

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

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This thesis is submitted in fulfilment of the requirements for the degree of Doctor of Philosophy, School of Life Sciences, The University of Technology, Sydney.



August, 2016

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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 where assistance has been duly acknowledged and reference has been made in the text.

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Date:

Acknowledgements

It has been a long journey. I have learnt so much about myself and strangely enough microRNAs along the way. I have had a unique and interesting PhD experience and I have many people to thank for that. I would like to start off by thanking both my supervisors.

To my primary supervisor Dr. Nham Tran who personally taught me all the basic molecular techniques required to become a versatile scientist. The hours you spent coaching me to become a great presenter paid off too as not only did I lose my fear of public speaking, I actually enjoy it now. I greatly appreciate your time and effort without which I wouldn't be the scientist I am today.

To my co-supervisor A/Prof. Gyorgy Hutvanger whose vast knowledge and experience in the microRNA field was admirable and inspirational to me. Your unwavering support helped me get through the finish line.

I would like to thank a couple of key researchers in UTS whose contributions at certain points of my PhD gave continued momentum to my project. Dr. Michael Johnson for the hours you spent helping me set up and analyse my scratch assays with absolutely nothing expected in return – that is rare in today's world. To Joyce To who sat behind me in the lab so I could always run to you for experimental insight and great conversations – your final piece of advice was what I needed to finish up my proliferation experiments.

I would like to thank the other PhD students at UTS including the students in my office who provided years of laughter and interesting times. I especially thank my now good friends who I met at UTS – Rob, Sam, Elliot, Marty and Peter. You guys served the dual purpose of not only being awesome friends but also sharing great technical advice based on your experiences in the lab.

I have to thank Jimmy and Roxby, past lab members with whom I shared countless 10 pm finishes in the lab with. The companionship made the hours fly by!

A big shout out to my best friends – Alvina, Roro, Jules, Marisa and Loraine. Honestly you girls were such strong emotional pillars of support for me and I have to thank all of you for the endless phone calls, DNMs and being the best type of friends a girl could ask for.

My gratitude to UTS for an APA scholarship and TCRN for an additional scholarship top up, both were essential for survival throughout my PhD and allowed me to focus on my research without worrying about finances.

Finally and most importantly, I thank my family. My sister Josephine and my little brother Hansel for always being supportive no matter what was going on in their lives. My father Asifo for being the ultimate role model not just as a father but also as a scientist. And my mother Pauline for the constant encouragement and almost daily phone calls. Thank you both for being there when I needed you the most.

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 miRNA¹⁶⁻¹⁸. 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 development²⁵⁻²⁸, 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 migration²⁹⁻³¹.

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.*

Abstracts associated with this thesis

P Ajuyah., G Hutvanger., N Tran (2015) The unique co-regulation of the tumour suppressor gene PDCD4 by miR-21 and miR-499. ICEI Conference, Shenzhen, China. April 18-20.

P Ajuyah., G Hutvanger., N Tran (2014) The unique co-regulation of the tumour suppressor gene PDCD4 by miR-21 and miR-499. New Horizons Conference. The Kolling Institute of Medical Research, Sydney, Australia. November 17-19.

P Ajuyah., G Hutvanger., N Tran (2014) The unique co-regulation of the tumour suppressor gene PDCD4 by miR-21 and miR-499. JaJRNA Conference. The University of Technology, Sydney, Australia. November 2-5.

P Ajuyah., G Hutvanger., N Tran (2014) The unique co-regulation of the tumour suppressor gene PDCD4 by miR-21 and miR-499. Lorne Genome Conference. Mantra Lorne, Australia. February 16-18.

P Ajuyah., G Hutvanger., N Tran (2014) The unique co-regulation of the tumour suppressor gene PDCD4 by miR-21 and miR-499. RNAUK 2014 Conference. Windermere, UK. January 24-26.

P Ajuyah., G Hutvanger., N Tran (2014) The unique co-regulation of the tumour suppressor gene PDCD4 by miR-21 and miR-499. RNA Silencing Keystone Symposia. Seattle, USA. January 31 – February 5.

P Ajuyah., G Hutvanger., N Tran (2013) The unique co-regulation of the tumour suppressor gene PDCD4 by miR-21 and miR-499. Scientific Research Meeting. The Kolling Institute of Medical Research. Sydney, Australia. November 18-20.

P Ajuyah., G Hutvanger., N Tran (2012) The unique co-regulation of the tumour suppressor gene PDCD4 by miR-21 and miR-499. Scientific Research Meeting. The Kolling Institute of Medical Research. Sydney, Australia. November 20-21.

P Ajuyah., G Hutvanger., N Tran (2012) The unique co-regulation of the tumour suppressor gene PDCD4 by miR-21 and miR-499. Networks in the Life Sciences. 14th International EMBL PhD Symposium. Heidelberg, Germany. October 25-27.

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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 |