

TABLE OF CONTENTS

DECLARATION.....	ii
PUBLICATIONS.....	iii
ABSTRACT.....	iv
DEDICATION.....	v
ACKNOWLEDGEMENTS.....	vi
ABBREVIATIONS	xii
LIST OF TABLES.....	xvi
LIST OF FIGURES	xvii
1. CHAPTER 1: INTRODUCTION.....	1
1.1 DWNN.....	1
1.2 APOPTOSIS	4
1.2.1 Extrinsic apoptosis pathway	4
1.2.1.1 TNF signalling pathway.....	6
1.2.1.2 TRAIL signalling pathway	9
1.2.2 Intrinsic apoptotic pathway.....	10
1.2.3 Genetic regulation of apoptosis	12
1.2.3.1 p53 tumour suppressor gene	12
1.2.3.2 Bcl-2 gene family.....	13
1.2.3.3 c-Myc	17
1.3 UBIQUITIN-LIKE PROTEINS.....	17
1.3.1 Ubiquitin-proteasome pathway	17
1.3.2 Significance of ubiquitin proteasome pathway in.....	19
(i) Cancer.	19
(ii) Apoptosis	19
1.4 CERVICAL CANCER.....	21
1.4.1 Prevalence	21
1.4.2 Risk factors	22

1.4.3	Morphology.....	22
1.4.4	Histopathology.....	23
1.4.5	Stem cells and stem-cancer cells (carcinogenesis)	24
1.5	HUMAN PAPILLOMAVIRUS.....	26
1.5.1	Structure.....	26
1.5.2	Viral Risk.....	29
1.6	GENOME AND GENOMICS	29
1.7	GENOMICS OF CERVICAL CANCER.....	30
1.8	ONCOGENES	31
1.8.1	HPV E7 and Rb protein	32
1.8.2	HPV-mediated immune evasion mechanism.....	34
1.8.3	Interaction of HPV E6 with transcription regulators	35
1.8.4	Interaction of HPV E6: small molecules-protein kinase, p21 and proliferating cell nuclear antigen (PCNA) bind E6.	35
1.8.5	HPV E6 and PDZ domain-containing protein	36
1.9	GENETIC REGULATION OF CERVICAL CANCER THROUGH APOPTOSIS	37
1.9.1	Degradation of HPV E6 oncogene by p53.....	37
1.9.2	HPV E7 interaction with Bak	39
1.9.3	HPV E6 and telomerase activity.....	39
1.9.4	HPV E2 and telomerase.....	40
1.10	INTERACTION OF HPV WITH CELL SURFACE RECEPTORS.....	42
1.11	THERAPEUTIC STRATEGIES FOR CERVICAL CANCER.....	46
1.11.1	Vaccination	46
1.11.2	Immunotherapy.....	48
1.12	AIM AND OBJECTIVES OF THE STUDY	49
2	CHAPTER 2: MATERIALS AND METHODS	50
2.1	SAMPLE COLLECTION	50
2.2	IN SITU HYBRIDIZATION	50
2.2.1	Probe Synthesis.....	50
2.2.1.1	RNA Extraction	50

2.2.1.2	Formaldehyde Agarose Gel Electrophoresis	52
2.2.1.2.1	Preparation of RNA-Agarose Gel	52
2.2.1.3	Reverse Transcription	52
2.2.1.4	Polymerase Chain Reaction (PCR).....	54
2.2.1.5	DNA Agarose Gel Electrophoresis of DNA.....	55
2.2.1.6	Cloning of the PCR product into a pGEM-T-Easy vector.....	55
2.2.1.6.1	Ligation	55
2.2.1.6.2	Transformation.....	56
2.2.1.7	Colony PCR	57
2.2.1.8	Miniprep – Plasmid DNA Extraction	58
2.2.1.9	Restriction Digestion	59
2.2.1.10	Linearization of the Clone	60
2.2.1.11	Purification of DNA from Agarose Gel.....	61
2.2.1.12	Digoxigenin (DIG) Labelling of DWNN Clones.....	62
2.2.1.13	Estimation of Minimal Probe Concentration	64
2.2.1.14	Concentration Estimation of the Probe	65
2.2.1.15	Localization of 5' 1.1 kb, 3' 1.1 kb, 3' 6.1 kb DWNN and Alternatively Spliced Exon 16 mRNA by In Situ Hybridization.....	66
2.2.1.15.1	Pre-Hybridization Treatment of Section.....	66
2.2.1.15.2	Hybridization	67
2.2.1.15.3	Post-Hybridization	67
2.2.2	Localization of the 13 kDa and 200 kDa DWNN Protein	69
2.2.2.1	Antibody Profiles	69
2.2.2.1.1	Anti-DWNN Antibody.....	69
2.2.2.1.2	Anti-Bcl-2 Antibody	70
2.2.2.2	Immunocytochemistry Controls.....	70
2.2.2.2.1	Negative Control.....	70
2.2.2.2.2	Positive Control	70
2.2.2.3	Immunocytochemistry	70
2.2.2.3.1	Colorimetric Method for Immunocytochemistry.....	71
2.2.3	Detection of Apoptosis	72

2.2.4	Proliferation Assay with Ki67 Antigen.....	74
2.2.5	Light Cycler RT-PCR.....	76
2.2.5.1	RNA Extraction.....	76
2.2.5.2	cDNA Synthesis.....	77
2.2.5.3	Real-time PCR with Light Cycler.....	77
	APPENDIX 1: pGEM-T-EASY VECTOR MAP.....	79
	APPENDIX 2: REAGENTS AND SOLUTIONS.....	80
	APPENDIX 3: STRUCTURE OF DWNN GENE WITH THE ARROWS INDICATING PRIMER SITES. A- 5' 1.1 KB, B- 3' 1.1 KB, C- 3'6.1 KB AND D- EXON 16.....	85
3	CHAPTER 3: RESULTS.....	86
3.1	HISTOPATHOLOGY.....	86
3.1.1	Normal Squamous Epithelium.....	86
3.1.2	Squamous cell carcinoma.....	86
3.1.2.1	Epidermoid cervical carcinoma.....	86
3.1.2.2	Verrucous carcinoma.....	86
3.1.2.3	Carcinoma in situ.....	87
3.2	DWNN mRNA EXPRESSION PATTERN IN HUMAN CERVICAL CANCER.....	90
3.2	INTRODUCTION.....	90
3.2.1	RNA Extraction.....	90
3.2.2	Reverse Transcription (RT) - Analysis.....	90
3.2.3	PCR amplification of DWNN gene sequence.....	91
3.2.4	Cloning of DWNN (3'6.1 kb, 3'1.1 kb, 5'1.1 kb and Exon 16) sequences into pGem-T-Easy.....	91
3.2.5	In situ hybridization.....	94
3.2.6	Summary.....	97
3.3	TISSUE DISTRIBUTION OF THE DWNN PROTEIN IN HUMAN CERVICAL CANCER.....	107
3.3.1	Introduction.....	107
3.3.2	Antibody profiles.....	108
3.3.2.1	Anti-DWNN antibody.....	108
3.3.2.2	Anti- Bcl-2 antibody.....	108
3.3.3	Immunocytochemical localisation of DWNN protein.....	109

3.3.3.1	DWNN-13 kDa	109
3.3.3.2	DWNN-200 kDa	111
3.3.3.3	Localisation of Bcl-2 protein	111
3.3.4	Summary	114
3.4	IMAGE ANALYSIS	115
3.5	APOPTOSIS DETECTION USING TUNEL.....	124
3.5.1	Introduction.....	124
3.5.2	Apoptotic activity.....	124
3.5.3	Summary	125
3.6	TUMOUR PROLIFERATION ASSAY USING KI67 ANTIGEN.	128
3.6.1	Introduction.....	128
3.6.2	Localisation of ki67 protein.....	129
3.6.3	Summary	130
3.7	LIGHTCYCLER PCR: CONFIRMS DWNN EXPRESSION LEVELS.....	133
4	Chapter 4: General Discussion	136
4.1	INTRODUCTION	136
4.2	DWNN EXPRESSION AND LOCALIZATION IN CERVICAL CANCER.	138
4.2.1	In situ hybridization	138
4.2.2	Immunocytochemistry	141
4.2.3	Image analysis.....	141
4.3	APOPTOSIS DETECTION USING TUNEL.	143
4.4	PROLIFERATION ASSAY USING KI67 EXPRESSION	145
4.5	BCL-2 EXPRESSION.....	146
4.6	LIGHTCYCLER RT-PCR.....	147
4.7	DWNN PARTIAL cDNAs.....	148
4.8	DWNN AS A UBIQUITIN LIGASE.....	149
5	CHAPTER 5: CONCLUSION	151
6	CHAPTER 6: REFERENCES	153