The Role of miR-21 and miR-499 in Head and Neck Cancer

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Declaration

I hereby state that all the investigations presented in this thesis were carried out under the supervisions of Dr. Nham Tran and A/Prof. Gyorgy Hutvagner. This thesis incorporates original research which has not been previously submitted for a higher degree to any other institution. The experimental investigations and analysis described in this thesis were completed by me, except were assistance has been duly acknowledged and reference has been made in the text.

Pamela Ajuyah **Date:**

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Abstract

Globally there are more than half a million new cases of head and neck cancer each year [1,2]. More than 90% of head and neck tumours are head and neck squamous cell carcinomas (HNSCCs) which originate in the lip/oral cavity, nasopharynx, oropharynx, hypopharynx and the larynx [1,3].

HNSCCs are inadequately diagnosed and as a result many head and neck cancer patients are diagnosed at the advanced stages of the disease [4]. The lack of biomarkers for HNSCC has resulted in this poor diagnosis of the cancer. Furthermore, a limited understanding of the molecular biology of the cancer has led to few treatment options. The future of HNSCC diagnosis and treatment can lie in the small non-coding RNAs called miRNAs. miRNAs function as gene regulators and have been implicated in the development and progression of various cancers [5-8]. In HNSCC, two miRNAs miR-21 and miR-499 have been found to be upregulated in tumours compared to normal tissues [9]. Furthermore, these miRNAs both regulate the tumour suppressor gene Programmed Cell Death 4 (PDCD4). PDCD4 has been found to be involved in oncogenic pathways including apoptosis, proliferation, angiogenesis and invasion [10,11]. PDCD4 is also downregulated in many HNSCC tumours [12-14]. This thesis endeavoured to determine the role of miR-21 and miR-499 in HNSCC through their regulation of PDCD4.

The first aim was to study the co-regulation of PDCD4 by miR-21 and miR-499. When genes are co-regulated by miRNAs this can lead to heavy regulation of the genes [15]. This is essential for genes critical to cancer initiation and progression [15]. Currently there are limited studies examining the various modes of regulation miRNAs can use to simultaneously regulate a single gene at its 3´ untranslated region (3´UTR). In this project, site mutants for miR-21 and miR-499 at the 3´UTR of PDCD4 were created and ligated to luciferase reporter vectors. Using luciferase assays it was revealed that miR-21 and miR-499 regulate the 3´UTR independently of each other. However,

miR-21 does aid miR-499 interactions with the PDCD4 3´UTR. Furthermore, the last two miR-499 sites are regulated in a co-dependent manner and mutating either site completely abolishes regulation of PDCD4 by miR-499. This is the first study detailing the regulatory dynamics of PDCD4.

The co-regulation of PDCD4 by miR-21 and miR-499 has an extra layer of complexity in that the miRNAs also have a regulatory relationship with each other. Overexpression of miR-21 was found to endogenously upregulate miR-499 expression in cells. There are few studies in the literature on miRNA mediated regulation of another miRNA. These studies show that miRNA mediated regulation usually occurs when a miRNA(s) has a binding site in the primary transcript of another miRNA or at the promoter region of the mature miRNA [16-18]. Further research into miR-21's upregulation of miR-499, found that the regulation was not reciprocal as overexpression of miR-499 did not affect miR-21 levels. A few models were designed and tested to investigate how miR-21 was able to regulate miR-499. Primary levels of miR-499 were unchanged by miR-21 overexpression. Thus regulation of miR-499 by miR-21 occurred post-transcription. The stability of miR-499 was measured when *de novo* synthesis of miRNAs was switched off. miR-499 was found like other miRNAs to degrade over 24 hours. However, if miR-21 was overexpressed in cells then miR-499 levels were stabilised. It was thought that perhaps miR-21 is able to stabilise miR-499 through target-mediated miRNA protection (TMMP). In this model the half-life of a miRNA can be increased by its interactions with a target mRNA [19,20]. It is predicted that through a gene like PDCD4 miR-21 is able to encourage miR-499 interactions with the gene. Perhaps miR-21 binding removes obtrusive secondary structure at the miR-499 binding sites on the 3´UTR. This allows miR-499 to interact with the gene thus protecting it from degradation.

A few studies have found that a single miRNA is able to alter the expression of multiple miRNAs [21,22]. However, the mechanism behind this or even if this is a common occurrence with miRNAs in general is still yet to be understood.

Therefore, the regulation of miR-499 by miR-21 was extended genome-wide to determine if other miRNAs were also affected by miR-21 overexpression. Affymetrix arrays revealed that not only were many miRNAs upregulated by miR-21 overexpression but also downregulated. Furthermore, miR-499 overexpression could also differentially regulate other miRNAs. The miRNAs that were most upregulated by miR-21 were found to have targets that could potentially be co-targeted by several of these miRNAs. miR-21 and miR-499 also had genes that they could potentially co-target together. Therefore, perhaps miRNAs that are regulated by other miRNAs are involved in regulating similar genes leading to an enhanced or differential regulation of these genes.

Finally, the function of miR-21 and miR-499 in HNSCCs were examined. miR-21 is involved in certain oncogenic pathways in HNSCCs [23,24], but no studies have investigated miR-499's role. Considering that miRNAs are at the forefront of gene dysregulation during cancer initiation and development [25-28], it is worth understanding how they are able to affect cancerous processes. This is useful for the identification of new biomarkers for HNSCC but also for the design of miRNA based therapeutics.

Using live cell imaging and scratch assays, it was found that miR-21 and miR-499 were able to promote migration in HNSCCs. It is predicted that this promoted migration most likely occurs through the downregulation of the tumour suppressor genes PDCD4, SRY (Sex Determining Region Y) Box 6 and Forkhead Box Protein 04 (FOXO4). These genes have been shown in other cancers to be directly involved in migration [29-31].

This thesis explores in depth the regulation of the tumour suppressor gene PDCD4 by miR-21 and miR-499 in a HNSCC context. It uncovers the type of regulation this gene undergoes, the relationship between the two miRNAs and other miRNAs and the function of these miRNAs in HNSCC. Studies such as these pave the way for designing new clinical therapeutics by

understanding the molecular aberrations that lead to head and neck cancer development.

Publications and abstracts associated with this thesis

Publications arising from this thesis

P Ajuyah., A Ahadi., J Lu., G Hutvagner., N Tran (2016) The unique co-regulation of the tumour suppressor gene PDCD4 by miR-21 and miR-499. *In Preparation.*

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Abbreviations

actD	actinomycin D
bp	base pair
BOT	base of tongue
CDS	coding sequence
CMM	cooperative miRNA module
DC	double transfection control siRNA
DK	double transfection siAgo2
DMEM	dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotides
ECL	enhanced chemiluminescence
EMT	epithelial-mesenchymal transition
FBS	fetal bovine serum
FOM	floor or mouth
FOXO4	forkhead box protein 04
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HITS-CLIP	high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
KD	knock down
KO	knock out
LB	luria-bertani
MAP4K1	mitogen-activated protein kinase kinase kinase kinase 1
miRNA	microRNAs
ncRNA	non-coding RNA
NEB	new england biolabs
nt	nucleotide
oncomiRs	oncogenic miRNAs

ORF	open reading frame
OSCC	oral squamous cell carcinoma
PAR-CLIP	photoactivatable ribonucleoside–enhanced cross-linking and immunoprecipitation
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDCD4	programmed cell death 4
piRNA	piwi-interacting RNAs
pri-miRNA	primary miRNA
qPCR	quantitative real-time PCR
RISC	RNA induced silencing complex
RNA	ribonucleic acid
RNA pol II	RNA polymerase II
SC	single transfection control siRNA
SDS	sodium dodecyl sulfate
SEER	surveillance, epidemiology and end results
shRNA	short hairpin RNA
siAgo2	ago2 specific targeting siRNA
siRNA	short interfering RNA
SK	single transfection siAgo2
SNP	single nucleotide polymorphism
SOX6	SRY (sex determining region Y) box 6
TMMP	target-mediated miRNA protection
UTR	untranslated region
WT	wild type