

**The impact of the Human papillomavirus type 16 on
non-coding RNAs in Head and Neck cancer**

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the degree of**

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under the supervision of

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Certificate of original authorship

I, Dayna Grace Sais, declare that this thesis is submitted in fulfilment of the requirements for the award of Doctor of Philosophy in the School of Biomedical Engineering, Faculty of Engineering, and Information Technology at the University of Technology Sydney.

This thesis is wholly my work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

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Statement & list of papers and conferences

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3. Mason, D., Zhang, X., Marques, T.M., Rose, B., Khoury, S., Hill, M., Deutsch, F., Lyons, J.G., Gama-Carvalho, M. and Tran, N., 2018. Human papillomavirus 16 E6 modulates the expression of miR-496 in oropharyngeal cancer. *Virology*, 521, pp.149-157

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1. Mason D, Zhang X, Marques TM, Gama-Carvalho M, Tran N, "MiR-496 expression is altered in Oropharyngeal cancers by HPV16 E6" *Combio*, Sydney, 2018
2. Mason D, Zhang X, Marques TM, Rose B, Khoury S, Hill M, Deutsch F, Lyons JG, Gama-Carvalho M, Tran N, "Human papillomavirus 16 E6 modulates the expression of miR-496 in oropharyngeal cancer" *International Papilloma virus conference*, Sydney, 2018
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6. Mason D, Tran N, “Using a viral interactome to uncover mechanistic links between HPV16 and specific microRNAs in Head and neck cancers” *Lorne Cancer*, Online Conference, 2021

7. Mason D, Tran N, “Uncovering mechanistic links between HPV16 and specific microRNAs in Head and Neck cancers” *RNA 2021, RNA Society*, Online Conference, 2021

COVID-19 impact statement:

Due to the COVID-19 pandemic and enforced restrictions, I experienced two extended periods of suspended experimental work during my candidature. These COVID-19 restrictions were due to NSW-wide lockdowns, the first period being from March 2020 to July 2020 and the second from July 2021 to November 2021. I was also under stricter COVID-19-related restrictions due to high transmission rates in my area of residence and residing with an immunocompromised individual.

During the two COVID-19 lockdowns, it was highly recommended that all research activities be conducted from home. Additionally, laboratory access was restricted to those with highly time-sensitive research and animal works that were unable to be reorganised or postponed. Due to my research not fitting within this category and travel restrictions, I was unable to conduct experiments during this time. This meant that a proportion of the experiments within this thesis were delayed, and particular experiments were not achieved.

These issues particularly affected Chapter 5 and Chapter 7 of this thesis. Due to delays, I was unable to complete the western blot validation for the SREBF2 and HPV16 E6/E7 knockdowns. I did optimise the SREBF2 antibody in control cells, but HPV16 E6/E7 antibodies need to be optimised, and experimental samples need to be tested. Due to the COVID-19 delays, I also was unable to repeat the RNA sequencing of the HPV16 E6/E7 knockdown to account for variation between biological replicates. Additionally, the decrease in air freight and the associated increase in boat freight due to COVID-19 prolonged the time for essential western blot reagents to arrive. In this case the shipment delay was greater than three months. The delivery of RNA samples for total RNA sequencing was also postponed due to travel restrictions. The sequencing service provider was also changed. As a result, the RNA sequencing results, and western blot reagents were received within the last four months of candidature. The differentially expressed lncRNAs were not followed up with confirmatory RT-qPCR or functional analysis, and antibody optimisation was delayed. To account for changes in the experimental timeline and available resources, the aims of this thesis were adjusted. The time delays were mitigated by shifting the main focus towards a more computational and bioinformatics approach for the synthesis of this thesis.

Table of Contents:

Certificate of original authorship	ii
Acknowledgements:	iii
Statement & list of papers and conferences	v
COVID-19 impact statement:	vii
Table of Contents:	viii
Supplementary/Appendix material	xiv
List of figures:	xvi
List of tables:	xix
Abbreviations:	xx
Abstract:	1
Chapter 1: Introduction and literature review	3
1.1. Head and Neck cancers	5
1.1.1. Prevalence of Head and Neck cancers	5
1.1.2. Clinical outcome and treatment	7
1.1.3. Risk factors from Head and Neck cancer	7
1.1.4. HPV positive vs. HPV negative Head and Neck cancers	8
1.2. Non-coding RNAs in cancers.....	10
1.3. Small non-coding RNAs: microRNAs.....	10
1.3.1. MicroRNAs and their Discovery.....	10
1.3.2. The biogenesis of microRNAs	11
1.4. Manuscript: The dynamic interactome of microRNAs and the Human papillomavirus in Head and Neck cancers	13
1.4.1. Copyright information:	13
1.4.2. Author contribution	14
1.4.3. Manuscript: Abstract.....	15
1.4.4. Manuscript: HPV rates are increased in Head and Neck cancers.....	15
1.4.5. Manuscript: Human Papillomavirus	16
1.4.6. Manuscript: MicroRNAs: tiny gene regulators	17
1.4.7. Manuscript: The dynamic regulation of the miRNAome by HPV16	19
1.4.8. Manuscript: Interactomes: mapping the system-wide impact of HPV	24
1.4.9. Manuscript: An HPV-interactome for understanding drug mechanisms of action	26
1.4.10. Manuscript: Conclusion	29
1.5. Long non-coding RNAs.....	31
1.5.1. Long non-coding RNAs: discovery, biogenesis, and function	31
1.5.2. The classification of long non-coding RNAs	33

1.5.3.	The role of long non-coding RNAs in cancer	35
1.5.4.	The impact of HPV on long non-coding RNAs expression	39
1.5.5.	Long non-coding RNAs and their role in Head and Neck cancer	40
1.6.	Hypothesis	45
1.7.	Aims	45
Chapter 2: Materials and methods.....		46
2.1.	Materials	47
2.2.	Methods	51
2.2.1.	Cell lines	51
2.2.2.	Designing HPV16 E6/E7 and TRINGS siRNAs and transient reverse transfection of siRNA	51
2.2.3.	HPV16 E6/E7 plasmid overexpression.....	52
2.2.3.1.	Plasmids preparation from bacterial stab cultures	52
2.2.3.2.	DNA midiprep preparation	54
2.2.3.3.	Transfection efficiency and fluorescence-activation sorting (FACS) flow cytometry	54
2.2.3.4.	Plasmid transient reverse transfection	55
2.2.4.	Harvesting cells	55
2.2.5.	Total RNA isolation	56
2.2.6.	Analysis of gene expression using quantitative Polymerase Chain Reaction.....	57
2.2.6.1.	Random primer and specific miRNA cDNA synthesis	57
2.2.6.2.	TaqMan qPCR approach and design.....	59
2.2.6.3.	SYBR green approach and design	59
2.2.7.	Protein isolation and western blot analysis.....	61
2.2.8.	The Cancer Genome Atlas	61
Chapter 3: Developing a virus-microRNA interactome using Cytoscape		62
3.1.	Copyright information.....	63
3.2.	Authors contribution	64
3.3.	Abstract.....	66
3.4.	Graphical Abstract	67
3.5.	Building a viral miRNA human interactome	68
3.6.	Downloading required viral dataset	68
3.7.	Importing data into Cytoscape	68
3.8.	Downloading the human interactome	69
3.9.	Merging the two networks to create a virus and human interactome	71
3.10.	Filtering the viral/human interactome	71
3.11.	Assigning gene names and transcription factors	73

3.12.	Downloading the miRNAs and their targets	73
3.13.	Addition of miRNAs and target genes to the virus and human interactome 74	
3.14.	Annotation of genes and final virus/human/miRNA interactome.....	77
3.15.	Additional information.....	80
Chapter 4: Human papillomavirus 16 E6 modulates the expression of miR-496 in oropharyngeal cancer		81
4.1.	Copyright information	82
4.2.	Author contribution.....	83
4.3.	Abstract.....	85
4.4.	Introduction	86
4.5.	Methods	88
4.5.1.	Study population	88
4.5.2.	HPV status.....	88
4.5.3.	E6*I and E6*II constructs and stable transfectants	90
4.5.4.	Cell culture and transfection	90
4.5.5.	RNA isolation and microarray.....	90
4.5.6.	Quantitative PCR (qPCR) and statistical analysis	91
4.5.7.	Luciferase assays.....	92
4.5.8.	HPV-Human interactome network	92
4.6.	Results.....	93
4.6.1.	Patient cohort and HPV status	93
4.6.2.	miRNA signatures can differentiate HPV positive and HPV negative oropharyngeal SCCs into two separate clusters.....	93
4.6.3.	HPV16 E6 regulates expression of miR-33 and miR-496	96
4.6.4.	Direct post-transcriptional regulation of E2F2 by miR-496 in Head and Neck cancer cell lines	98
4.6.5.	HPV16 interactome analysis identified potential mechanisms regulating miR expression	100
4.7.	Discussion.....	104
4.8.	Appendix	107
Chapter 5: HPV16 E6 and E7 oncogenes can alter the expression of SREBF2 and miR-33a in Head and Neck cancers		110
5.1.	Introduction	111
5.2.	Methods	112
5.2.1.	Mining: The Cancer Genome Atlas	112
5.2.2.	Cell lines	112
5.2.3.	Cell culture and transfections	112

5.2.4.	Designing TaqMan assays for miRNA detection	114
5.3.	Results	116
5.3.1.	The regulation of SREBF2 and specific miRNAs in HPV16 positive oropharyngeal cancers	116
5.3.2.	Optimisation of PCR TaqMan assays for HPV16 E6 and E7 and miR-33a	119
5.3.3.	SREBF2 and miR-33a are overexpressed in HPV16 positive Head and Neck cancer cell lines	121
5.3.4.	Decrease in SREBF2 expression does not always lead to an alteration in miR-33a expression in Head and Neck cancer cell lines	123
5.3.5.	HPV16 major viral oncogenes E6 and E7 can influence the expression of SREBF2 and miR-33	125
5.3.6.	The regulation of E2F2 by HPV16 E6/E7	129
5.3.7.	There is a positive correlation between SREBF2 and miR-33a across different cancer types.....	130
5.4.	Discussion.....	131
5.4.1.	Co-expression of SREBF2 and miR-33a in cancer cells	131
5.4.2.	Depletion of SREBF2 may not affect miR-33a levels	132
5.4.3.	E6 and E7 oncogenes can potentially regulate SREBF2/miR-33a expression.....	134
5.4.4.	Significance and Future directions	135
5.5.	Appendix.....	141
Chapter 6: The lncRNA TRINGS influences cell viability of HPV16 positive cervical cancers despite glucose starvation.....		149
6.1.	Introduction	150
6.2.	Methods	153
6.2.1.	RNA interference	153
6.2.2.	RNA isolation and quantitative PCR.....	154
6.2.3.	Cell viability assay	154
6.2.4.	Interactome and gene ontology analysis	155
6.3.	Results.....	156
6.3.1.	LncRNA TRINGS expression level is independent of glucose starvation but is dependent on p53 levels	156
6.3.2.	Modulation of TRINGS expression can influence cell viability of cancer cells	160
6.3.3.	The modulation of TRINGS in cells with wild type p53.....	166
6.3.4.	TRINGS expression in Head and Neck cancers	168
6.3.5.	Determining the relationship between TRINGS and HPV16 using an interactome	172
6.4.	Discussion.....	175

6.4.1.	The impact of glucose starvation on TRINGS expression	176
6.4.2.	The effect of TRINGS on normal cells	177
6.4.3.	The expression of TRINGS in Head and Neck cancers	178
6.4.4.	Mechanistic models for the TRINGS – p53 network	179
6.4.5.	Future directions:.....	180
6.5.	Appendix	182
Chapter 7: The interaction between microRNAs and long non-coding RNAs in HPV16 related Oropharyngeal cancers		184
7.1.	Introduction	185
7.2.	Methods	187
7.2.1.	TCGA RNA-seq data analysis	187
7.2.2.	LncRNA-miRNA sponging prediction and HPV16 non-coding RNA interactome analysis	189
7.2.3.	Gene Ontology and KEGG analysis	189
7.2.4.	Cell culture and transfections	190
7.2.5.	Quantitative-PCR (qPCR) and statistical analysis.....	190
7.2.6.	RNA sequencing of HPV16 E6/E7 knockdown	191
7.3.	Results	193
7.3.1.	Identifying differentially expressed lncRNAs, miRNAs and mRNAs between HPV16+ and HPV16- Oropharyngeal cancers	193
7.3.2.	Interactome analysis of differentially expressed lncRNAs-miRNAs-mRNAs in HPV16 OPC.....	198
7.3.3.	Knockdown of HPV16 viral oncogenes E6/E7 reveal lncRNAs to be targeted in OPC	205
7.4.	Discussion.....	211
7.4.1.	The characterisation of lncRNAs in HPV16 positive OPC	211
7.4.2.	Building an RNA axis interactome to gauge functionality	212
7.4.3.	Predicting the impact on gene regulation by these DE-lncRNAs/miRNAs interactions.....	214
7.4.4.	In-vitro knockdown of HPV16 E6/E7 to confirm the regulation of these DE-lncRNAs	215
7.5.	Conclusion	216
7.6.	Appendix	217
Chapter 8: Overall discussion		221
8.1.	An HPV-RNA interactome is a valuable tool for uncovering novel mechanistic insights	221
8.2.	The human papillomavirus and its viral oncogenes E6 and E7 can influence the expression of miRNAs in Oropharyngeal cancers: Determining the role SREBF2 and miR-33a play in HPV16 positive OPC	225

8.3. LncRNAs can play an important role in cancer cell survival in HPV related cancers: an in-depth investigation into the lncRNA TRINGS	230
8.4. Exploring the impact of HPV16 on long non-coding RNA expression in Oropharyngeal cancers.	234
8.5. Overall conclusions.....	237
References	239

Supplementary/Appendix material

The supplementary tables listed below can be found via:

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Supplementary Table 4.1

Supplementary Table 7.1

Supplementary Table 7.2

Supplementary Table 7.3

Supplementary Table 7.4

Chapter 4: Appendix Figure 4. 1: Fluorescently sorted GFP/E6s and GFP HN6 cells. 107

Appendix Figure 4. 2 108

Appendix Figure 4. 3: E2F2 and p16 fold change with miR-496 overexpression. .. 109

Chapter 5:

Appendix Table 5. 1: The cancer gene atlas, Head and Neck cancer cohort: patient barcode IDs and relevant clinical information: 141

Appendix Table 5. 2: Correlation between SREBF2 and miR-33a expression in different cancer types from the TCGA..... 143

Appendix Figure 5. 1: The HPV16 status of the Head and Neck cancer cell lines panel. 144

Appendix Figure 5. 2: Knockdown of SREBF2 using siRNA in HPV16+ HNC cell lines SCC154 and SCC090 at 24hrs post-transfection. 145

Appendix Figure 5. 3: Knockdown of HPV16 E6/E7 using siRNA in HPV16+ HNC cell lines SCC154 and SCC090 at 24hrs post-transfection..... 146

Appendix Figure 5. 4: Knockdown of HPV16 E6/E7 using siRNA in HPV16+ HNC cell lines SCC154 at 24hrs and 48hrs post-transfection. 147

Appendix Figure 5. 5: Expression levels of specific genes in HPV+ and HPV- Oropharyngeal cancer from TCGA database..... 148

Appendix Figure 5. 6: Western blot optimisation for SREBF2 protein analysis in HUH7 cells: 148

Chapter 6:

Appendix Figure 6. 1: Knockdown of TRINGS using two siRNA in HPV16 positive cell line SiHa at 24hrs, 48hrs, 72hrs and 96hrs post transfection. 182

Appendix Figure 6. 2: Knockdown of TRINGS siRNA in HCT116 TP53 null cells at 24hrs, 48hrs, 72hrs and 96hrs post transfection under A) normal glucose conditions and B) glucose starved conditions. 182

Appendix Figure 6. 3: Trypan blue staining of SiHa knockdown of TRINGS siRNA at 24hrs, 48hrs, 72hrs and 96hrs post transfection under normal glucose conditions and glucose starved conditions..... 183

Chapter 7:

Appendix Figure 7. 1: Plot PCA of RNA sequencing data on HPV16 E6/E7 siRNA knockdown in SCC090:.....	217
Appendix Figure 7. 2: Differentially expressed lncRNAs between HPV16 E6/E7 siRNA knockdown and control SCC090 cells.	218
Appendix Figure 7. 3: Heatmaps representing differentially expressed lncRNAs in HPV16 E6/E7 siRNA knockdown with A) siRNA 1 and B) siRNA 2 vs control, p-value <0.05	219
Appendix Figure 7. 4: Differentially expressed lncRNAs from HPV16 E6/E7 siRNA knockdown in TCGA cohort:	220

List of figures:

Chapter 1:

Figure 1. 1: Head and Neck squamous cell carcinoma subtypes:	6
Figure 1. 2: The relationship between HPV16 oncogenes and miRNA biogenesis. .	18
Figure 1. 3: HPV-miRNA interactome for oropharyngeal cancers:	25
Figure 1. 4: Using interactomes to map chemotherapeutic drug associations with mRNAs targets.	28
Figure 1. 5: The main archetypes of long non-coding RNA classification:	34

Chapter 2:

Figure 2. 1 Linear representation of all plasmid vectors:	53
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Chapter 3:

Figure 3. 1: Graphical abstract depicting the methodology for the development of a viral-miRNA interactome	67
Figure 3. 2 Importing Raw Human and HPV Data.	70
Figure 3. 3: Merged and Filtered Network.....	72
Figure 3. 4 Screenshots of the. txt files created for the miRNAs of interest.....	75
Figure 3. 5: Selecting nodes	76
Figure 3. 6: Annotating the network	78
Figure 3. 7: The final HPV16-miRNA interactome created using the described Cytoscape methodology, indicating both the impact of transcription factors and genes on mRNA and miRNA expression.	79

Chapter 4:

Figure 4. 1: Deregulation of specific miRNAs in HPV positive and HPV negative oropharyngeal (tonsillar) cancers.	94
Figure 4. 2: Overexpression of E6 can regulate miR-33 and miR-496.	97
Figure 4. 3: Regulation of E2F2 by miR-496 in various head and neck cancer cells.	99
Figure 4. 4 Building an HPV interactome.	102

Chapter 5:

Figure 5. 1: Expression levels of specific genes and miRNAs in HPV16+ and HPV- Oropharyngeal cancer from TCGA database.....	117
Figure 5. 2: Expression levels of specific genes and miRNAs in HPV+ (HPV16 and HPV 18) and HPV- Cervical cancer from TCGA database.	118
Figure 5. 3: Detection of HPV16 E6 and E7 in cell lines panel.	119
Figure 5. 4: Primer optimisation for miR-33a detection.	120
Figure 5. 5: SREBF2 and miR-33a levels in HPV16+ Head and Neck cancer cell lines.....	122
Figure 5. 6: Knockdown of SREBF2 using siRNA in the HPV16+ Head and Neck cancer cell lines SCC154 and SCC090 at 48 hours post-transfection.....	124
Figure 5. 7: Knockdown of HPV16 E6 and E7 using several siRNA in the HPV16+ Head and Neck cancer cell lines SCC154 and SCC090 at 48 hours post-transfection.	127

Figure 5. 8: The overexpression of HPV16 E6 and E7 led to an increase in the expression levels of SREBF2 at 48 hours post-transfection in the Head and Neck cancer cell line SCC4.....	128
Figure 5. 9: The knockdown of HPV16 E6 and E7 led to a decrease in the expression levels of E2F2 at 48 hours post-transfection, in the Head and Neck cancer cell lines.....	129
Figure 5. 10: Proposed model for miR-33a and miR-496 dysregulation by HPV16.	140

Chapter 6:

Figure 6. 1: TRINGS regulation of E6 degradation of p53 and its impact on the necrotic cell pathway.....	152
Figure 6. 2: Relative expression levels of TRINGS in a cell lines panel	157
Figure 6. 3: Relative expression levels of TRINGS over time in immortalised keratinocyte cell lines comparing control (Ctl) cells to cells expressing HPV16 E6/E7.	157
Figure 6. 4: Relative expression levels of TRINGS over time in cervical cancer cell lines under glucose starved conditions.	159
Figure 6. 5: Relative expression levels of TRINGS over time in colorectal cancer cell line HCT116 under glucose starved conditions comparing cells A) Wild type P53 and B) Null P53.	159
Figure 6. 6: Knockdown of TRINGS using siRNA in HPV16 positive cell line SiHa at 24hrs, 48hrs, 72hrs, and 96hrs post-transfection under normal glucose and glucose starved conditions.	162
Figure 6. 7: Knockdown of TRINGS using siRNA in HPV16 positive cell line Caski at 24hrs, 48hrs, 72hrs, and 96hrs post-transfection under normal glucose and glucose starved conditions.	163
Figure 6. 8: Knockdown of TRINGS using siRNA in iNOK cells at 24hrs, 48hrs, 72hrs, and 96hrs post-transfection comparing control cells and cells expressing HPV16 E6/E7	164
Figure 6. 9: Knockdown of TRINGS using siRNA in iHFK cells at 24hrs, 48hrs, 72hrs and 96hrs post-transfection comparing control cells and cells expressing HPV16 E6/E7.	165
Figure 6. 10: Knockdown of TRINGS using siRNA in the cell line HCT116 with wild type p53 at 24hrs, 48hrs, 72hrs, and 96hrs post-transfection under normal glucose and glucose starved conditions.....	167
Figure 6. 11: Relative expression levels of TRINGS in a Head and Neck cancer cell line panel.....	169
Figure 6. 12: Relative expression levels of TRINGS in a Head and Neck cancer patient tumours	171
Figure 6. 13: Expression levels of TRINGS in comparing tumours that are HPV+ and HPV- in cervical and oropharyngeal cancers from TCGA database:	171
Figure 6. 14: Interactome depicting the lncRNA TRINGS, HPV16 E6, p53, each of their targets and how they interact with each other.....	173
Figure 6. 15: Gene Ontology of TRINGS/p53/HPV16 E6 interactome:.....	174

Chapter 7:

Figure 7. 1: Flow diagram representing the methodology for processing RAW RNA-seq data from TCGA and development of lncRNA-miRNA-mRNA interactome	188
Figure 7. 2: Flow chart representing the processing and analysis of RNA sequencing data from HPV16 E6/E7 knockdown.....	192
Figure 7. 3: Differentially expressed lncRNAs between HPV16+ and HPV- OPC patients.....	194
Figure 7. 4: Differentially expressed miRNAs between HPV16+ and HPV- OPC patients.....	196
Figure 7. 5: Differentially expressed mRNAs between HPV16+ and HPV negative OPC patients.....	197
Figure 7. 6: HPV16 upregulated lncRNA and the predicted miRNAs they sponge:	200
Figure 7. 7: HPV16 down regulated lncRNA and the predicted miRNAs they sponge:	201
Figure 7. 8 Gene ontology and KEGG analysis of mRNAs targeted by upregulated miRNA sponged by downregulated lncRNAs in HPV16+ Oropharyngeal cancers:	204
Figure 7. 9 Quantitative PCR validation of HPV16 E6/E7 knockdown using several siRNA in the HPV16+ HNC cell line, SCC090:	207
Figure 7. 10: A constructed heat map representing differentially expressed lncRNAs in common between siRNAHPV16 E6/E7 1 and 2 (pink bar) compared to the control (blue bar) in SCC090 cell line.	208
Figure 7. 11: RNF157-AS1 and sponged miRNA interactome:.....	210

Chapter 8:

Figure 8. 1A model depicting HPV16 E6 and E7 interactions with TP53 and the lncRNA TRINGS.....	232
--	-----

List of tables:

Chapter 1:

Table 1. 1: Comparison of HPV positive and HPV negative HNSCC	9
Table 1. 2: Summary of microRNA expression profiles in HPV+ oropharyngeal and oral cancers.....	21
Table 1. 3: Examples of specific lncRNAs and their associated impact on the hallmarks of cancer	37
Table 1. 4: lncRNAs dysregulation in Head and Neck Squamous Cell Carcinomas and their function	43

Chapter 2:

Table 2. 1: Commercially available kits and related reagents used in this study	47
Table 2. 2: Reagents used in this study	48
Table 2. 3: TaqMan probes used in this study (Applied Biosystems, ThermoFisher Scientific, USA).....	50
Table 2. 4: Antibodies used in this study for western blotting	50
Table 2. 5: Random Primer cDNA synthesis protocol	57
Table 2. 6: Specific microRNA primer cDNA Synthesis protocol.....	58
Table 2. 7: TaqMan [®] quantitative Polymerase Chain reaction protocol.....	59
Table 2. 8: Fast SYBR [™] Green quantitative Polymerase Chain reaction protocol.....	60

Chapter 4:

Table 4. 1: Cohort for the study included 30 tumour samples.....	89
Table 4. 2: Identification of miRNAs in HPV+ tonsil SCC were compared to HPV- tonsil SCC tissue.	95
Table 4. 3: Enrichment analysis of miR targets	103

Chapter 5:

Table 5. 1: Sequences for siRNA used in this study.....	113
Table 5. 2: miRNA step loop and primer sequences	114
Table 5. 3: Stem-loop microRNA cDNA Synthesis protocol	115
Table 5. 4: TaqMan [®] quantitative Polymerase Chain reaction protocol.....	115
Table 5. 5: Correlation between SREBF2 and miR-33a expression in different cancer types from the TCGA.....	130

Chapter 6:

Table 6. 1: siRNAs used in the knockdown of TRINGS	153
Table 6. 2 :qPCR primers used in this study:.....	154

Chapter 7:

Table 7. 1: Sequences for siRNA used in this study.....	190
Table 7. 2: Summary of DE lncRNAs from siRNA knockdown compared to TCGA.....	209

Abbreviations:

Abbreviation	Term
ABCC1	ATP Binding Cassette transporter sub-family C
ACTB	Actin Beta
Ago	Argonaut protein
Air	Antisense of IGF2R non protein coding RNA
ANRIL	Antisense Non-Coding RNA in the INK4 locus
AS	Antisense
ATP	Adenosine triphosphate
B2M	Beta-2-Microglobulin
BACH1	BTB Domain and CNC homolog 1
BAK1	BCL2 Antagonist/Killer 1
BAN	4-Bromoanisole
BEND4	BEN domain containing 4
BRCA1	Breast Cancer type 1 susceptibility protein
C19MC	Chromosome 19 microRNA cluster
CAV1	Caveolin 1
CCEPR	Cervical Carcinoma Expressed PCNA Regulator
CDC	Centre for Disease Control
CDK	Cyclin-dependent kinase
CDKN1A	Cyclin-dependent kinase inhibitor 1
CDKN2B	Cyclin dependent kinase inhibitor 2B
cDNA	complementary DNA
CEBP	CCAAT Enhancer Binding Protein
ceRNA	competitive endogenous RNA
CO2	Carbon dioxide
COLCA	Colorectal Cancer Associated RNA
CREBBP	CREB Binding Protein
CTCF	CCCTC Finding factor
CTDSP	CTD small phosphatase like 2
Ctl	Control
DAVID	Database for Annotation Visualisation and Integrated Discovery
DGCR8	Di George critical region 8
DE	Differential Expression
dH2O	deionised H2O
DINO	Damage induced noncoding
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxy ribonucleic acid
dNTP	deoxyribonucleotide triphosphates
DOX	Doxycycline
E2F	E2F transcription factor
EBV	Epstein Barr virus
ECT2	Epithelial Cell Transforming 2 oncogene

EGFL7	EGF like domain multiple 7
EGFR-AS1	Epidermal Growth Factor Receptor antisense RNA 1
EGOT	Eosinophil Granule Ontogeny Transcript
EMT	Epithelial Mesenchymal Transition
EP300	Histone acetyltransferase p300
FACS	Fluorescence Activated Cell Sorting
FAM83H-AS1	Family with sequence similarity 83 member H antisense RNA transcript 1
FC	Fold Change
FCS	Foetal calf serum
FFPE	Fresh Frozen paraffin embedded
FOXC1	Foxhead Box C1
FOXCUT	FOXC1 promoter upstream transcript
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAS5	Growth Arrest Specific 5
GATA2	GATA binding protein 2
GFP	Green Fluorescent Protein
GO	Gene ontology
GSk3B	Glycogen synthase kinase 3 beta
HERC5	HECT and RLD Domain containing E3 Ubiquitin Protein Ligase 5
HFKs	Human Foreskin Keratinocytes
HMGCR	3-Hydroxy-2-Methylglutaryl-CoA Reductase
HMGCS	3-Hydroxy-2-Methylglutaryl-CoA Synthase
HNC	Head and Neck cancer
HNSCC	Head and Neck Squamous cell carcinomas
HOTAIR	HOX Transcript Antisense intergenic RNA
HOTS2	H19 opposite tumour suppressor
HPV	Human papilloma virus
HPV+	HPV positive
HPV-	HPV negative
HRP	horseradish peroxidase
hrs	hours
HSC70	Heat shock cognate 70
HSCP90	Heat shock protein 90
IDT	Integrated DNA Technologies
IGF2	Insulin like growth factor 2
iHFK	Immortalised human foreskin keratinocytes
iNOK	Immortalised normal oral keratinocyte
IRF3	Interferon regulatory factor 3
ISH	Immunohistology chemistry
kb	Kilobase
Kcnq1ot1	KCNQ1 Opposite strand/Antisense transcript 1

KEGG	Kyoto Encyclopedia of Genes and Genomes
KSFM	Keratinocyte serum free medium
LDLR	Low density lipoprotein receptor
lncRNA	Long non-coding RNAs
MAFK	MAF BZIP transcription factor K
MALAT1	Metastasis Associated Lung Adenocarcinoma transcript 1
MCM7	Minichromosomal maintenance complex component 7
MEG3	maternally expressed gene 3
miRNA or miR	MicroRNA
MRE	miRNA response element
mRNA	messenger RNA
mTOR	Mechanistic target of rapamycin kinase
MYC	MYC proto-oncogene, BHLH transcription factor
NCAN	Neurocan
ncRNA	Non-coding RNA
NEAT1	Nuclear paraspeckle assembly transcript 1
oncomiR	Oncogenic miRNA
OPC	Oropharyngeal cancer
OSCC	Oral squamous cell carcinoma
PANDA	Promoter of CDKN1A antisense DNA damage activated RNA
PANDAR	Promoter of CDKN1A antisense DNA damage activated RNA
PBS	Phosphate buffered saline
PCAT7	Prostate Cancer associated transcript 7
PCNA	Proliferative cell nuclear antigen
PCR	polymerase chain reaction
PI3K	Phosphatidylinositol-3-Kinase
piRNAs	PIWI interacting RNAs
pRB	Retinoblastoma protein
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PRINS	Psoriasis Associated non protein coding RNA induced by stress
PRNCR1	Prostate Cancer associated non coding RNA 1
PTEN	Phosphatase and tensin homolog
PTENP1	Phosphatase and tensin homolog pseudogene 1
PTOV1-AS1	PTOV1 antisense RNA 1
PTPN1	Tyrosine-protein phosphatase non receptor type 1
PURPL	P53 upregulated regulator or p53 levels
PVT1	Pvt1 oncogene
RAN	Ras-related nuclear protein
RBL1	Retinoblastoma like protein 1
RISC	RNA induced silencing complex
RNA	Ribonucleic acid

RNA pol	RNA polymerase
RNA-GTP	RNA guanosine triphosphate
RNF157	Ring Finger Protein 157
RNU6B	RNA, U6 small nuclear 6
RPM	Reads per million
RT	Reverse transcription
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SAM	Significance Analysis of Microarrays
SCC	Squamous cell carcinoma
siRNA	Small interfering RNA
SIRT7	Sirtuin 7
SLC7A5	Solute Carrier Family 7-member 5
SNHG12	Small Nucleolar RNA host gene 12
snoRNA	Small nucleolar RNA
SNPs	Single nucleotide polymorphisms
SREBF2	Sterol regulatory binding factor 2
STAT5A	Signal Transducer and activator of transcription 5A
STRAP	Serine/Threonine kinase receptor associated protein
T-UCR	Transcribed ultra-conserved regions
TCGA	The Cancer Genome Atlas
TFBS	Transcription factor binding sites
TFs	Transcription factors
TIRARP	TIR domain containing adaptor protein
TMPOP2	Thymopoietin pseudogene 2
TP53	Tumour protein p53
TRINGS	TP53 regulated inhibitor of necrosis under glucose starvation
TSCC	Tongue squamous cell carcinoma
TTY15	Testis-Specific Transcript, Y-linked 15
UBR4	Ubiquitin Protein Ligase E3 Component N-Recognin-4
UCA1	Urothelial cancer associated 1
UCSC	University of California Santa Cruz
UNSW	university of new south wales
USF1	Upstream Transcription factor 1
UTR	untranslated region
WT	Wild type
XIST	X-inactive specific transcripts
XPO	Exportin
YY1	Yin yang 1

Abstract:

The Human Papillomavirus is a major risk factor for Head and Neck cancers, having a strong association with the Oropharyngeal cancer (OPC) subtype. The high-risk variant, HPV type 16 (HPV16), is the cause for 90% of all HPV related OPCs. Non-coding RNAs, such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), have been shown to have dysregulated expression in OPC. The dysregulated expression on non-coding RNAs has severe consequences on a cell and can lead to tumour development. The aim of this study was to identify specific non-coding RNAs that HPV16 and its viral oncogenes E6 and E7 target in HNC, specifically OPC, and the mechanisms behind their altered expression.

To further our understanding of HPV16s impact on miRNAs, we investigated the expression levels of miRNAs in OPC tissue. We compared miRNA expression between HPV16+ and HPV16- samples using an LNA miRNA array. MiR-496 and miR-33a were found to be the most significantly deregulated. Using a bioinformatics approach, we constructed a novel HPV16-non-coding RNA interactome to identify mechanistic links between miR-33a, SREBF2 and miR-496. Interestingly, SREBF2 harbours miR-33a, and we believe there is a correlation whereby E6 and E7 regulate the expression of SREBF2/miR-33a, which then has a downstream impact on miR-496. TCGA analysis showed that SREBF2 was significantly higher in the HPV+ OPC and not in cervical samples. This suggests this pathway is unique to OPCs. We knocked out HPV16 E6/E7 and SREBF2 using siRNAs and showed that the viral oncogenes could regulate SREBF2 and miR-33a. Although with the knockdown of SREBF2, we saw no change in miR-33a expression, suggesting that miR-33a is not co-expressed with its host gene and is under its own form of regulation by HPV16. This study has identified a regulatory pathway involving HPV16 E6/E7 and specific miRNAs. We also sought to expand our research to include other regulatory RNAs such as long non-coding RNAs.

The lncRNA, TRINGS (TP53 regulated induced by glucose stress), was investigated in HPV16 E6/E7 expressing cells. It was determined that TRINGS expression is decreased with the presence of the viral genes. Using a siRNA knockdown system, we demonstrated that the modulation of TRINGS expression will reduce cell viability

of cervical cancer cell lines and that TRINGS expression could not be stimulated by glucose starvation alone. TRINGS was also determined to be specific for HPV related cervical cancers. We believe that HPV16 targets a different cohort of lncRNAs in HPV related OPC. Given this, we assessed raw RNA-sequencing data from TCGA to identify differentially expressed lncRNAs in HPV16+ OPC. In future research we aim to investigate the function of these lncRNAs in HPV related OPC.

This study has identified the complexity of molecular pathways between HPV16 E6/E7 and non-coding RNAs and the consequences in Head and Neck cancers. The construction of viral interactomes combined with in vitro approaches provided novel insights for discovering mechanistic links in HPV16+ Head and Neck cancers.