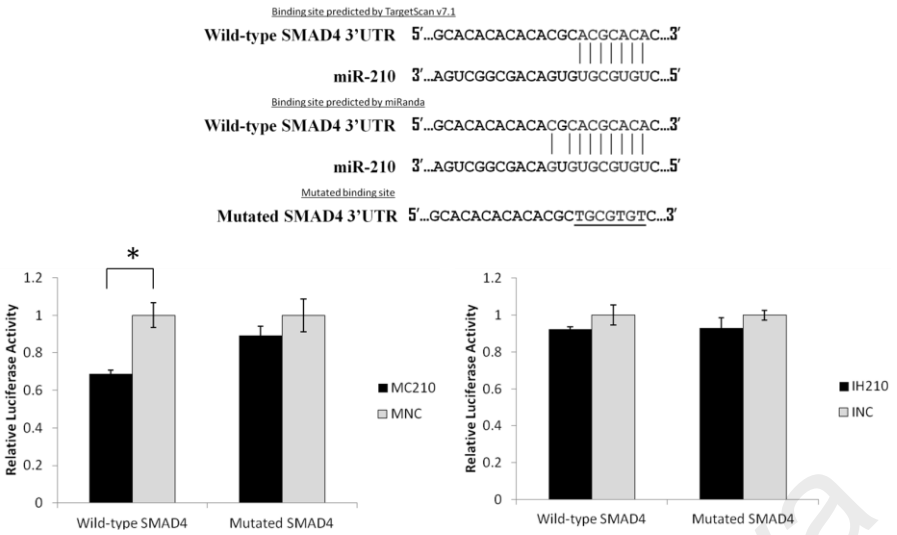

Figure 4.14: SMAD4 is a direct target of miR-210.

Wild-type miR-210 binding site in SMAD4 as predicted by TargetScan v7.0 and miRanda. Site-directed mutation was carried out on miR-210 binding site in SMAD4 as underlined (top). SiHa cells were co-transfected with 40 ng of vector containing wild-type or mutated miR-210 binding site with 100 nM of miR-210 mimic, inhibitor or negative controls for 48 h. Data are presented as changes relative to negative controls after normalization to *Renilla* luciferase activity. The data presented are representative of three independent experiments (bottom). MC210: cells transfected with miR-210 mimic; MNC: cells transfected with mimic negative control; IH210: cells transfected with miR-210 inhibitor; INC: cells transfected with inhibitor negative control; * denotes $p<0.05$.

4.5.2.2 Confirmation of miR-629 direct targets

BIK was chosen as a putative target of miR-629 from the list of predicted targets (Table 4.2). BIK is a pro-apoptotic protein that induces apoptosis through mitochondrial release of cytochrome c and activation of caspases (caspase-3, -7 and -9) (Tong *et al.*, 2001). Further investigations revealed that BIK promotes apoptosis by binding to the pro-survival proteins BCL2 and BCL-xL, allowing BAX to activate the mitochondrial apoptosis pathway (Gillissen *et al.*, 2003). Although the effects of ACA on BIK expression has not been investigated, previous studies have shown that ACA also mediated apoptosis through mitochondrial release of cytochrome c and activation of caspases (Ito *et al.*, 2004; Ito *et al.*, 2005). Furthermore, the direct interaction between BIK and miR-629 has not been reported previously.

To confirm the direct interaction between BIK and miR-629, a fragment of the 3'UTR from BIK containing the wild-type binding site for miR-629 was cloned into luciferase reporter vector. Co-transfection of this wild-type construct (40 ng) with 100 nM of miR-629 mimic, inhibitor and negative controls was then carried out in SiHa cells and changes in the luciferase activity were assayed after 48 h. No significant changes in luciferase activity were observed in cells co-transfected with wild-type construct and miR-629 mimic, suggesting that BIK is not a direct target of miR-629. No significant differences were found following co-transfection of miR-629 inhibitor and the wild-type construct (Figure 4.15). Since BIK was not confirmed as the direct target of miR-629, there was no need to carry out site-directed mutation on the miR-629 binding site in BIK to confirm binding specificity.

The RSU1 was then selected as a putative target for miR-629 from the list of predicted targets (Table 4.2). It was previously demonstrated that ectopic expression of RSU1 inhibited cell growth by inducing the expression of CDKI $p21^{Cip1}$ (Masuelli *et al.*, 1999; Vasaturo *et al.*, 2000). Besides that, apoptosis was also induced by RSU1 by inhibiting pro-survival PINCH-1 and activating pro-apoptotic protein PUMA (Giotopoulou *et al.*, 2015). Loss of PINCH-1 increased the level of pro-apoptotic protein BIM, resulting in activation of mitochondrial apoptosis pathway (Chen *et al.*, 2008). Furthermore, it was also reported that over-expression of RSU1 reduced cell proliferation and migration through inhibition of Rho GTPase, namely RhoA, Rac1 and cdc42 (Donthamsetty *et al.*, 2013). Besides reducing cell proliferation, inducing apoptosis and cell cycle arrest, studies have demonstrated that ACA is able to inhibit angiogenesis, cell migration and invasion too. Notably, activation of Rac1 and cdc42 were suppressed by ACA (Pang *et al.*, 2011). These findings suggest that ACA could mediate its anti-cancer effects through RSU1. Furthermore, the direct interaction between RSU1 and miR-629 has not been reported previously.

To investigate the direct interaction between RSU1 and miR-629, a fragment of the 3'UTR from RSU1 containing the wild-type binding site for miR-629 was cloned into luciferase reporter vector to generate a wild-type construct. Site-directed mutation was carried out at the miR-629 binding site in RSU1 to generate a mutated construct. SiHa cells were co-transfected with 40 ng of wild-type or mutated construct together with 100 nM of miR-629 mimic, inhibitor or negative controls for 48 h. A significant reduction in relative luciferase activity was observed in cells co-transfected of miR-629 mimic and wild-type construct (by 28.54%), indicating that RSU1 is a direct target of miR-629. On the other hand, no significant differences in relative luciferase activity were seen in cells co-transfected with miR-629 mimic and mutated construct, indicating specificity of the miR-629 binding to RSU1. Co-transfection of wild-type or mutated construct with miR-629 inhibitor or inhibitor negative control did not resulted in any significant changes in relative luciferase activity (Figure 4.16).

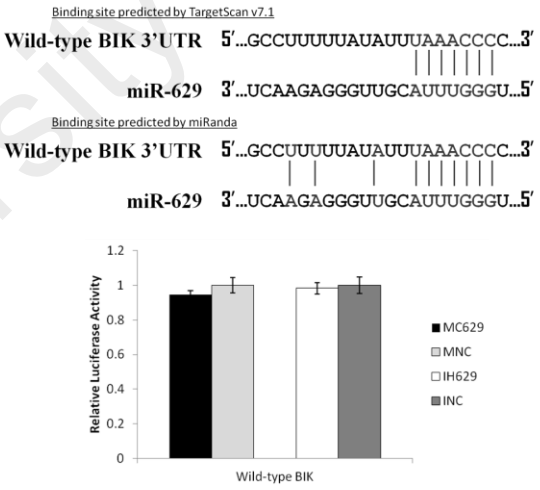

Figure 4.15: BIK is not a direct target of miR-629.

Wild-type miR-629 binding site in BIK as predicted by TargetScan v7.0 and miRanda (top). SiHa cells were co-transfected with 40 ng of vector containing wild-type miR-629 binding site with 100 nM of miR-629 mimic, inhibitor or negative controls at 48 h post-transfection. Data are presented as changes relative to negative controls after normalization to *Renilla* luciferase activity. The data presented are representative of three independent experiments (bottom). MC629: cells transfected with miR-629 mimic; MNC: cells transfected with mimic negative control; IH629: cells transfected with miR-629 inhibitor; INC: cells transfected with inhibitor negative control.

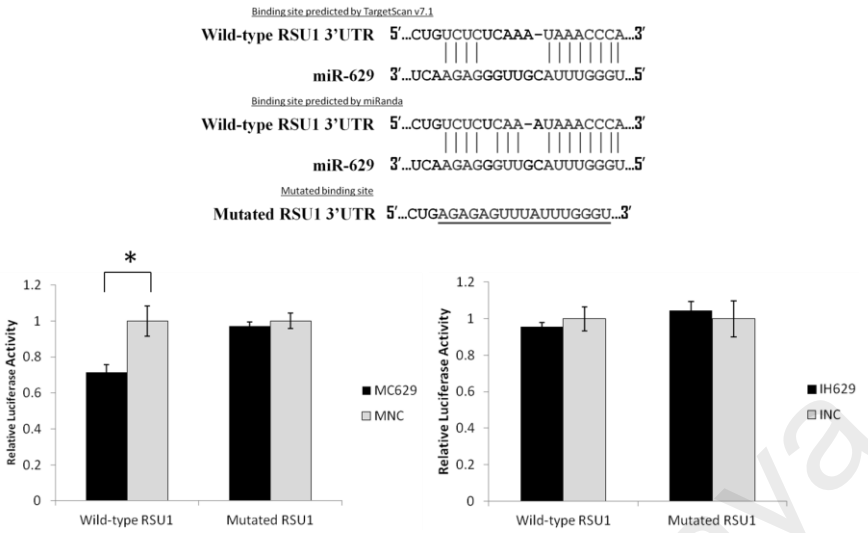

Figure 4.16: RSU1 is a direct target of miR-629.

Wild-type miR-629 binding site in RSU1 as predicted by TargetScan v7.0 and miRanda. Site-directed mutation was carried out on miR-629 binding site in RSU1 as underlined (top). SiHa cells were co-transfected with 40 ng of vector containing wild-type or mutated miR-629 binding site with 100 nM of miR-629 mimic, inhibitor or negative controls at 48 h post-transfection. Data are presented as changes relative to negative controls after normalization to *Renilla* luciferase activity. The data presented are representative of three independent experiments MC629: cells transfected with miR-629 mimic; MNC: cells transfected with mimic negative control; IH629: cells transfected with miR-629 inhibitor; INC: cells transfected with inhibitor negative control; * denotes $p<0.05$.

4.5.3 Effects of ACA and miR-210 or miR-629 on gene target protein level

Since SMAD4 and RSU1 were confirmed as direct targets of miR-210 and miR-629, the effects of ACA on these gene targets were investigated next. Given that miRNAs regulate genes post-transcriptionally, it was hypothesized that over-expression of miR-210 and miR-629 would reduced the protein levels in SMAD4 and RSU1, while inhibition of miR-210 and miR-629 expression would increased the protein levels in SMAD4 and RSU1.

4.5.3.1 Effects of ACA on gene target protein level

To investigate the effects of ACA on SMAD4 and RSU1 protein expression, both CaSki and SiHa cells were treated with 20 µM and 30 µM of ACA for 12 and 24 h. The

changes in the protein levels of SMAD4 and RSU1 at 12 and 24 h compared to untreated cells were then determined using western blots. Compared to the untreated cells, western blot analyses showed higher protein levels of SMAD4 following treatment with ACA for 24 h in both CaSki (increased by 1.54 fold) and SiHa (increased by 1.73 fold) cells (Figure 4.17). Similarly, higher protein levels of RSU1 were also observed at 24 h post-treatment with ACA in both CaSki (increased by 1.52 fold) and SiHa (increased by 1.44 fold) cells, compared to untreated cells (Figure 4.18). In summary, western blot analyses showed that ACA increased the expression of SMAD4 and RSU1 in both CaSki and SiHa cells upon treatment for 24 h.

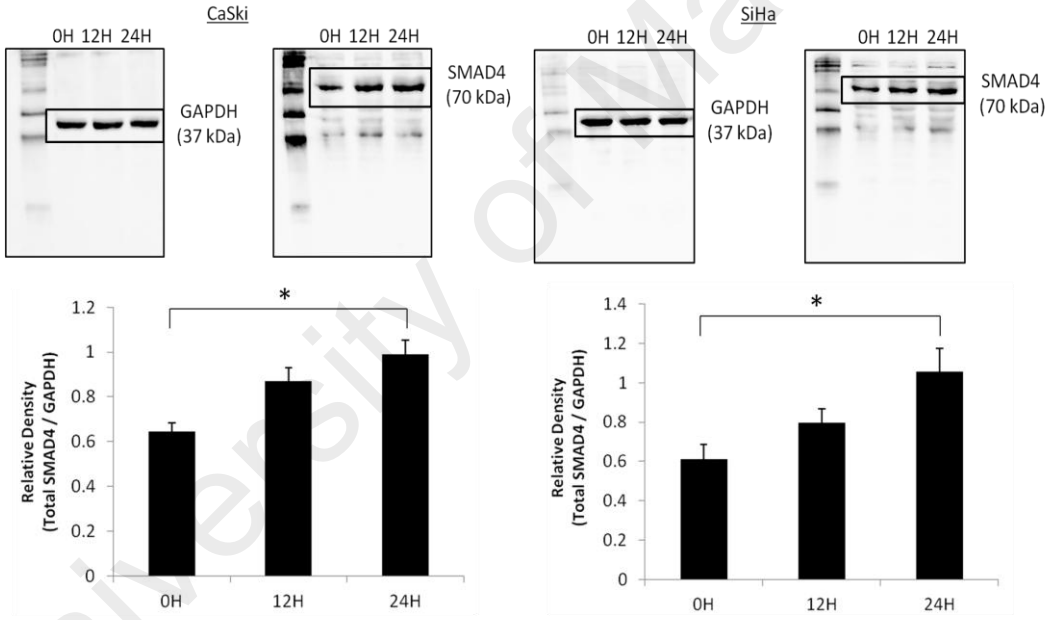

Figure 4.17: ACA increased protein levels of SMAD4 in cervical cancer cells.

Cells were treated with ACA (20 µM in CaSki cells and 30 µM in SiHa cells) for 12 and 24 h. Changes in the protein levels of SMAD4 were then compared to untreated cells using western blots. Representative western blot images of three independent experiments are presented. The densitometric data are expressed as the mean relative density (normalised to GAPDH) ± SEM from three independent experiments. OH: untreated cells; 12H: cells treated with ACA for 12 h; 24H: cells treated with ACA for 24H; * denotes $p<0.05$.

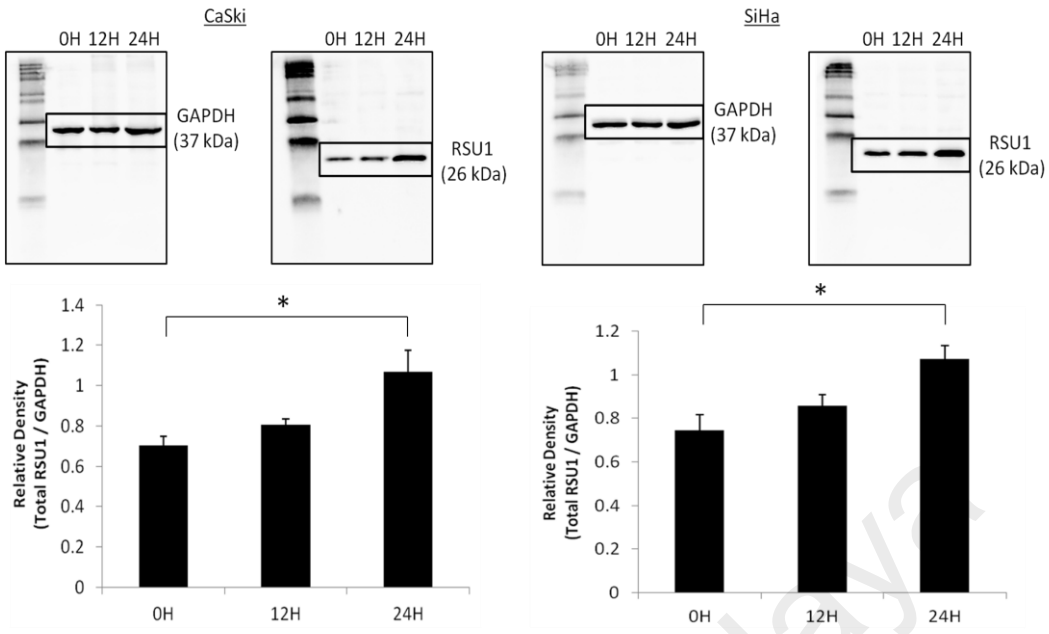

Figure 4.18: ACA increased protein levels of RSU1 in cervical cancer cells.

Cells were treated with ACA (20 µM in CaSki cells and 30 µM in SiHa cells) for 12 and 24 h. Changes in the protein levels of RSU1 were then compared to untreated cells using western blots. Representative western blot images of three independent experiments are presented. The densitometric data are expressed as the mean relative density (normalised to GAPDH) ± SEM from three independent experiments. OH: untreated cells; 12H: cells treated with ACA for 12 h; 24H: cells treated with ACA for 24H; * denotes $p<0.05$.

4.5.3.2 Effects of miR-210 or miR-629 on gene target protein level

To investigate if miR-210 or miR-629 can regulate the expression of SMAD4 or RSU1 at translational level, both CaSki and SiHa cells were transfected with 100 nM of miR-210 or miR-629 mimic, inhibitor or negative controls. Western blot analyses were then carried out on protein lysates at 48 h post-transfection. Results showed that transfection with miR-210 inhibitor increased the protein levels of SMAD4 by 1.45 fold in CaSki cells and 1.39 fold in SiHa cells compared to cells transfected with inhibitor negative control. Unexpectedly, although there were slight reduction in the protein levels of SMAD4 in CaSki and SiHa cells transfected with miR-210 mimic compared to cells transfected with mimic negative control, they were found to be not significant (Figure 4.19). Compared to cells transfected with inhibitor negative control, the protein

levels of RSU1 increased by 1.28 fold in CaSki cells and 1.33 fold in SiHa cells following transfection with miR-629 inhibitor. Surprisingly, transfection with miR-629 mimic also did not resulted in significant changes in protein levels of RSU1, although there were also slight reduction in the protein levels of RSU1 compared to cells transfected with mimic negative control (Figure 4.20). Notably, no significant differences were observed in the protein levels of SMAD4 and RSU1 in CaSki and SiHa cells transfected with mimic or inhibitor negative controls compared to non-transfected cells.

In summary, western blot analyses showed that inhibition of miR-210 or miR-629 expression increased the protein levels of SMAD4 or RSU1 in both cervical cancer cells. However, over-expression of miR-210 or miR-629 did not resulted in significant changes in the protein levels of SMAD4 or RSU1 in both cervical cancer cells. These unexpected results are discussed in Section 5.4.

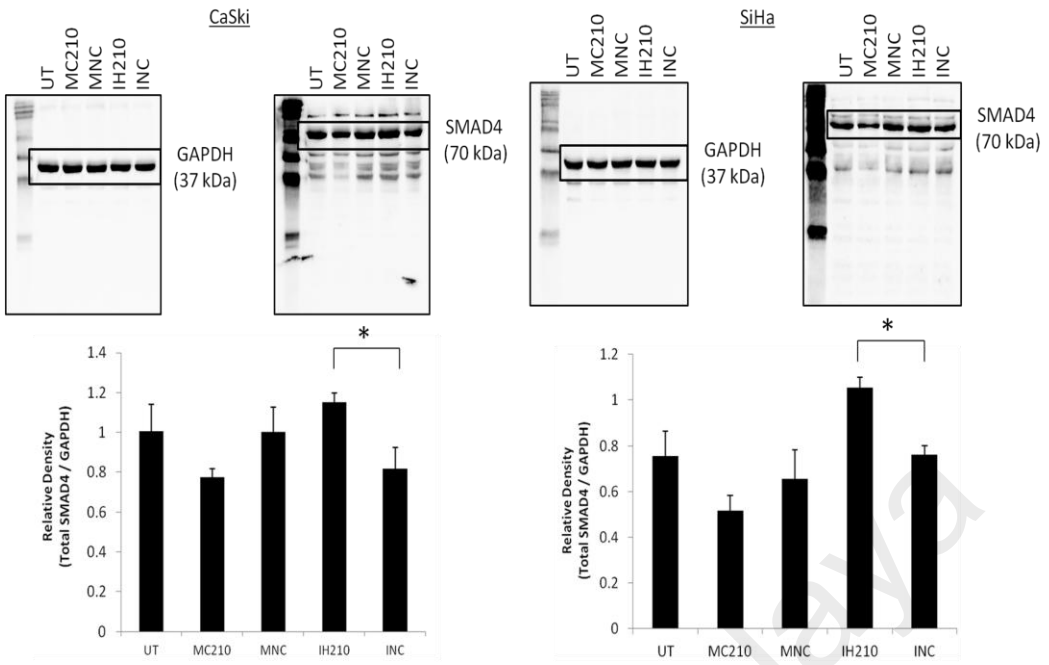

Figure 4.19: Inhibition of miR-210 expression increased the protein levels of SMAD4 in both cervical cancer cells.

Cells were transfected with 100 nM of miR-210 mimic, inhibitor or negative control for 48 h. Representative western blot images of three independent experiments are presented. The densitometric data are expressed as the mean relative density (normalised to GAPDH) ± SEM from three independent experiments. UT: untreated cells; MC210: cells transfected with miR-210 mimic; MNC: cells transfected with mimic negative control; IH210: cells transfected with miR-210 inhibitor; INC: cells transfected with inhibitor negative control; * denotes $p<0.05$.

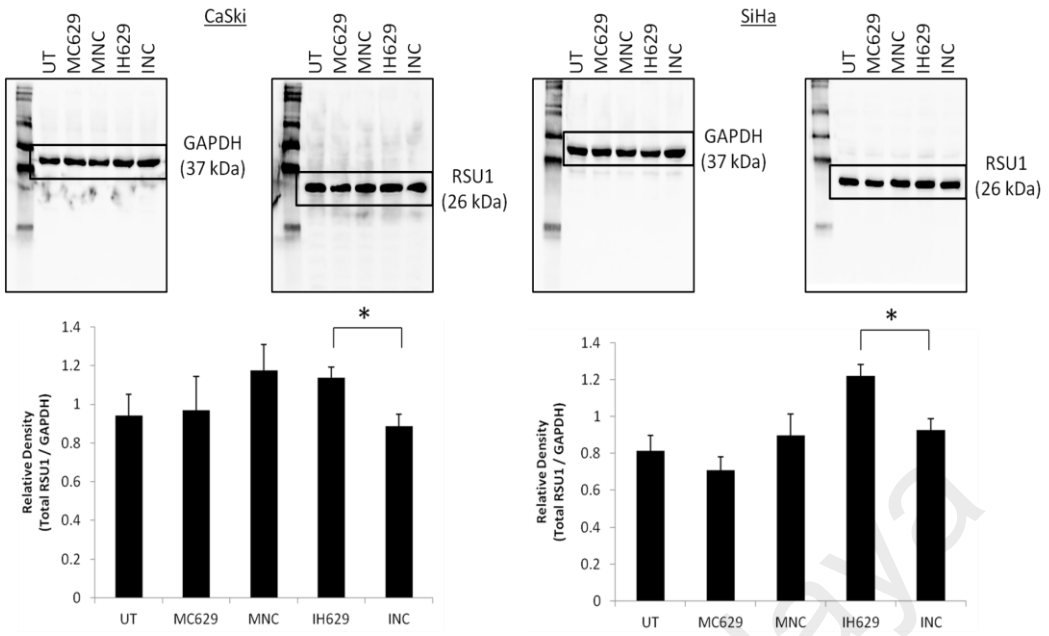

Figure 4.20: Inhibition of miR-629 expression increased the protein levels of RSU1 in both cervical cancer cells.

Cells were transfected with 100 nM of miR-629 mimic, inhibitor or negative controls for 48 h. Representative western blot images of three independent experiments are presented. The densitometric data are expressed as the mean relative density (normalised to GAPDH) ±SEM from three independent experiments. UT: untreated cells; MC629: cells transfected with miR-629 mimic; MNC: cells transfected with mimic negative control; IH629: cells transfected with miR-629 inhibitor; INC: cells transfected with inhibitor negative control; * denotes $p<0.05$.

4.6 Effects of SMAD4 and RSU1 over-expression on sensitivity towards ACA

Chemotherapy is widely used in treating various cancers and has led to significant improvement in the overall response and survival rates in cancer patients. However, its effectiveness is often impeded by drug toxicities and development of drug resistance in cancer cells (Gottesman, 2002; Crafton & Salani, 2016). One of the emerging approaches to address these issues is chemo-gene therapy, which aims to sensitize (or re-sensitize) the cancer cells to the chemotherapeutic drugs through over-expression of tumour suppressors and/or suppression of oncogenes. Various studies have demonstrated the potential of chemo-gene therapy over the years. In one such study, it was demonstrated that over-expression of E2F1 increased sensitivity towards

camptothecin in colorectal cancer cells, resulting in stronger inhibition of cancer cell growth in both *in vitro* and *in vivo* models (Dong *et al.*, 2003). Another group also reported that over-expression of ezrin-radixin-moesin-binding phosphoprotein-50 (EBP50), a tumour suppressor in several cancer types, enhanced 5-fluorouracil-induced anti-proliferative and apoptosis-inducing effects in gastric cancer cells compared to non-transfected cells and cells transfected with empty vector. It was further demonstrated that this was mediated through down-regulation of BCL2, up-regulation of BAX and activation of caspase-3 and -9 (Lv *et al.*, 2012).

Hence, it was also investigated in the current study if over-expression of SMAD4 and RSU1 can affect the anti-proliferative and apoptosis-inducing effects of ACA in cervical cancer cells. To evaluate whether over-expression of SMAD4 and RSU1 can affect sensitivity towards ACA in cervical cancer cells, cell proliferation was examined using MTT reagent while apoptotic induction was determined using Annexin V/PI and caspase 3/7 assays.

4.6.1 Over-expression of SMAD4 and RSU1 in cervical cancer cells

To over-express SMAD4 and RSU1, both CaSki and SiHa cells were transfected with 50 ng of mammalian expression plasmids containing the coding region of SMAD4 or RSU1. The empty plasmid was included as negative control in this study to ensure that the effects observed is specific. Western blot analyses were then carried out at 24 h post-transfection to confirm over-expression of SMAD4 and RSU1 in the cervical cancer cells. Compared to the negative control (empty plasmid), western blot analyses showed increased in the protein levels of SMAD4 and RSU1 in both cervical cancer cells following transfection with the expression plasmids. The protein levels of SMAD4 increased by 1.31 fold in CaSki cells and 1.52 fold in SiHa cells (Figure 4.21). On the other hand, the protein levels of RSU1 increased by 1.34 fold in CaSki cells and 1.60

fold in SiHa cells (Figure 4.22). In summary, higher protein levels of SMAD4 and RSU1 were observed following transfection with mammalian expression plasmids containing the coding region of SMAD4 or RSU1.

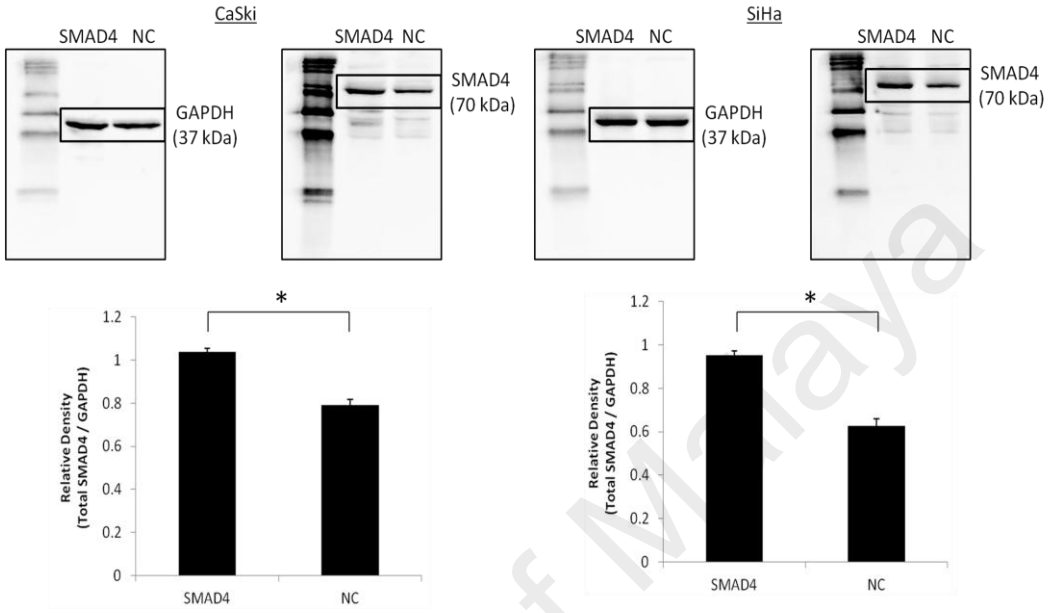

Figure 4.21: Over-expression of SMAD4 increased its protein levels in both cervical cancer cells.

Cells were transfected with 50 ng of plasmid that expressed the coding region of SMAD4 or empty plasmid for 24 h. Representative western blot images of two independent experiments are presented. The densitometric data are expressed as the mean relative density (normalised to GAPDH) ± SEM from two independent experiments. SMAD4: cells transfected with plasmid that expressed the coding region of SMAD4; NC: cells transfected with empty plasmid; * denotes $p<0.05$.

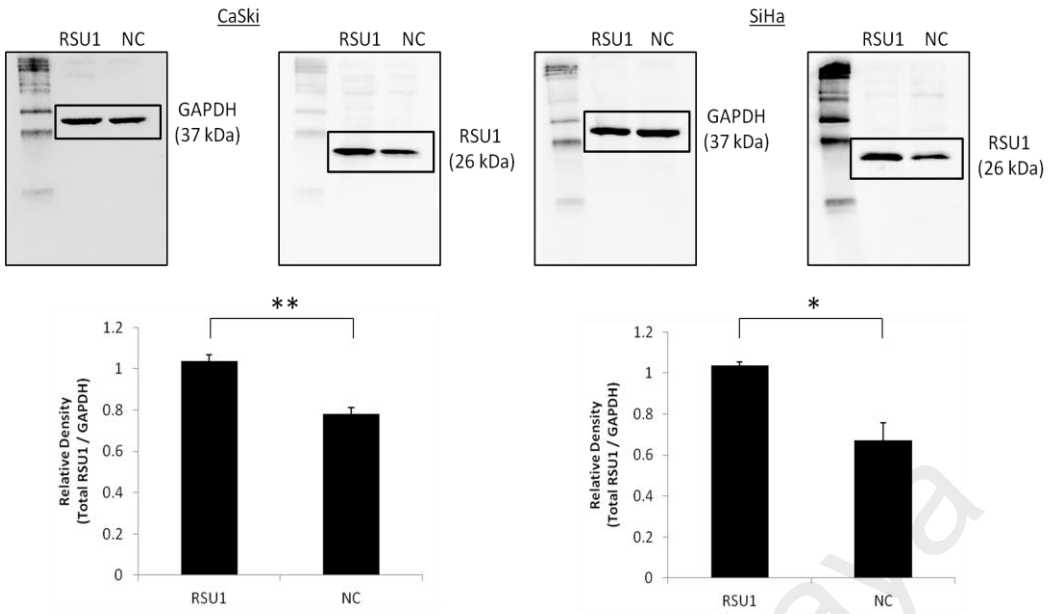

Figure 4.22: Over-expression of RSU1 increased its protein levels in both cervical cancer cells.

Cells were transfected with 50 ng of plasmid that expressed the coding region of RSU1 or empty vector for 24 h. Representative western blot images of three independent experiments are presented. The densitometric data are expressed as the mean relative density (normalised to GAPDH) ±SEM from three independent experiments. RSU1: cells transfected with plasmid that expressed the coding region of RSU1; NC: cells transfected with empty plasmid; ** denotes $p<0.01$; * denotes $p<0.05$.

4.6.2 Effects of SMAD4 and RSU1 over-expression on cell proliferation in ACA treated cervical cancer cells

Both cervical cancer cells were transfected with 5 ng of mammalian expression plasmids containing the coding region of SMAD4 or RSU1 or empty plasmid for 12 h followed by treatment with ACA (10 µM in CaSki cells and 15 µM in SiHa cells) for another 12 h. Results from MTT assays showed a reduction in cell proliferation when SMAD4 and RSU1 were over-expressed in both cervical cancer cells compared to cells transfected with empty plasmid. Compared to negative controls, over-expression of SMAD4 decreased cell proliferation by 44.25% in CaSki cells and 32.79% in SiHa cells while over-expression of RSU1 reduced cell proliferation by 49.55% in CaSki cells and 54.09% in SiHa cells, following treatment with ACA (Figure 4.23).

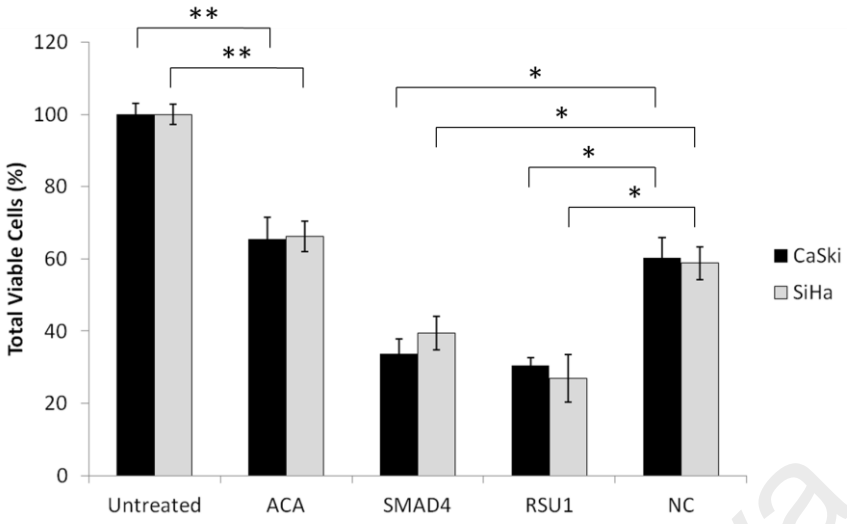

Figure 4.23: Over-expression of SMAD4 and RSU1 reduced cell proliferation in cervical cancer cells treated with ACA.

Cells were transfected with 5 ng of mammalian expression plasmids containing the coding region of SMAD4 or RSU1 or empty plasmid for 12 h followed by treatment with ACA (10 µM in CaSki cells and 15 µM in SiHa cells) for another 12 h. The data presented are representive of three independent experiments. SMAD4: cells transfected with plasmid that expressed the coding region of SMAD4; RSU1: cells transfected with plasmid that expressed the coding region of RSU1; NC: cells transfected with empty plasmid; ** denotes $p<0.01$; * denotes $p<0.05$.

4.6.3 Effects of SMAD4 and RSU1 over-expression on apoptosis in ACA treated cervical cancer cells

Both cervical cancer cells were transfected with 50 ng of mammalian expression plasmids containing the coding region of SMAD4 or RSU1 or empty plasmid for 12 h followed by treatment with ACA (20 µM in CaSki cells and 30 µM in SiHa cells) for another 12 h. The number of apoptotic cells were then determined using Annexin V/PI assay. Upon treatment with ACA, over-expression of SMAD4 increased the number of apoptotic cells by 1.48 fold in CaSki cells and 1.69 fold in SiHa cells while over-expression of RSU1 increased the number of apoptotic cells by 1.63 fold in CaSki cells and 1.96 fold in SiHa cells, compared to cells transfected with empty plasmid (Figure 4.24).

To confirm apoptotic induction, both cervical cancer cells were transfected with 50 ng of mammalian expression plasmids containing the coding region of SMAD4 or RSU1 or empty plasmid for 6 h followed by treatment with ACA (20 µM in CaSki cells and 30 µM in SiHa cells) for another 6 h. The caspase-3 and -7 activities were then measured using caspase 3/7 assay. Over-expression of SMAD4 increased the caspase-3 and -7 activities by 1.28 fold in CaSki cells and 1.55 fold in SiHa cells compared to cells transfected with empty plasmid, when these cells were treated with ACA. Following treatment with ACA, caspase-3 and -7 activities increased by 1.63 fold in CaSki cells and 1.96 fold in SiHa cells when RSU1 were over-expressed compared to cells transfected with empty plasmid (Figure 4.25).

In summary, findings from this section demonstrated that over-expression of SMAD4 and RSU1 enhanced the anti-proliferative and apoptosis-inducing effects of ACA in cervical cancer cells.

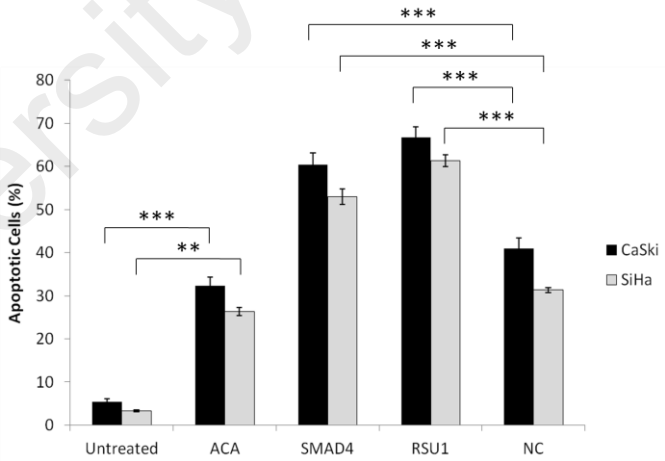

Figure 4.24: Over-expression of SMAD4 and RSU1 increased percentage of apoptotic cells in cervical cancer cells treated with ACA.

Cells were transfected with 50 ng of mammalian expression plasmids containing the coding region of SMAD4 or RSU1 or empty plasmid for 12 h followed by treatment with ACA (20 µM in CaSki cells and 30 µM in SiHa cells) for another 12 h. The data presented are representive of two independent experiments. SMAD4: cells transfected with plasmid that expressed the coding region of SMAD4; RSU1: cells transfected with plasmid that expressed the coding region of RSU1; NC: cells transfected with empty plasmid; *** denotes $p<0.001$; ** denotes $p<0.01$.

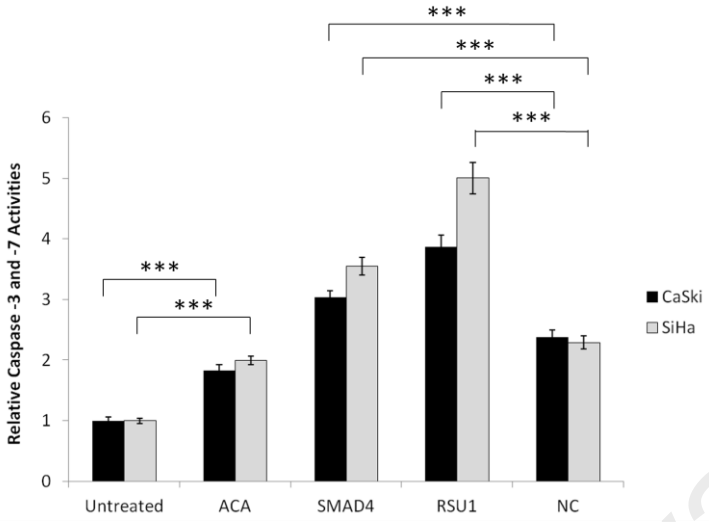

Figure 4.25: Over-expression of SMAD4 and RSU1 augmented caspase-3 and -7 activites in cervical cancer cells treated with ACA.

Cells were transfected with 50 ng of mammalian expression plasmids containing the coding region of SMAD4 or RSU1 or empty plasmid for 6 h followed by treatment with ACA (20 µM in CaSki cells and 30 µM in SiHa cells) for another 6 h. The data presented are representive of three independent experiments. SMAD4: cells transfected with plasmid that expressed the coding region of SMAD4; RSU1: cells transfected with plasmid that expressed the coding region of RSU1; NC: cells transfected with empty plasmid; *** denotes $p<0.001$.

CHAPTER 5: DISCUSSION

5.1 ACA down-regulated miR-210 and miR-629 expression in cervical cancer cells

Natural compounds have been widely used in the treatment of various diseases including cancers for many years. Systemic reviews conducted have also indicated the cost-effectiveness of medicine derived from natural products compared to conventional medicine (Herman *et al.*, 2005; Kennedy *et al.*, 2009). These findings have definitely encouraged the development of natural compounds into anti-cancer drugs. In fact, a study reported in 2016 estimated that majority of the anti-cancer drugs approved by the FDA from 1981 to 2014 were either natural compounds or their derivatives (Newman & Cragg, 2016). The use of natural compounds is promising because of their lower toxicity profiles and ability to target multiple signalling pathways simultaneously (Millimouno *et al.*, 2014; Cragg & Pezzuto, 2016), compared to conventional medicine that is often more specific and targets particular gene or signalling pathway (Li *et al.*, 2010b). Seeing that miRNAs are widely distributed across human tissues in abundance and a single miRNA can regulate the expression of many gene targets, they are one of the largest classes of epigenetic regulators in human cells (Wu *et al.*, 2010; Ludwig *et al.*, 2016). Since studies have shown that natural compounds exert their anti-cancer effects through epigenetic regulation (Wang *et al.*, 2013b), the possible involvement of miRNAs in mediating natural compounds anti-cancer activities have been widely investigated (Phuah & Nagoor, 2014).

Investigations have revealed that many miRNAs were differentially expressed in response to treatment with natural compounds, suggesting that these miRNAs may play a role in regulating response towards natural compounds. For example, miR-15a and miR-16 were found to be up-regulated by curcumin in both leukemia (Gao *et al.*, 2012) and breast cancer cells (Yang *et al.*, 2010), as well as in liver cancer cells treated with

EGCG (Tsang & Kwok, 2010). Besides that, reduced miR-21 expression was observed in colorectal and lung cancer cells treated with curcumin (Mudduluru *et al.*, 2011; Zhang *et al.*, 2014), and in prostate, pancreatic and bladder cancer cells treated with resveratrol (Sheth *et al.*, 2012; Liu *et al.*, 2013a; Zhou *et al.*, 2014a). The expression of miR-27a was also found to be down-regulated following treatment with curcumin in colon cancer cells (Gandhy *et al.*, 2012), and in melanoma, ovarian and pancreatic cancer cells treated with genistein (Sun *et al.*, 2009; Xu *et al.*, 2013; Xia *et al.*, 2014). On the other hand, miR-221 was found to be differentially regulated: down-regulated in prostate cancer cells treated with genistein (Chen *et al.*, 2011), but up-regulated in liver cancer cells treated with EGCG (Arffa *et al.*, 2016).

In the current study, both miR-210 and miR-629 were found to be down-regulated at 12 and 24 h post-treatment with ACA in CaSki and SiHa cells compared to untreated cells, although a slight increase in the expressions of these miRNAs were observed at 24 h post-treatment compared to 12 h post-treatment (Figure 4.1).

The suppression of miR-210 and miR-629 expression by ACA observed in this study is in stark contrast compared to the earlier study (Phuah *et al.*, 2013). One possible reason for the discrepancy in the results could be due to the different treatments used in these two studies. miR-210, which was up-regulated in cervical cancer cells treated with cisplatin alone and in cells treated with cisplatin in combination with ACA in the previous study, was found to be down-regulated in cervical cancer cells treated with ACA alone in the current study. The cytotoxicity of cisplatin is primarily ascribed to its ability to interact covalently with DNA to form DNA-protein and DNA-DNA intrastrand and interstrand crosslinks (Eastman, 1987, 1990). On the other hand, ACA mediate its effects by suppressing the constitutive activation of NF-κB through inhibition of IKKα/β phosphorylation (Ichikawa *et al.*, 2005; In *et al.*, 2012). While

studies on the mechanisms of ACA's anti-cancer activities are still in its infancy compared to cisplatin, the different miR-210 expression pattern observed between the past and current study is likely due to the different mechanisms involved in the anti-cancer activities of cisplatin and ACA.

However, seeing that cervical cancer cells were treated with ACA alone in both studies, this would not explain the different miR-629 expression observed between these two studies. Hence, the most plausible explanation for this observation is the temporal expression pattern of miRNAs in response to treatment with anti-cancer drugs, since the expression of these miRNAs were determined at different time points following treatment. The results from this study, which showed a slight increase in the expression of miR-210 and miR-629 from 12 h to 24 h post-treatment with ACA, further reinforce this notion.

Several studies have demonstrated that miRNAs were differentially expressed in response to treatment concentration and/or duration in the past. In a study carried out by Manavalan and his colleagues, it was reported that miR-10a, miR-21, miR-22, miR-125b, miR-181a, miR-200a, miR-221 and miR-222 exhibited different expression at different time points in breast cancer cells treated with 4-hydroxytamoxifen (4-OHT) for 1, 4, 6, and 8 h. More interestingly, miR-181a that was initially down-regulated at 1 and 4 h post-treatment, was found to be up-regulated at 6 h post-treatment, and no significant changes were observed at 8 h post-treatment. Likewise, both miR-125b and miR-200a were down-regulated at 4 h post-treatment with 4-OHT, but were subsequently up-regulated at 6 h post-treatment (Manavalan *et al.*, 2011). Meanwhile, another study reported that miR-100 was up-regulated in breast cancer cells treated with 0.010 µg/ml, 0.10 µg/ml and 1.00 µg/ml paclitaxel compared to untreated cells. However, the expression of this miRNA fluctuated at different concentrations of

paclitaxel, with the highest fold change observed in cells treated with 0.10 µg/ml paclitaxel (Zhang *et al.*, 2016b). These studies served to show that miRNA expression is not necessarily dose or time dependent, but a dynamic process. For example, a miRNA that is involved in DNA repair mechanisms would be up-regulated when the cells were first treated with anti-cancer drugs as the cells tried to repair the damage induced by the anti-cancer drugs. However, if the damage is too severe (prolonged exposure or at high dosage), the DNA repair mechanisms will be switched off for the cells to undergo cell death. Hence, this miRNA, which was initially up-regulated to promote DNA damage repair, will now be down-regulated to facilitate cell death.

Nevertheless, the inhibition of miR-210 and miR-629 expression following treatment with ACA in this study indicated the possible involvement for these miRNAs in mediating ACA's anti-cancer activities. However, these results (dysregulation in miRNA expression patterns) are of little value at this point and thus, required further investigations. This is because alteration in the miRNAs expression could be a direct response to ACA, which consequently inhibits cell growth (causal), or in response to other changes in the cells following treatment with ACA (resultant). Hence, the effects of miR-210 and miR-629 expression on sensitivity towards ACA in cervical cancer cells were subsequently investigated.

5.2 Inhibition of miR-210 and miR-629 expression conferred sensitivity towards ACA in cervical cancer cells

Being one of the largest classes of gene regulators, studies have shown that miRNAs are involved in regulating diverse biological processes such as cell proliferation, migration, invasion, apoptosis, cell cycle and angiogenesis (Lin & Gregory, 2015; Bracken *et al.*, 2016). Studies have also demonstrated that miRNAs are involved in regulating response towards anti-cancer drugs, including natural compounds. For

example, over-expression of miR-9 and miR-192-5p enhanced the apoptotic effects of curcumin in ovarian (Zhao *et al.*, 2014) and lung cancer cells (Jin *et al.*, 2015). Meanwhile, over-expression of miR-21 abrogated the anti-proliferative and apoptotic effects of curcumin in lung cancer cells (Zhang *et al.*, 2014) and resveratrol in in bladder cancer cells (Sheth *et al.*, 2012). Besides that, inhibition of miR-23a increased ACA-induced apoptosis in head and neck cancer cells, while over-expression of this miRNA attenuated ACA-induced apoptosis (Wang *et al.*, 2013a).

Various studies have reported that both miR-210 and miR-629 are up-regulated in different cancers such as breast, pancreatic, prostate, lung, kidney and cervical cancer (Camps *et al.*, 2008; Greither *et al.*, 2010; Rao *et al.*, 2012; Liu *et al.*, 2015b; Xie *et al.*, 2015). It was previously demonstrated that miR-210 is over-expressed in both HPV type 16 positive CaSki and SiHa cells, compared to normal cervical tissues and HPV negative cervical cancer cells (C-33A) (Martinez *et al.*, 2008). Meanwhile, it was reported that miR-629 is over-expressed in HPV positive cervical cancer cells (SiHa cells and HPV type 18 positive HeLa cells) due to the continuous expression of the E6 and E7 oncoproteins in these cells, indicating that miR-629 could similarly be over-expressed in CaSki cells (Yablonska *et al.*, 2013; Honegger *et al.*, 2015).

Since both miR-210 and miR-629 are over-expressed in cervical cancer cells, indicating possible oncogenic roles for these miRNAs in cervical cancer cells, it was hypothesized that inhibition of these miRNAs may increased sensitivity towards ACA. Accordingly, results from the cell proliferation and apoptosis assays demonstrated that inhibition of miR-210 and miR-629 expression enhanced the anti-proliferative and apoptosis-inducing effects of ACA in cervical cancer cells, although no significant effects were observed when miR-210 and miR-629 were over-expressed (Figures 4.6 to 4.9).

The results observed here could be due to the interaction between these miRNAs and its molecular targets that have been confirmed previously. Among the targets that have been validated for miR-210 previously are SIN3A (Shang *et al.*, 2014), AIFM3 (Yang *et al.*, 2012), CASP8AP2 (Kim *et al.*, 2009) and MNT (Zhang *et al.*, 2009). It was previously reported that SIN3A is involved in inhibiting cell proliferation and inducing apoptosis (McDonel *et al.*, 2009), AIFM3 is involved in inducing apoptosis by mediating the release of cytochrome c from mitochondria (Xie *et al.*, 2005) and CASP8AP2 is involved in the cleavage and activation of caspase-8 to activate apoptosis (Lee *et al.*, 2012). On the other hand, MNT is a transcriptional repressor that functions as c-MYC antagonist, and plays a role in suppressing cell cycle and proliferation (Hurlin *et al.*, 2003). Since miRNAs are negative gene regulators, inhibition of miR-210 would increased the expression of these targets, and subsequently reduced cell proliferation and/or increased apoptosis in these cells, leading to the enhanced anti-proliferative and apoptotic effects observed in cervical cancer cells transfected with miR-210 inhibitor and treated with ACA.

Other than these targets, several other targets of miR-210 have also been identified such as E2F3 (Giannakakis *et al.*, 2008; Gou *et al.*, 2012), which promotes cell proliferation by allowing the cell cycle progression from G1 to S phase and initiation of DNA replication (Leone *et al.*, 1998). However, studies have also demonstrated that deregulated pRB pathway can lead to apoptotic induction by the E2Fs. Expression of the E7 oncoprotein from HPV type 16, which disrupted the pRB/E2F complexes, resulted in transcriptional up-regulation of pro-apoptotic proteins PUMA, NOXA, BIM, and Hrk/DP5 by E2F1 (Hershko & Ginsberg, 2004). In addition, other studies have also shown that E2F3 can also induced apoptosis in response to deregulated pRB pathway, although this was found to be E2F1-dependent (Ziebold *et al.*, 2001; Lazzerini Denchi & Helin, 2005). Notably, although both CaSki and SiHa cells have wild-type pRB, its

activity is greatly reduced by the E7 oncoprotein produced from HPV type 16 (Scheffner *et al.*, 1991), suggesting that apoptosis can also be induced by E2F1 and E2F3 in response to the deregulated pRB pathway. Hence, inhibition of miR-210 expression and subsequent increased E2F3 expression might lead to increased apoptosis, instead of cell proliferation in these cervical cancer cells.

Besides E2F3, HOXA1 was also confirmed as direct target of miR-210 (Huang *et al.*, 2009). It was previously demonstrated that E-cadherin increased the expression of HOXA1 through Rac1 (Zhang *et al.*, 2006), resulting in decreased apoptosis due to transcriptional up-regulation of BCL2 (Zhang *et al.*, 2003). Although inhibition of miR-210 might increase the expression of HOXA1 resulting in reduced apoptosis, this might not be so since ACA has been reported to suppress the expression of Rac1 and BCL2 previously (Ichikawa *et al.*, 2005; Pang *et al.*, 2011).

Furthemore, ACVR1B (Mizuno *et al.*, 2009), IGF (Tang *et al.*, 2016) and FGFLR1 (Tsuchiya *et al.*, 2011) were also confirmed to be directly targeted by miR-210 previously. ACVR1B is a type I receptor, which acts as transducer of activin, and play a role in repressing osteoblastic differentiation (Miyazono *et al.*, 2005). IGF2 is a growth-promoting hormone involved in cell proliferation, cell growth, differentiation, and survival (Nakae *et al.*, 2001). On the other hand, contradictory roles have been proposed for FGFLR1. One study showed that it negatively regulate cell proliferation (Trueb, 2011), while another study demonstrated that it does not affect cell growth or proliferation, but induces cell adhesion (Yang *et al.*, 2016).

Although these targets were all confirmed to be directly targeted by miR-210, care should be taken when interpreting the effects of these targets in mediating ACA's anti-cancer activities. Due to the cell type specific differences that might exist in different cells, inhibition of miR-210 in cervical cancer cells might not necessarily lead to

increased expression of these targets. This was previously demonstrated by a group, whereby over-expression of miR-210 did not affect the expression of CASP8AP2 and MNT, which were confirmed to be directly targeted by miR-210 (Tsuchiya *et al.*, 2011). Furthermore, the functional roles for these targets have not been investigated in cervical cancer cells. In addition, since each miRNA is capable of regulating many other targets, the possible involvement of other targets, which have yet to be validated, could also be involved in response towards ACA.

As for miR-629, only one target have been confirmed thus far, TRIM33, (Jingushi *et al.*, 2015). Different groups have proposed contradictory roles played by TRIM33 in regulating TGF-β signalling pathway previously. It was suggested that TRIM33 is a negative regulator of TGF-β signalling by targeting SMAD4 for ubiquitination and degradation (Dupont *et al.*, 2005). Meanwhile, another group showed that TRIM33 compete with SMAD4 to bind with activated R-SMADs, and the abundance of R-SMADs/TRIM33 and R-SMADs/SMAD4 complexes formed was proportional to the relative abundance of TRIM33 and SMAD4 in the cells. Contradictory to the earlier study, no evidence was found that TRIM33 induced degradation of SMAD4. Instead, it exhibited stronger binding preference for R-SMADs compared to SMAD4. Since the affinity of the binding was carried out in cervical cancer cells, and validated in keratinocytes and colorectal cancer cells in this study, it was suggested that the previous system reported could be unique to TGF-β signalling in *Xenopus* (He *et al.*, 2006). Besides that, another study showed that the R-SMADs/TRIM33 complexes can function as chromatin reader, by making target genes accessible to R-SMADs/SMAD4 complexes in response to nodal stimulation to trigger differentiation of mammalian embryonic stem cells (Xi *et al.*, 2011).

However, it is highly unlikely that the increased anti-proliferative and apoptotic effects seen in cervical cancer cells transfected with miR-629 inhibitor and treated with ACA were due to the effect of TRIM33. Although only one target was confirmed for miR-629 thus far, analyses with miRNA target prediction tools revealed many potential targets of miR-629. Hence, it is more likely that other targets of miR-629 that has yet to be validated mediated the effects observed.

Since inhibition of miR-210 and miR-629 expression enhanced the anti-proliferative and apoptotic effects of ACA in cervical cancer cells, it was highly expected that over-expression of these miRNAs would reversed the sensitivity towards ACA, and reduced the anti-proliferative and apoptotic effects of ACA in these cells. However, no significant changes in cell proliferation and apoptotic induction were observed in cervical cancer cells treated with ACA when miR-210 or miR-629 expression was inhibited. One possible reason could be that both of these miRNAs were already highly expressed in these cervical cancer cells prior to transfection with miR-210 or miR-629 mimic (Martinez *et al.*, 2008; Yablonska *et al.*, 2013; Honegger *et al.*, 2015). Hence, increasing the expression of these miRNAs might not result in significant effects since the targets or pathways affected by these miRNAs might already be activated or repressed by the endogenous miRNAs. Although results from RT-qPCR showed high levels of these two miRNAs following transfection with miRNA mimics (Figures 4.4 and 4.5), the miRNA must be incorporated into RISC for it to be functional. While RT-qPCR is convenient and commonly used to detect miRNA level, it does not distinguish between miRNAs in functional or non-functional pools (Thomson *et al.*, 2013). Furthermore, it was reported that majority of the transfected miRNA mimic localised with or adjacent to lysosomes, similar to reports on lipid-based siRNA transfection (Barreau *et al.*, 2006). Therefore, the high level of miRNA detected by RT-qPCR following transfection with miRNA mimic could be due to their retention within

vesicles and subsequent amplification by RT-qPCR following lysis. Another way of measuring miRNA levels would be the use of a reporter system containing reporter gene such as luciferase or green fluorescent protein and its recognition site. The specific reporter for each miRNA produces low reporter expression when miRNA level is high and vice-versa (Kiriakidou *et al.*, 2005).

5.3 miR-210 inhibition reduced cell viability and induced apoptosis in SiHa cells

Previous studies have reported that alterations in specific miRNA expression alone were sufficient to induce significant effects on biological processes such as cell proliferation or apoptosis. For example, ectopic expression of miR-7, which was induced by curcumin, inhibited cell proliferation, migration and invasion as well as promoted apoptosis in bladder cancer cells (Saini *et al.*, 2011). It was also shown that inhibition of miR-186*, which was down-regulated by curcumin, suppressed proliferation and induced apoptosis in lung cancer cells (Zhang *et al.*, 2010). Meanwhile, over-expression of miR-622 that was up-regulated by resveratrol in lung cancer cells inhibited cell proliferation and colonies formation (Han *et al.*, 2012). Functional analyses on miR-663 and miR-744, which was induced by resveratrol, revealed that up-regulation of these miRNAs suppressed cell proliferation in breast cancer cells (Vislovukh *et al.*, 2013). It was also reported that suppression of miR-27a, which was down-regulated by genistein, inhibited cell growth and migration in ovarian and pancreatic cancer cells (Xu *et al.*, 2013; Xia *et al.*, 2014). Another group demonstrated that cell growth was inhibited and apoptosis was induced following repression of miR-223, which was down-regulated by genistein (Ma *et al.*, 2013).

Hence, it was also investigated in this study if alterations of miR-210 and miR-629 expression, which were down-regulated by ACA, can similarly affect cell proliferation and apoptosis in CaSki and SiHa cells. Results showed that changes in miR-210 and

miR-629 expression did not affect cell proliferation or apoptosis in CaSki cells. On the other hand, inhibition of miR-210 expression reduced cell proliferation and induced apoptosis in SiHa cells. Changes in miR-629 expression did not affect cell proliferation or apoptosis in both CaSki and SiHa cells (Figures 4.10 to 4.12).

While these results appeared to be contradictory compared to other studies discussed earlier, a search in the literature revealed several similar findings. In one such study, it was reported that over-expression of miR-126 by itself did not exhibited any significant effects on cell viability or apoptosis in drug resistant gastric cancer cells. However, cell viability was greatly reduced while apoptosis was significantly induced when these miR-126 over-expressing cells were treated with vincristine and adriamycin, indicating that miR-126 sensitized these cells to the anti-cancer drugs (Wang *et al.*, 2016b). Another study that investigated the effect of miR-192 suppression in *in vivo* models demonstrated that inhibition of miR-192 by itself did not significantly affect tumour volume in lung cancer xenografts. However, tumour volume was greatly reduced in miR-192 inhibited cells when treated with cisplatin and gemcitabine, implying that inhibition of miR-192 expression enhanced sensitivity towards these anti-cancer drugs (Cao *et al.*, 2015). Similarly, another group also reported that inhibition of miR-93 expression did not affect tumour weight and volume in bladder cancer xenografts, but both tumour weight and volume were significantly decreased following treatment with cisplatin when miR-93 was inhibited (Liu *et al.*, 2016a). Meanwhile, another study demonstrated that over-expression of miR-519d reduced cell proliferation and induced apoptosis in A2780 cells, but not in SKOV3 cells, even though they are both ovarian cancer cells. However, ectopic expression of miR-519d significantly decreased cell proliferation and induced apoptosis in both ovarian cancer cells treated with cisplatin (Pang *et al.*, 2014).

These findings indicated that while certain miRNAs might affect sensitivity towards anti-cancer drugs, it may not be able to result in phenotypic changes on its own. Although it was not discussed in these studies as to what could be the possible reasons, this could be due to the context-specific effects that are involved in miRNA mediated regulation, which can be found in the literature. For example, the RNA binding protein ELAVL1 was identified as a derepressor for miR-122 regulation of SLC7A1 in hepatoma cells. Under normal conditions, SLC7A1 is repressed by miR-122 since ELAVL1 is sequestered in the nucleus. However, under stress conditions, the ELAVL1 translocates from nucleus into cytoplasm, and abolishes SLC7A1 repression by miR-122 (Bhattacharyya *et al.*, 2006). This study showed that miRNA mediated regulation is not only dependent on miRNA expression or its functional target site, but also on other factors. In addition, this study also demonstrated that miRNA repression of target is a reversible process.

On the other hand, the different effects observed in CaSki and SiHa cells following inhibition of miR-210 expression, suggest that there could be a different mechanisms involved in miR-210 regulation in these two cell lines. Studies have shown that miRNA composition in cancer cells can vary significantly even in the same cancer type, depending on the histological background or differentiation status (Lu *et al.*, 2005; Yanaihara *et al.*, 2006). Although CaSki and SiHa cells are both squamous cell carcinomas of cervical origin, CaSki cells were derived from metastatic site in the small intestine and SiHa cells were derived from the primary site in the cervix.

5.4 SMAD4 and RSU1 are direct targets of miR-210 and miR-629, respectively

Since it was demonstrated that inhibition of miR-210 and miR-629 expression enhanced the anti-proliferative and apoptosis-inducing effects of ACA in cervical cancer cells, next would be to identify the molecular targets of these miRNAs to

delineate how ACA mediate its anti-cancer effects. These putative targets were selected based on several criteria. First, since miRNAs are negative gene regulators, it was hypothesized that these miRNAs mediate their effects by targeting tumour suppressors. Second, targets are involved in regulating cell proliferation and/or apoptosis. Third, context score, since lower context score indicates higher probability of the predicted targets being biologically validated. Fourth, similarities in the molecular targets of the predicted targets and ACA based on past literature (described in Sections 4.5.2.1 and 4.5.2.2).

Based on these criteria, SMAD4 and RSU1 were identified as putative targets of miR-210 and miR-629 from the list of predicted targets (Tables 4.1 and 4.2). The direct interaction between SMAD4 with miR-210 and RSU1 with miR-629 were subsequently confirmed for the first time using luciferase reporter assays (Figures 4.14 and 4.16). Although the direct interaction between miR-210 and SMAD4 for the first time was demonstrated for the first time, previous studies have shown that SMAD4 is also targeted by many other miRNAs. Among these miRNAs are miR-18a (Li *et al.*, 2012b), miR-19 (Fuziwara & Kimura, 2014), miR-20a-5p (Cheng *et al.*, 2016a), miR-26b (Dong *et al.*, 2014; Liu *et al.*, 2014), miR-130a-3p (Liu *et al.*, 2016b), miR-135b (Song *et al.*, 2016), miR-144-3p (Huang *et al.*, 2016), miR-146a (Zhong *et al.*, 2010; Li *et al.*, 2012a; Xiao *et al.*, 2012), miR-146b-5p (Geraldo *et al.*, 2012; Wang *et al.*, 2016a), miR-181a-5p (Ouyang *et al.*, 2016), miR-182-5p (Hirata *et al.*, 2012), miR-199a (Zhang *et al.*, 2012), miR-224 (Zhang *et al.*, 2013a), miR-483-3p (Shen *et al.*, 2015) and miR-1260b (Hirata *et al.*, 2013). All these published studies showed that regulation of SMAD4 by miRNAs have been extensively investigated and proved that a single target can be regulated by multiple miRNAs. Contrastingly, only one miRNA, miR-409-5p, has been confirmed to directly target RSU1 (Yu *et al.*, 2017), implying that miRNA mediated regulation of RSU1 activity is still at its infancy.

The findings from the luciferase reporter assays suggested that ACA could mediate its anti-cancer effects through these targets. Hence, it was next investigated if treatment with ACA could induce changes in SMAD4 and RSU1 protein levels in cervical cancer cells. Western blot analyses revealed increased protein levels of SMAD4 and RSU1 following treatment with ACA in CaSki and SiHa cells (Figures 4.17 and 4.18). Since miRNAs are known to regulate gene targets at translational level, it was next investigated if changes in the expression of miR-210 and miR-629 could affect the protein levels of SMAD4 and RSU1. Western blot analyses showed increased protein levels of SMAD4 and RSU1 following inhibition of miR-210 and miR-629 in both cervical cancer cells. However, over-expression of miR-210 and miR-629 did not resulted in significant reduction in the protein levels of SMAD4 and RSU1 in both CaSki and SiHa cells (Figures 4.19 and 4.20). Although these results were unexpected, they appeared to concur with the earlier findings, whereby over-expression of miR-210 and miR-629 did not resulted in significant changes in cell proliferation and apoptotic induction in CaSki and SiHa cells treated with ACA (Figures 4.6 to 4.9).

Studies have also reported that the interactions between miRNAs and their targets are dependent on cellular context: the relative abundances of miRNA and its targets that are available in the cells (Mukherji *et al.*, 2011). One possible reason for this could be that since both of these miRNAs were already over-expressed in these cells, increasing the miRNA levels further would not result in significant changes since the availability of the miR-210 and miR-629 binding site on SMAD4 and RSU1 would already be "saturated". Besides that, SMAD4 have been reported to be down-regulated in cervical cancer cells previously. Hence, the reduced availability of the target for miR-210 could also explain the results observed. The same might also applied for RSU1. However, since there is currently no literature on RSU1 expression in cervical cancer cells, it

would be highly speculative to think so, even though RSU1 was found to be down-regulated in various other cancers thus far.

On the other hand, the increased protein levels in SMAD4 and RSU1 observed following inhibition of miR-210 and miR-629 expression can be considered as relatively modest. This could be because both SMAD4 and RSU1 contain only one binding site for miR-210 and miR-629, respectively. Previous studies have reported that multiplicity of binding sites would exert a stronger effect in the interaction between miRNA and its specific target (Doench *et al.*, 2003; Selbach *et al.*, 2008).

Furthermore, each gene target can be targeted by other different miRNAs. For example, SMAD4 is also targeted by other miRNAs such as miR-19 (Fuziwara & Kimura, 2014), miR-20a-5p (Cheng *et al.*, 2016a), miR-26b (Dong *et al.*, 2014; Liu *et al.*, 2014), miR-130a-3p (Liu *et al.*, 2016b), miR-135b (Song *et al.*, 2016), while RSU1 is also targeted by miR-409-5p (Yu *et al.*, 2017).

Hence, altering the expression of one miRNA may only cause a subtle change in the protein expression of its targets. Given that each miRNA can have many gene targets, it is the simultaneous down-regulation or up-regulation of multiple targets by the miRNA that results in the phenotypic changes seen. In other words, although both SMAD4 and RSU1 were confirmed as direct targets of miR-210 and miR-629 using luciferase reporter assays, the increased sensitivity towards ACA following inhibition of miR-210 and miR-629 may not be solely due to the effects of these targets alone.

5.5 SMAD4 in cervical cancer cells

Many studies have identified SMAD4 as a tumour suppressor that is frequently inactivated or down-regulated in various cancers including cervical cancer (Baldus *et al.*, 2005; Kloth *et al.*, 2008). Studies have also demonstrated that SMAD4 over-

expression promoted apoptosis in cervical cancer cells in the absence of TGF-β (Lee *et al.*, 2001a) and suppressed cervical cancer xenografts in nude mice (Klein-Scory *et al.*, 2007), demonstrating its tumour suppressive role in cervical cancer cells. However, the function of SMAD4 in cervical cancer cells, especially in SiHa cells, remained somewhat controversial.

Both CaSki and SiHa cells were first reported to be resistant to the growth-inhibitory effects of TGF-β in 1990 (Braun *et al.*, 1990). Similar results were also reported by another group later. Subsequent investigations by this group found that SiHa cells expressed lower levels of SMAD4 mRNA compared CaSki cells. Furthermore, mutation was also detected in SMAD4 gene in SiHa cells, but not in CaSki cells. However, over-expression of SMAD4 in SiHa cells restored responsiveness towards TGF-β and was even able to induce apoptosis in the absence of TGF-β (Lee *et al.*, 2001a).

In contrast to these two studies, Kang and his colleagues reported that CaSki and SiHa cells are responsive to the growth inhibitory effects of TGF-β. In addition, the presence of TGF-β RI and RII mRNA were confirmed in CaSki and SiHa cells without mutations, and transcriptional responsiveness to TGF-β were maintained in these cells (Kang *et al.*, 1998). Meanwhile another study showed that SiHa cells, which was earlier reported to exhibit no mutation in SMAD4 (Maliekal *et al.*, 2003), was found to be highly responsive towards TGF-β. Furthermore, it was demonstrated that following TGF-β treatment, SiHa cells exhibited high SMAD DNA binding activity (with active complex containing SMAD2 and SMAD4), reduced cell proliferation and cell cycle was arrested in G0/G1 phase (Maliekal *et al.*, 2004). Another study reported that the SMAD4 mRNA and protein were readily detected in both CaSki and SiHa cells. In addition, although no clear correlation was observed between status of SMAD4 with

TGF-β responsiveness, both CaSki and SiHa cells were found to be responsive to TGF-β (Baldus *et al.*, 2005).

In summary, although early studies reported that both CaSki and SiHa cells were resistant towards growth-inhibitory effects of TGF-β, subsequent studies demonstrated otherwise. Furthermore, the mutation reported in SMAD4 gene in SiHa cells were not detected in two other studies.

5.6 Over-expression of SMAD4 and RSU1 enhanced sensitivity towards ACA in cervical cancer cells

The negative correlation between SMAD4 expression and sensitivity towards anti-cancer drugs have been reported such as cetuximab in head and neck squamous cell carcinoma (Cheng *et al.*, 2015), TGF-β1 inhibitor in pancreatic cancer (Chen *et al.*, 2014), DNA topoisomerase inhibitors in lung cancer (Haeger *et al.*, 2016) and 5-fluorouracil in breast and colorectal cancer (Papageorgis *et al.*, 2011; Yu *et al.*, 2013a; Zhang *et al.*, 2016a). Recently, it was reported that over-expression of SMAD4 enhanced sensitivity of colorectal cancer cells towards 5-fluorouracil in both *in vitro* and *in vivo* models (Zhang *et al.*, 2016a). These findings suggest that over-expression of SMAD4 could also potentially enhanced sensitivity towards ACA in cervical cancer cells. Although there is currently no literature that reported the correlation between RSU1 expression to anti-cancer drugs response, and its role in mediating sensitivity or resistance towards anti-cancer drugs, different studies have demonstrated its role as tumour suppressor in various cancers capable of reducing cell proliferation, migration, invasion and inducing apoptosis. Furthermore, findings from the current study indicated that sensitivity towards ACA following inhibition of miR-629 is mediated through RSU1. Hence, the effects of SMAD4 and RSU1 over-expression on sensitivity towards ACA in cervical cancer cells were investigated next.

Results from the cell proliferation and apoptosis assays showed that over-expression of SMAD4 and RSU1 augmented the anti-proliferative and apoptosis-inducing effects of ACA in cervical cancer cells (Figures 4.23 to 4.25). These results indicated that ACA could mediate its anti-cancer activities, at least in part, through SMAD4 and RSU1. Notably, these experiments were carried without TGF-β stimulation. Although SMAD4 is a component of TGF-β signalling pathway, and activation of this pathway requires the binding of TGF-β to the constitutively active TGF-β type II receptor, several studies have demonstrated that over-expression of SMAD4 was able to exert its effects in the absence of TGF-β.

In one such study, it was demonstrated that over-expression of SMAD4 increased $p21^{Waf1}$ and inhibited cell proliferation with or without the presence of TGF-β. Furthemore, it was demonstrated that over-expression of SMAD4 was able to function as transactivators by inducing $p21^{Waf1}$, independent of TGF-β receptor activation (hence, without the requirement of ligand stimulation) (Hunt *et al.*, 1998). It was also shown that ectopic expression of SMAD4 were able to induce apoptosis in SiHa cells in absence of TGF-β (Lee *et al.*, 2001a). Since the experiments were carried out without the use of ligand in this study, it is speculated that the effects observed are ligand independent.

5.7 Pathway model

Based on the findings from this study and past literature that have reported the molecular targets for ACA, SMAD4 and RSU1, the following pathway model was proposed to demonstrate the link between ACA's anti-cancer activities with miR-210, miR-629, SMAD4 and RSU1 (Figure 5.1). From the proposed pathway model, it can be seen that ACA can mediate its anti-proliferative and apoptosis-inducing effects through miR-210 and miR-629 and their confirmed targets. Treatment with ACA would result in

down-regulation of miR-210 and miR-629, increasing the protein levels of SMAD4 and RSU1. This is consistent with earlier findings in this study, whereby SMAD4 and RSU1 protein levels were found to be up-regulated following treatment with ACA. Increased SMAD4 expression then inhibit the expression of cMyc or induce p21 expression, which will then inhibit the cyclin D/CDK 4/6 complex, resulting in cell proliferation suppression. Increased SMAD4 expression can also reduce the expression of Bcl-xL directly, or indirectly through up-regulation of BIK, resulting in apoptosis induction. Previous studies have shown that treatment with ACA reduced the expression of cMyc, cyclin D and Bcl-xL, but induce the expression of p21. On the other hand, increased in RSU1 expression would down-regulate the expression of Rac1 and cdc42, resulting in inhibition of cell proliferation. It can also similarly induce p21 expression to suppress cell proliferation by inhibiting cyclin D/CDK 4/6 complex. Both Rac1 and cdc42 have also been reported to be inhibited by ACA previously. Increased RSU1 expression can also result in apoptotic induction by down-regulating PINCH1, which will then increase the expression of pro-apoptotic BIM, or by increasing the expression of PUMA, which will then inhibit Bcl-xL.

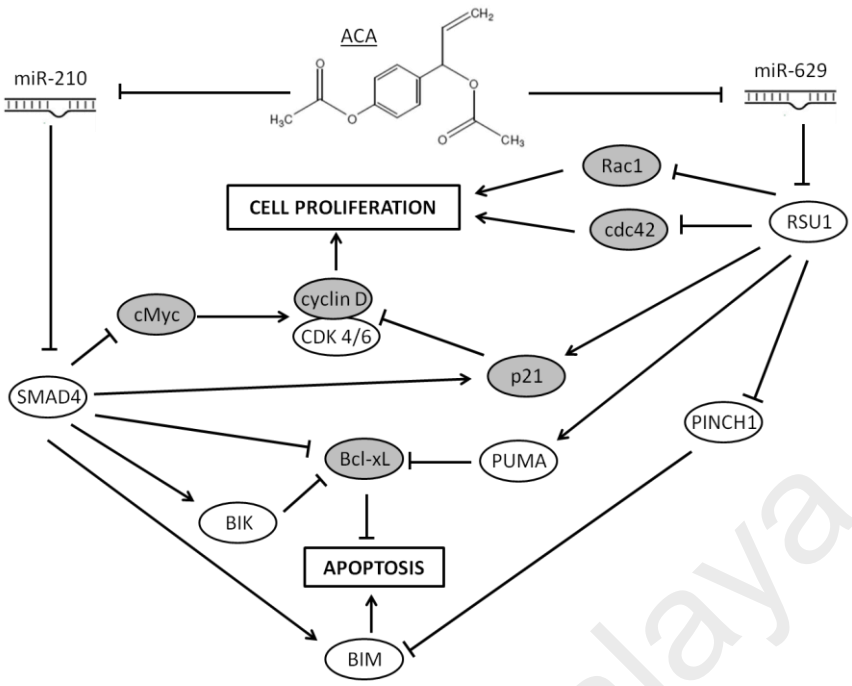

Figure 5.1: Proposed pathway model depicting the interaction between ACA with miR-210, miR-629, SMAD4 and RSU1.

Grey coloured symbols denoted targets affected by ACA; Inhibitory relationship are denoted as flat arrow heads; Positive relationship are denoted as open arrow heads.

5.8 Limitations of the study

Although the present study was carefully designed to address specific research objectives, several limitations could have influence the conclusions drawn from the data.

First, the method used in identifying the putative targets of miR-210 and miR-629. Although initial targets were predicted using TargetScan v7.0, which is considered as one of the most robust miRNA target prediction tool, the decision to subject the list of predicted targets from TargetScan v7.0 to gene annotation enrichment analyses using DAVID v6.7 have resulted in a number of predicted targets being left out. There is possibility that the predicted targets that were left out might have potentially play a more significant role in mediating ACA's anti-cancer effects.

Second, functional activity of SMAD4 and responsiveness to TGF-β signalling pathway in cervical cancer cells. Although there are contradictory reports on the functional activity of SMAD4 and responsiveness to the growth-inhibitory effects of TGF-β signalling in these cells, the functional activity of SMAD4 and responsiveness to TGF-β were not confirmed in the cervical cancer cells used in this study.

Third, the effects of ACA on TGF-β signalling pathway were not investigated. The identification of SMAD4 as a direct target of miR-210, which was found to be involved in regulating sensitivity towards ACA, suggest that ACA could possibly affect TGF-β signalling in cervical cancer cells. Furthermore, the experiments were carried out without the use of TGF-β ligand, hence it cannot be concluded if the effects are ligand dependent or otherwise.

Fourth, only one gene target for each miRNA was identified in this study. This might not provide a very accurate overall picture since miRNAs are capable of regulating multiple gene targets simultaneously, and more often than not, it is the simultaneous changes in the expression of multiple gene targets that result in the phenotype seen.

5.9 Future works

Given that one of the key limitations to the current study is the functional status of SMAD4 and responsiveness towards growth-inhibitory effects of TGF-β in cervical cancer cells, one of the first steps that should be taken is to asssess TGF-β responsiveness in these cells using p6SBE and p3TPluc reporter. The p6SBE promoter, constructed to reflect SMAD-dependent transcriptional induction, contained concatemerised SBE (SMAD binding element) sites is often used to assess the involvement of SMAD proteins in TGF-β responsiveness. The p3TPluc promoter is a hybrid promoter carrying sequences derived from the fibronectin and PAI-1 promoters is used to assess TGF-β responses.

Besides that, whole transcriptome analyses on cells transfected with miRNA and/or treated with ACA can be carried out using whole transcriptome microarray analysis or NGS RNA sequencing, which can detect both coding and multiple forms of noncoding RNA to provide a more comprehensive view of the entire transcriptome. The use of CytoScape or Ingenuity Pathway Analysis (IPA) to analyze molecular interaction networks and biological pathways would then provide an overall picture on the effects of ACA and/or miRNA on cellular processes.

The effects of ACA on TGF-β signalling pathway and its key members should also be investigated since SMAD4 was identified as a direct target of miR-210 that can play a role in mediating the anti-cancer effects of ACA. Ideally, experiments should be carried out with and without ligand, to determine whether the effects are ligand dependent or otherwise.

Studies have reported that although miRNAs are able to regulate multiple gene targets, the effects induced by the miRNAs on its targets may only cause a subtle change in the protein expression. Furthermore, it is widely expected that each targets harbour binding sites that could be targeted by several other miRNAs. Hence, changing the expression of one miRNA might not result in significant changes. In light of this, perhaps different methods can be used to measure protein levels in the cells such as ELISA, flow cytometry based assay or expression vectors.

Since the current study highlighted the potential of using miRNAs and natural compounds in cancer therapy, this study should eventually be extended into *in vivo* settings. Preclinical studies using *in vivo* models needs to be conducted to evaluate the pharmacokinetics and pharmacodynamics effects of this treatment approach. This is to identify the optimal treatment approach, which maximizes therapeutic effect with minimal physiological side effects.

CHAPTER 6: CONCLUSION

The main aims of the present study were to investigate the functional role of miR-210 and miR-629 in regulating sensitivity towards ACA in cervical cancer cells, and identify the novel targets of these miRNAs. Results from this study showed that inhibition of miR-210 and miR-629, which were down-regulated by ACA, enhanced the anti-proliferative and apoptotic effects of ACA in cervical cancer cells. Both SMAD4 and RSU1 were also identified and confirmed to be novel targets of miR-210 and miR-629, respectively. Western blot analyses revealed that treatment with ACA and inhibition of miR-210 and miR-629 expression up-regulated the expression of SMAD4 and RSU1. Furthermore, ectopic expression of SMAD4 and RSU1 augmented anti-proliferative and apoptotic effects of ACA in cervical cancer cells.

Collectively, this study demonstrated that ACA mediates its anti-proliferative and apoptosis-inducing effects in cervical cancer cells through miR-210 and miR-629, which directly targets SMAD4 and RSU1. These findings suggest that combinatorial treatment involving natural compounds and miRNAs could provide promising approach in treating cervical cancer.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

List of publications:

1. Phuah, N. H., & Nagoor, N. H. (2014). Regulation of microRNAs by natural agents: new strategies in cancer therapies. *BioMed Research International, 2014*, 804510.

2. Phuah, N. H., Azmi, M. N., Awang, K., & Nagoor, N. H. (2017). Suppression of microRNA-629 enhances sensitivity of cervical cancer cells to 1'S-1'-acetoxychavicol acetate (ACA) via regulating RSU1. *OncoTargets and Therapy, 10*, 1695-1705.

3. Phuah, N. H., Azmi, M. N., Awang, K., & Nagoor, N. H. (2017). Down-regulation of microRNA-210 confers sensitivity towards 1'S-1'-acetoxychavicol acetate (ACA) in cervical cancer cells by targeting SMAD4. *Molecules and Cells*, *40*(4): 291-298.

Paper presented:

Conference: 21st MSMBB Annual Scientific Meeting 2014

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Title: Down-regulation of miR-210 enhances sensitivity towards 1'S-1'-acetoxychavicol acetate (ACA) in human cervical carcinoma cells.