

BIOCHEMICAL CHARACTERIZATION OF THE INTERACTION BETWEEN
CTCF/YB-1 TRANSCRIPTION FACTORS WITH HPV 16 AND 18 E7
ONCOPROTEIN AND THEIR INVOLVEMENT DURING C-MYC GENE
REGULATION IN CELL PROLIFERATION PATHWAY

by

VENUGOPAL BALAKRISHNAN

Thesis submitted in the fulfillment of the requirements
for the degree of
Doctor of Philosophy

August 2008

ACKNOWLEDGEMENTS

First and foremost I would like to express my deepest gratitude to my main supervisor, Dr. Shaharum Shamsuddin, who has contributed fruitful ideas, comments, continuous motivation and excellent technical assistance towards the completion of this thesis. I would also like to thank my co-supervisor, Prof. (Dr.) Nor Hayati Othman for her assistance, guidance and supervision throughout this PhD study. Furthermore, I would like to extend my gratitude to Institute of Postgraduate Studies, USM, for offering me with Pasca-siswazah scholarship as the financial assistance.

I am thankful to Assoc. Prof. Nik Soriani Yaacob and Prof. Norazmi Mohd Nor for allowing me to use the cell culture facilities at PPSK, USM. The excellent graphic work and precious time spent on producing few of the illustration in the thesis by Mr. Muhammad Ismail is greatly appreciated. I would like to express my sincere gratitude to my lab mates and friends Syaiful, Arnida, Hadi, Kuru, Khairul and Zul for the motivation and help during my PhD program.

This thesis is dedicated to my beloved wife, Kogila Vaani and my mother, Pushpavathi for their love, encouragement and support in completing this thesis. I hope both of their prayers and hard times have paid off. Finally, I am grateful to my family members too for cheering me to pass the finishing line.

TABLE OF CONTENT

ACKNOWLEDGEMNT	ii
CONTENT	iii
LIST OF TABLES	xii
LIST OF FIGURES	xiv
ABSTRACT	xx
ABSTRAK	xxii
Chapter 1: Introduction.....	1
1.1 Research Background and Hypothesis.....	1
1.1.1 Human Papillomavirus and cervical cancer.....	1
1.1.2 CTCF transcription factor.....	3
1.1.3 YB-1 transcription factor.....	4
1.1.4 Synergistic effect of CTCF and YB-1 towards <i>c-myc</i> oncogene.....	5
1.2.1 Cervical cancer and cellular mechanism.....	10
1.2.1.1 The p16 ^{INK4A} -cyclin D1-CDK4/6- pRb-E2F pathway.....	11
1.2.1.1.1 p16 ^{INK4A}	11
1.2.1.1.2 Cyclin D1.....	13
1.2.1.1.3 CDK4.....	15
1.2.1.1.4 pRb/E2F.....	16
1.2.1.2 The p21 ^{WAF1/CIP1} -p27 ^{KIP1} -cyclin E-CDK2 pathway.....	18
1.2.1.2.1 p21 ^{WAF1/CIP1}	18
1.2.1.2.2 p27 ^{KIP1}	19
1.2.1.2.3 Cyclin E.....	21
1.2.1.2.4 CDK2.....	22
1.2.1.3 The p14 ^{ARF} -MDM2-p53 pathway.....	24
1.2.1.3.1 P14 ^{ARF}	24
1.2.1.3.2 MDM-2.....	25
1.2.1.3.3 p53.....	27
1.2.1.4 Other Cellular Factors.....	28
1.2.1.4.1 Cyclin A.....	28
1.2.1.4.2 Cyclin B.....	29
1.3 Objectives of the project.....	30
Chapter 2: General Material and Methods.....	31
2.1 DNA Materials and vectors.....	31
2.2 Recombinant DNA Methods.....	32
2.2.1 Oligonucleotide synthesis.....	32

2.2.2	Extraction of the plasmid DNA with the QIAprep Spin Miniprep Kit	32
2.2.3	Extraction of the plasmid DNA: Alkaline lysis method	33
2.2.4	Restriction enzyme digestion of plasmid DNA	34
2.2.5	Agarose gel electrophoresis of plasmid DNA	35
2.2.6	Purification of DNA from agarose gel using QIAquick gel extraction kit	35
2.2.7	Nucleic acid concentration determination	36
2.2.8	Dephosphorylation of plasmid DNA	36
2.2.9	Filling the 5' end of the DNA with the Klenow enzyme	37
2.2.10	Blunt end ligation of plasmid DNA	38
2.2.11	Addition of synthetic adapter to protruding termini	38
2.2.12	Directional Cloning into Plasmid vector.....	39
2.2.13	Polymerase Chain Reaction (PCR).....	39
2.2.13.1	Determination of annealing parameters for PCR.....	41
2.2.13.2	Purification of PCR product using Qiaquick PCR purification	41
2.2.13.3	Cloning of PCR product into pCR 2.1 TOPO (Invitrogen Inc.)	42
2.3	Microbiological methods	43
2.3.1	Media and solutions	43
2.3.2	Bacterial strains.....	43
2.3.3	Freezing and storage of <i>Escherechia coli</i> (<i>E. coli</i>) cells.....	43
2.3.4	Preparation of competent <i>Escherechia coli</i> (<i>E. coli</i>) cells and transformation	44
2.3.5	Transformation of bacterial cell using XL1-Blue <i>E. coli</i> (Stratagene, USA) ..	45
2.4	Bacterial protein expression and analysis	48
2.4.1	Expression of recombinant protein using pET bacterial system (Novagen,....	48
2.4.2	Expression of recombinant proteins using GST system	49
2.5	Protein Analysis	50
2.5.1	Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis.....	50
2.5.2	Staining of SDS PAGE	53
2.5.2.1	Coomassie Brilliant Blue staining	53
2.5.2.2	Silver staining (Heukeshoven and Dernick, 1995)	53
2.5.3	Western blotting assay	54
2.5.4	Estimation of protein concentration (BioRad).....	55
2.6	Biochemical and Immunochemical Methods.....	55
2.6.1	Immobilized Metal Affinity Chromatography (IMAC) (Sulkowski, 1985)	55
2.6.1.1	Preparation of IDA-Agarose resin (Sigma, Germany)	55
2.6.1.2	Preparation of IDA-Sepharose (SIGMA, Germany)	56
2.6.1.3	His-tagged protein purification: lysate preparation, washing and elution	57
2.6.1.4	Dialysis of His-tagged proteins.....	58
2.6.2	Covalent coupling of proteins.....	58
2.6.2.1	Coupling of bacterially expressed protein to thiol-activated sepharose ...	59
2.6.2.2	Thiol groups.....	59
2.6.2.3	Thiol-thiol coupling via disulfide exchange	60
2.6.2.4	Preparation of thiol-activated Sepharose	62
2.6.2.5	Preparation of His-tagged protein for coupling	63
2.6.2.6	Desalting Procedure	63
2.6.2.7	Coupling of protein to thiol-activated Sepharose	64
2.6.2.8	Blocking procedure for coupled protein	64

2.6.3 Coupling of bacterially expressed GST fused protein to Glutathione-Sepharose	65
2.6.3.1 Preparation of Glutathione-Sepharose 4B	65
2.6.3.2 Binding of GST fused protein to Glutathione-Sepharose 4B	65
2.6.4 Interaction assay.....	66
2.6.4.1 <i>In-vitro</i> pull down interaction assay	66
2.6.4.2 <i>In-vivo</i> interaction assay: Coimmunoprecipitation (Co-IP).....	66
2.6.4.3 Phosphorylation of proteins with CK2 <i>in-vitro</i>	67
2.7 Cellular Biology techniques.....	68
2.7.1 Cell lines	68
2.7.2 Preparation of medium.....	68
2.7.3 Thawing cells.....	69
2.7.4 Sub-culturing of cells.....	71
2.7.5 Freezing cells	71
2.7.6 Preparation of total cell lysates	72
2.7.6.1 Lysis Method 1 (single-step lysis for extraction of total cellular protein) 72	
2.7.6.2 Lysis Method 2 (nuclear and cytoplasmic protein extraction).....	73
2.7.6.3 Lysis Method 3 (2-step lysis).....	74
2.7.6.4 Lysis Method 4 (RIPA lysis buffer).....	74

Chapter 3: Production truncated and full domain of E7 oncoproteins and truncated E6 oncoproteins of HPV 16 and 18 in bacterial expression system

3.1 Introduction.....	76
3.1.1 Human Papillomavirus (HPV).....	76
3.1.1.1 Progression to malignancies	80
3.1.1.2 E2 Protein.....	82
3.1.1.3 E6 Protein.....	83
3.1.1.4 Protein E7.....	88
3.1.2 Objectives	92
3.2 Materials and Methods.....	93
3.2.1 Cloning of HPV16 and 18, E6 and E7 truncated and E7 full domain proteins 93	
3.2.1.1 Establishment of pET16bSH3(Cys) vector.....	96
3.2.1.2 Genomic DNA preparation from HeLa and CasKi cells	96
3.2.1.3 Primer design for HPV-16 and -18, E6 and E7 truncated and E7 full domain genes	98
3.2.1.4 PCR amplification of HPV-16 and -18, E6 and E7 truncated and E7 full domain genes	99
3.2.1.5 Cloning of amplified fragments of 16 E7FL, 16 E6Trunc, 16 E7Trunc, 18 E7FL, 18 E6Trunc and 18 E7Trunc genes.....	100
3.2.1.6 Subcloning of fragments of 16 E7FL, 16 E6Trunc, 16 E7Trunc, 18 E7FL, 18 E6Trunc and 18 E7Trunc genes into pET16SH3(Cys) vector	101
3.2.2 Expression of 16 E7FL, 16 E6Trunc, 16 E7Trunc, 18 E7FL, 18 E6Trunc and 18E7Trunc domains in bacterial expression system	101
3.2.3 Purification of the bacterially expressed 16 E7FL, 16 E6Trunc, 16 E7Trunc, 18E7FL, 18 E6Trunc and 18 E7Trunc proteins by IMAC	102
3.3 Results.....	103

3.3.1 PCR amplification of 16 E7FL, 16 E6Trunc, 16 E7Trunc, 18 E7FL, 18 E6Trunc and 18 E7Trunc domains	103
3.3.2 Cloning of the amplified fragments of 16 E7FL, 16 E6Trunc, 16 E7Trunc, 18E7FL, 18 E6Trunc and 18 E7Trunc domains	103
3.3.2.1 Cloning and insert digestion in pCR [®] 2.1 TOPO [®] vector	103
3.3.2.2 Sub-cloning of PCR product into pET16bSH3(Cys) clones and sequence verification	104
3.3.3 Expression of 16 E7FL, 16 E6Trunc, 16 E7Trunc, 18 E7FL, 18 E6Trunc and 18E7Trunc domains of HPV in bacterial system.....	104
3.3.4 Purification of the bacterially expressed 16 E7FL, 16 E6Trunc, 16 E7Trunc, 18E7FL, 18 E6Trunc and 18 E7Trunc proteins by IMAC	105
3.4 Discussion	118
3.4.1 Construction of the Human Papillomavirus genes.....	118
3.4.2 Optimization of the expression condition for the truncated and full domains HPV.....	119
3.4.3 Bacterially expressed proteins of HPV migrate aberrantly in SDS-PAGE ...	120
3.4.4 IMAC purification of bacterially expressed truncated and full domains of HPV proteins.....	122
3.5 Conclusion	125

Chapter 4: Production of YB-1 CSD and YB-1 CDTrunc in PET expression system and expression of YB-1FL, YB-1(3) and YB-1 (4) in GST expression system

4.1 Introduction.....	126
4.1.1 YB-1.....	126
4.1.1.1 dbpA : organization, structure and function	130
4.1.1.2 dbpB (YB-1) : organization, structure and function.....	131
4.1.2 Objectives	133
4.2 Materials and Methods.....	134
4.2.1 Production of the YB-1 protein in a bacterial system.....	134
4.2.1.1 Cloning of YB-1 CSD and YB-1 CDTrunc domain proteins.....	134
4.2.1.2 Primer design for YB-1 CSD and YB-1 CDTrunc domain genes.....	134
4.2.1.3 PCR amplification of YB-1 CSD and YB-1 CDTrunc domain genes....	135
4.2.1.4 Cloning of amplified fragments of YB-1 CSD and YB-1 CDTrunc	136
4.2.1.5 Subcloning of fragments of YB-1 CSD and YB-1 CDTrunc genes into pET16bSH3(Cys) vector.....	136
4.2.1.6 Expression of YB-1 CSD and YB-1 CDTrunc domains in bacterial expression system	137
4.2.1.7 Purification of the bacterially expressed YB-1 CSD and YB-1 CDTrunc proteins by Immobilized Metal Affinity Chromatography (IMAC).....	137
4.2.2 Glutathione-S-Transferase (GST) gene fusion system	137
4.2.2.1 Expression of the full length YB-1 protein using GST system	138
4.2.2.1.1 Double digestion of pGEX-2TK~YB1 (YB-1 FL).....	139
4.2.2.1.2 Identification of GST fused YB-1 expressed in <i>E. coli</i>	139
4.2.2.2 Expression of the truncated versions of the YB-1 protein using the GST bacterial system.....	142

4.2.2.2.1 Digestion of pGEX-2T~YB-1 truncated versions	142
4.2.2.3 Expression of the truncated YB-1 in <i>E. coli</i>	142
4.2.3 Purification of full length and truncated YB-1 by immobilization	143
4.3 Results.....	145
4.3.1 Amplification, cloning, expression and purification of YB-1 CSD and YB-1CDTrunc domains	145
4.3.1.1 PCR amplification of YB-1 CSD and YB-1 CDTrunc domains	145
4.3.1.2 Cloning of the amplified fragments of YB-1 CSD and YB-1 CDTrunc domains	145
4.3.1.2.1 Cloning and insert digestion in pCR [®] 2.1 TOPO [®] vector	145
4.3.1.2.2 Sub-cloning, insert digestion and sequencing of pET16bSH3(Cys) clones	146
4.3.1.3 Expression of YB-1 CSD and YB-1 CDTrunc domains of YB-1 in bacterial expression system.....	150
4.3.1.4 Purification of the bacterially expressed YB-1 CSD and YB-1 CDTrunc proteins by IMAC	150
4.3.2 Production of YB-1 FL, YB-1 (3) and YB-1 (4) proteins using GST bacterial expression system.....	153
4.3.2.1 Characterization of YB-1 FL, YB-1 (3) and YB-1 (4) plasmids.....	153
4.3.2.2 Expression of YB-1 FL, YB-1 (3) and YB-1 (4) domains in bacterial expression system	153
4.3.2.3 Purification of YB-1 FL, YB-1 (3) and YB-1 (4) proteins using immobilization on Glutathione-Sepharose 4B.....	156
4.4 Discussion.....	158
4.4.1 Production of the YB-1 CSD and YB-1 CDTrunc proteins	158
4.4.1.1 Construction of the YB-1 CSD and YB-1 CDTrunc genes in pET16SH3(Cys) bacterial expression vector.....	158
4.4.1.2 Expression and IMAC purification of YB-1 CSD and YB-1 CDTrunc domain proteins.....	159
4.4.2 Expression and purification of YB-1 FL, YB-1 (3) and YB-1 (4) proteins...	161
4.5 Conclusion	162

Chapter 5: Expression and purification of truncated human CTCF domains in bacterial expression system.....	163
5.1 Introduction.....	163
5.1.1 CTCF.....	163
5.1.1.1 General characteristic features of CTCF, a multivalent transcription factor	163
5.1.1.2 The biological role of CTCF in the cell	171
5.1.1.2.1 CTCF as a transcriptional factor.....	171
5.1.1.2.2 CTCF is an insulator protein: boundary controller.....	174
5.1.1.2.3 Histone acetylation/deacetylation and transcription regulation: link with CTCF	176
5.1.1.3 Genetic imprinting: New role of CTCF	177
5.1.1.3.1 Role of CTCF in genetic imprinting.....	178
5.1.1.3.2 Cancer epigenetics: Potential link of CTCF to cancer development	181

5.1.1.4	CTCF regulation by post-translational modifications.....	181
5.1.1.4.1	CTCF regulation by phosphorylation	181
5.1.1.5	CTCF regulation by acetylation and Poly(ADP-ribosyl)ation.....	183
5.1.1.6	Proteins partners of CTCF	184
5.1.2	Objectives	184
5.2	Materials and Methods.....	185
5.2.1	Expression of CTCF-N (amino terminal domain), CTCF-Zn (Zinc finger domain) and CTCF-C (carboxyl terminal domain) truncated domains of hCTCF using pET expression system.....	185
5.2.1.1	Digestion of CTCF-N, CTCF-Zn and CTCF-C with restriction enzymes.....	185
5.2.1.2	Expression of CTCF-N, CTCF-Zn and CTCF-C domains	185
5.2.2	Purification of the bacterially expressed CTCF-N, CTCF-Zn, CTCF-C proteins by IMAC	186
5.3	Results.....	187
5.3.1	Characterization of CTCF-N, CTCF-Zn and CTCF-C plasmids.....	187
5.3.2	Expression of CTCF-N, CTCF-Zn, CTCF-C domains of human CTCF in bacterial expression system.....	187
5.3.3	Purification of the bacterially expressed CTCF-N, CTCF-Zn, CTCF-C domains of human CTCF proteins by IMAC	189
5.4	Discussion	192
5.5	Conclusion	195

Chapter 6: Production of HPV16 E7, HPV18 E7, YB-1 CSD, YB-1 CDTrunc and CTCF-Zn Polyclonal antibody	196
6.1 Introduction.....	196
6.1.1 Background.....	196
6.1.2 Objectives	199
6.2 Materials and Methods.....	200
6.2.1 Preparation of the purified CTCF-Zn, YB-1 CSD, YB-1 CDTrunc, HPV16 E7Trunc and HPV18 E7Trunc domains for immunization	200
6.2.2 Preparation of nuclear extracts from 293T and HeLa cells	200
6.2.3 Immunization protocol (Harlow and Lane, 1988)	200
6.2.4 Immunization and blood collection	201
6.2.5 Purification of HPV16 E7 and HPV18 E7 antibody (Montage [®] Antibody Purification Kit, Millipore, USA).....	202
6.3 Results.....	203
6.3.1 Preparation of the purified and dialysed CTCF-Zn, YB-1 CSD, YB-1 CDTrunc, HPV16 E7Trunc and HPV18 E7Trunc domains	203
6.3.2 Analysis of the sera by western blotting.....	203
6.3.3 Purification of α -16E7-HPV and α -18E7-HPV antibodies.....	205
6.3.4 Characterization of the specificity and efficiency of the α -Zn-CTCF, α -CSD-(YB-1), α -CD-(YB-1), α -16E7-HPV and α -18E7-HPV antibodies	210
6.4 Discussion.....	216
6.4.1 Preparation of bacterially expressed truncated proteins: CTCF-Zn, YB-1 CSD, YB-1 CDTrunc, HPV16 E7Trunc and HPV18 E7Trunc	216

6.4.2 Production of polyclonal antibody.....	217
6.4.3 Purification of α -16E7-HPV and α -18E7-HPV	219
6.4.4 Characterization of the α -Zn-CTCF, α -CSD-(YB-1), α -CD-(YB-1), α -16E7-HPV and α -18E7-HPV antibodies	220
6.5 Conclusion	223

Chapter 7: *In-vivo* and *in-vitro* interaction of CTCF/YB-1 with HPV 16 and 18 E7 oncoproteins.....

7.1 Introduction.....	224
7.1.1 Protein-protein interactions in biological processes	224
7.1.1.1 Methods and basic concepts for the study of protein-protein Interaction.....	226
7.1.1.2 Protein-protein interactions in transcriptional regulation	228
7.1.2 Oncogene or proto-oncogene.....	230
7.1.2.1 c-Myc	231
7.1.2.2 c-Myc and the cell cycle	237
7.1.2.2.1 Control of c-Myc protein expression in G ₁ -S phase of cell cycle.....	238
7.1.2.2.2 Control of G ₁ progression of cell cycle by c-Myc protein.....	242
7.1.2.2.2 (a) Cyclin E complexes.....	242
7.1.2.2.2 (b) p27 ^{KIP1} and p21 ^{WAF1/CIP1} (KIP/CIP Protein).....	243
7.1.2.2.2 (c) Cyclin D complexes	244
7.1.2.2.2 (d) Cdc25a.....	245
7.1.2.2.2 (e) Rb and Id2	246
7.1.2.2.3 c-Myc protein inhibit cell differentiation (cell cycle arrest).....	246
7.1.2.3 c-Myc and cancer	249
7.1.2 Objectives	250
7.2 Materials and Methods.....	251
7.2.1 Coupling of bacterially expressed CTCF-N, CTCF-Zn, CTCF-C, HPV16 E7FL, HPV18 E7FL and YB-1 CSD protein to thiol-activated sepharose... 251	
7.2.2 Determination of native capability of bacterially expressed protein: <i>In-vitro</i> Pull-down Interaction Assay for TS-HPV16 E7FL, TS-HPV18 E7FL and YB-1 CSD	251
7.2.3 <i>In-vivo</i> Interaction: Co-Immunoprecipitation (Co-IP).....	252
7.2.4 <i>In-vitro</i> Interaction: Pull-down Interaction Assay	252
7.2.5 <i>In-vitro</i> interaction assay between HPV16 E7 and HPV18 E7.....	254
7.2.5.1 Co-Immunoprecipitation (Co-IP).....	254
7.2.5.2 <i>In-vitro</i> Pull-down analysis.....	254
7.2.5.2.1 Interaction of coupled protein TS-HPV16 E7FL with HeLa cell lysate	254
7.2.5.2.2 Interaction of Coupled Protein TS-HPV18 E7FL with Bacterially Expressed Protein HPV16 E7FL	255
7.2.6 <i>In-vitro</i> Phosphorylation of CTCF-Zn, YB-1 CSD, HPV16 E7FL and HPV18 E7FL using Casien Kinase II enzyme (CK2)	255
7.2.7 Pull-down Experiments using <i>in-vitro</i> phosphorylated HPV16 E7FL and HPV18 E7FL domains with CTCF-Zn and YB-1 CSD.....	255
7.2.8 Electro Mobility Shift Assay (EMSA).....	256

7.3 Result	259
7.3.1 Coupling of Bacterially Expressed Proteins to Thiol-Activated Sepharose ..	259
7.3.2 Determination of native capability of bacterially expressed and coupled protein TS-HPV16 E7FL, TS-HPV18 E7FL and YB-1 CSD.....	260
7.3.2.1 Pull-down assay of TS-HPV16 E7FL and TS-HPV18 E7FL with HeLa lysate	260
7.3.2.2 Pull-down assay of TS-YB-1 CSD with 293T lysate	261
7.3.3 <i>In-vivo</i> Interaction: Co-Immunoprecipitation (Co-IP).....	263
7.3.3.1 Co-IP using α -HPV16 E7 and α -HPV18 E7: detected with α -CTCF antibody (monoclonal).....	263
7.3.3.2 Co-IP using α -HPV16 E7 and α -HPV18 E7: detected with α -YB-1 antibody (polyclonal).....	264
7.3.4 <i>In-vitro</i> Interaction: Pull-down Interaction Assay	266
7.3.4.1 Pull-down Interaction Assay: Immobilised CTCF domains with HPV16 E7FL and HPV18 E7FL.....	266
7.3.4.2 Pull-down Interaction Assay: Immobilised YB-1 domains with HPV16 E7FL and HPV18 E7FL.....	267
7.3.4.3 Pull-down Interaction Assay: Immobilised CTCF-Zn domain with HPV16 E7FL, HPV18 E7FL and YB-1 CSD.....	269
7.3.5 <i>In-vivo</i> and <i>in-vitro</i> interaction between HPV16 E7FL and HPV18 E7FL ...	272
7.3.5.1 Co-Immunoprecipitation (Co-IP).....	272
7.3.5.2 <i>In-vitro</i> interaction of HPV16 E7FL with HPV18 E7 from HeLa cells lysate	272
7.3.5.3 <i>In-vitro</i> interaction of HPV18 E7FL and HPV16 E7FL.....	273
7.3.6 <i>In-vitro</i> Phosphorylation of CTCF-Zn, YB-1 CSD, HPV16 E7FL and HPV18 E7FL using Casien Kinase II enzyme (CK2)	275
7.3.7 Effects of phosphorylation on HPV16 E7FL and HPV18 E7FL interaction to CTCF-Zn and YB-1 CSD	277
7.3.8 Protein DNA interaction assay: Electro Mobility Shift Assay (EMSA).....	277
7.3.8.1 EMSA: Gradual introduction of HPV16 E7FL and HPV18 E7FL proteins effect the binding of CTCF~YB-1 at FpV cognate site	278
7.3.8.2 EMSA: HPV16 E7FL and HPV18 E7FL proteins was added to the mixture	283
7.4 Discussion	286
7.4.1 Coupling of bacterially expressed protein to thiol-activated sepharose	286
7.4.2 Determination of native capability of bacterially expressed and coupled protein TS-HPV16 E7FL, TS-HPV18 E7FL and TS-YB-1 CSD.....	287
7.4.2.1 <i>In-vitro</i> interaction of HPV16 E7FL and HPV18 E7FL with pRb	287
7.4.2.2 <i>In-vitro</i> interaction of YB-1 CSD with CTCF	288
7.4.3 <i>In-vivo</i> Interaction of (HPV16 and HPV18) E7 proteins to CTCF/YB-1: Co-Immunoprecipitation (Co-IP).....	289
7.4.3.1 Co-IP using α -HPV16 E7 and α -HPV18 E7: detection with α -CTCF ...	290
7.4.3.2 Co-IP using α -HPV16 E7 and α -HPV18 E7: detection with α -YB-1.....	291
7.4.4 <i>In-vitro</i> Interaction: Pull-down Interaction Assay	292
7.4.4.1 Pull-down Interaction Assay: Immobilised CTCF domains with HPV16 E7FL and HPV18 E7FL (bacterially expressed)	293

7.4.4.2 Pull-down Interaction Assay: Immobilised YB-1 domains with HPV16 E7FL and HPV18 E7FL (bacterially expressed)	294
7.4.4.3 Pull-down Interaction Assay: Immobilised CTCF-Zn domain with HPV16 E7FL, HPV18 E7FL and YB-1 CSD.....	295
7.4.5 Interaction assay of HPV16 E7FL and HPV18 E7FL	302
7.4.5.1 <i>In-vivo</i> interaction of HPV16 E7 and HPV18 E7: Co-IP	303
7.4.5.2 Interaction of coupled protein TS-HPV16 E7FL with HeLa cell lysate	303
7.4.5.3 <i>In-vitro</i> interaction HPV18 E7FL with HPV16 E7FL (bacterially expressed)	304
7.4.6 <i>In-vitro</i> Phosphorylation of CTCF-Zn, YB-1 CSD, HPV16 E7FL and HPV18 E7FL using Casien Kinase II enzyme (CK2)	306
7.4.7 Pull-down assay with <i>in-vitro</i> phosphorylated HPV16 E7FL and HPV18 E7FL domains with immobilised CTCF-Zn and YB-1 CSD.....	307
7.4.8 Effects of E7 proteins physical interaction towards CTCF~YB-1 binding: (EMSA)	309
7.4.8.1 Gradual introduction of HPV16 E7FL and HPV18 E7FL proteins.....	310
7.4.8.2 Effects of simultaneous addition of both HPV16 E7FL and HPV18 E7FL proteins towards the CTCF~YB-1 binding.....	311
7.4.9 Possible role of CTCF~HPV16 E7~HPV18 E7 interactions in tumourigenesis	313
7.5 Conclusion	315
Chapter 8: General discussion and conclusion	316
8.1 General discussion of this study	316
8.2 Conclusion	319
8.3 Future work.....	320
8.4 Limitations in this study.....	322
8.5 Clinical Significance.....	322
 REFERENCES	 323
 APPENDIX	 375

LIST OF TABLES

Table 2.1:	DNA and vectors used in this research.....	31
Table 2.2:	Restriction enzyme digestion mixture.....	34
Table 2.3:	Procedure for ligation using T ₄ DNA Ligase.....	39
Table 2.4:	Components for PCR mixture.....	40
Table 2.5:	Components for direct cloning of PCR product with pCR 2.1 TOPO	42
Table 2.6:	Standard media and solutions used in this study	46
Table 2.7:	List of bacterial strains and their features.....	47
Table 2.8:	Stock solution for SDS-PAGE preparation (Laemmli, 1970)	51
Table 2.10:	Formulation for SDS-PAGE Separating Gels [Recipes prepared solution_sufficient for 2 minigels, 10 mL each (1.5 mm thick)].....	52
Table 2.11:	Stacking gel recipe (for 2 gels preparation).....	52
Table 2.14:	List of cells and growth requirements.....	70
Table 2.15:	Recipes for three commonly used lysis buffer.....	72
Table 2.16:	List of antibodies used in this study.....	75
Table 3.1:	Annealing temperature of HPV 16 and 18, E6 and E7 truncated and E7 full domains.....	100
Table 3.2:	HPV domains: experimental vs theoretical molecular weights	121
Table 4.1:	Proposed functions for Y-Box proteins in different organisms.....	129
Table 4.2:	Annealing temperature of YB-1 CSD and YB-1 CDTrunc domains	135
Table 4.3:	YB-1 domains: experimental vs theoretical molecular weights.....	160
Table 5.1:	Truncated version of hCTCF domains: experimental vs theoretical molecular weights.....	193
Table 6.1:	Schematic table indicating the regime for antigen injections and bleeding for polyclonal antibody production using rabbit.....	201

Table 7.1:	Comparison and brief information about physical protein-protein interaction methods.....	227
Table 7.2:	Pull-down experiments to determine the direct interaction capabilities of the coupled proteins with interacting partner proteins.....	253
Table 7.4:	Phosphorylation reaction mixture for forward reaction using T ₄ polynucleotide kinase.....	257
Table 8.1:	A Model, illustrating different types of interaction between CTCF, HPV16 E7, HPV18 E7 and YB-1 at the promoter region of <i>c-myc</i> . Thus, regulating or deregulating the <i>c-myc</i> gene.....	318

LIST OF FIGURES

Figure 1.1: The binding of CTCF~YB-1 complex to the promoter of <i>c-myc</i> gene and down-regulates the expression of <i>c-myc</i> gene.....	6
Figure 1.2: Pathophysiology of squamous cell carcinoma of the cervix	9
Figure 2.1: Structure of Iminodiacetic acid (IDA)	56
Figure 2.2: Schematic representation of the reaction used to chemically couple thiol-containing proteins to thiol-activated sepharose.....	61
Figure 3.1: Schematic diagram showing the genomic organization of HPV type 16 and 18, and indicating the general function of their respective proteins	79
Figure 3.2: Integration of HPV 16 and 18 genomes into host cell DNA.....	81
Figure 3.3: Schematic diagram showing activation of p53 by DNA damage	86
Figure 3.4: Schematic diagram showing the interaction of high risk HPV E7 with pRb, p107 and p130.....	91
Figure 3.5: Schematic diagram showing the effect of HPV E6 and E7 on cell cycle control	91
Figure 3.6: Plasmid map the parental vector pET16bSH3	94
Figure 3.7: The pET16b expression vector.....	95
Figure 3.8: Amplification of HPV 16 E7FL (lane 1) and 18 E7FL (lane 2) using PCR with respective primers	106
Figure 3.9: Amplification of HPV16 E6Trunc (lane 1), HPV16 E7Trunc (lane 2), HPV18 E6Trunc (lane 3) and HPV18 E7Trunc (lane 4) using PCR with respective primers	106
Figure 3.10: <i>Nde</i> I and <i>Age</i> I double digestion of recombinant HPV16 E6Trunc and HPV16 E7Trunc genes in pCR [®] TOPO [®] 2.1 vector.....	107
Figure 3.11: <i>Nde</i> I and <i>Age</i> I double digestion of recombinant HPV18 E6Trunc and HPV18 E7Trunc genes in pCR [®] TOPO [®] 2.1 vector.....	107
Figure 3.12: <i>Nde</i> I and <i>Age</i> I double digestion of recombinant HPV16 E7FL and HPV18 E7FL genes in pCR [®] TOPO [®] 2.1 vector.....	108

Figure 3.13: <i>Nde</i> I and <i>Age</i> I double digestion of pET16bSH3(Cys) recombinant HPV16 E6Trunc and HPV16 E7Trunc	108
Figure 3.14: <i>Nde</i> I and <i>Age</i> I double digestion of pET16bSH3(Cys) recombinant HPV18 E6Trunc and HPV18 E7Trunc.....	109
Figure 3.15: <i>Nde</i> I and <i>Age</i> I digestion of pET16bSH3(Cys) recombinant HPV16 E7FL and HPV18 E7FL.....	109
Figure 3.16: The nucleotide sequence for 16 E6Trunc in pET16bSH3(Cys) vector.....	110
Figure 3.17: The nucleotide sequence for 16 E7Trunc in pET16bSH3(Cys) vector.....	110
Figure 3.18: The nucleotide sequence for 18 E6Trunc in pET16bSH3(Cys) vector.....	111
Figure 3.19: The nucleotide sequence for 18 E7Trunc in pET16bSH3(Cys) vector.....	111
Figure 3.20: The nucleotide sequence for 16 E7FL in pET16bSH3(Cys) vector.....	112
Figure 3.21: The nucleotide sequence for 18 E7FL in pET16bSH3(Cys) vector.....	112
Figure 3.22: Coomassie stained SDS-PAGE of expression of the HPV16 E6Trunc, HPV16 E7Trunc, HPV18 E6Trunc and HPV18 E7Trunc of Human Papillomavirus in <i>E. coli</i> BL-21 (DE 3) by IPTG induction	113
Figure 3.23: Coomassie stained SDS-PAGE of expression of the HPV16 E7FL and HPV18 E7FL of Human Papillomavirus in <i>E. coli</i> BL-21 (DE 3) by IPTG induction.....	113
Figure 3.24: Western analysis of the 16 E6Trunc, 16 E7Trunc, 18 E6Trunc and 18 E7Trunc of Human Papillomavirus in <i>E. coli</i> BL-21 (DE 3) by using α -Histag antibody	114
Figure 3.25: Western analysis of the 16 E7FL and 18 E7FL of Human Papillomavirus in <i>E. coli</i> BL-21 (DE 3) by using α -Histag antibody	114
Figure 3.26: IMAC purification of HPV 16 E7Trunc protein	115
Figure 3.27: IMAC purification of HPV 18 E7Trunc protein	115
Figure 3.28: IMAC purification of HPV 16 E6Trunc protein	116
Figure 3.29: IMAC purification of HPV 18 E6Trunc protein	116
Figure 3.30: IMAC purification of HPV 16 E7FL protein	117

Figure 3.31: IMAC purification of HPV 18 E7FL protein	117
Figure 3.32: Chemical structures of histidine and imidazole	124
Figure 4.1: Domain structure of human dbpB (YB-1) and dbpA.....	132
Figure 4.2: Schematic diagram of parental vector pGEX series used in this study.....	140
Figure 4.3: Schematic diagram of pGEX-2TK~YB1	141
Figure 4.4: Schematic representation of the YB-1 and truncated versions of GST-YB-1	144
Figure 4.5: Amplification of YB-1 CSD and YB-1 CDTrunc by using PCR	147
Figure 4.6: Restriction enzyme <i>Nde</i> I and <i>Age</i> I double digestion of YB-1 CSD and YB-1 CDTrunc genes in pCR [®] TOPO [®] 2.1 vector.....	147
Figure 4.7: Restriction enzyme <i>Nde</i> I and <i>Age</i> I double digestion of YB-1 CSD and YB-1 CDTrunc genes in pET16bSH3(Cys) vector.....	148
Figure 4.8: The nucleotide sequence for YB-1 CSD in pET16bSH3(Cys) vector	149
Figure 4.9: The nucleotide sequence for YB-1 CDTrunc in pET16bSH3(Cys) vector	149
Figure 4.10: Expression of the YB-1 CSD and YB-1 CDTrunc of human YB-1 in <i>E. coli</i> BL-21 (DE 3) by 1.0mM IPTG induction.....	151
Figure 4.11: IMAC purification YB-1 CSD protein.....	151
Figure 4.12: IMAC purification YB-1 CDTrunc protein	152
Figure 4.13: Restriction enzyme <i>Bam</i> HI and <i>Eco</i> RI double digestion of YB-1 FL	154
Figure 4.14: Expression of YB-1 domains in <i>E.coli</i> DH5- α by IPTG induction.....	154
Figure 4.15: Western analysis of the expressed YB-1 domains in <i>E.coli</i> DH5- α by using anti-YB-1 polyclonal antibody.....	155
Figure 4.16: Immobilization on Gluthathione-sepharose 4B of pEGX-2TK vector, YB-1 FL, YB-1 [3] and YB-1 [4]	157
Figure 5.1: CTCF binds to the promoter region of the chicken c-myc gene	164
Figure 5.2: The wide range of dissimilar CTCF DNA target sites and their recognition by the ZF domain.....	165

Figure 5.3: The map of human CTCF gene located on the chromosome segment 16q22.1 SRO.....	167
Figure 5.4: Genomic organization of the chicken (a) and human (b) CTCF genes.....	168
Figure 5.5: Structural features of the human CTCF protein showing the three domains of CTCF	170
Figure 5.6: Synergistic cooperation between hormone receptor and CTCF.....	173
Figure 5.7: Schematic model of how gene repression can be mediated by CTCF by several mechanisms	175
Figure 5.8: The <i>Igf2</i> and <i>H19</i> genes are expressed monoallelically from the paternal and maternal alleles, respectively	180
Figure 5.9: Restriction enzyme <i>Nde</i> I and <i>Age</i> I digestion of CTCF-N, CTCF-Zn and CTCF-C genes in pET16bSH3(Cys) vector.....	188
Figure 5.10: Expression of human CTCF domains in E.coli BL-21 (DE3) by IPTG induction	188
Figure 5.11: IMAC purification of CTCF-N protein.....	190
Figure 5.12: IMAC purification of CTCF-Zn protein	190
Figure 5.13: IMAC purification of CTCF-C protein.....	191
Figure 6.1: Schematic diagram how does B cells produces plasma cells with the help of T cells in order to produce antibody when there is an invasion of antigen or pathogen.....	197
Figure 6.2: Schematic diagram of an antibody structure showing two identical heavy chains domains and two identical light chain domains.....	197
Figure 6.3: Western blotting of the 2nd bleeding.....	206
Figure 6.4: Western blotting of the 3rd bleeding.....	207
Figure 6.5: Western blotting of the final bleeding.....	208
Figure 6.6: Purification of α -16E7-HPV and α -18E7-HPV antibodies	209
Figure 6.7: Testing of the α -Zn-CTCF antibody by western assay	212

Figure 6.8: Testing of the α -CSD-(YB-1) antibody by western assay	212
Figure 6.9: Testing of the α -CD-(YB-1) antibody by western assay.....	213
Figure 6.10: Testing of the α -16E7-HPV-Purified antibody by western assay	213
Figure 6.11: Testing of the α -18E7-HPV-Purified antibody by western assay	214
Figure 6.12: Testing of the α -16E7-MoAb antibody by western assay	214
Figure 6.13: Testing of the α -18E7-MoAb antibody by western assay.....	215
Figure 7.1: Two genetics methods are the Phage Display and Two Hybrid System.....	229
Figure 7.2: Functional domains of human c-Myc protein	235
Figure 7.3: MYC–MAX and MAD–MAX complexes regulate gene activation through chromatin remodelling.....	236
Figure 7.4: Western assay of thiol-activated coupled protein using α -Histag.....	262
Figure 7.5: Western assay of <i>in-vitro</i> pull-down assay	262
Figure 7.6: Western blotting using α -CTCF monoclonal antibody.....	265
Figure 7.7: Western blotting using α -YB-1 polyclonal antibody	265
Figure 7.8: Interaction of immobilized CTCF domains with HPV16 E7FL and HPV18 E7FL	268
Figure 7.9: Interaction of immobilized YB-1 domains with HPV16 E7FL and HPV18 E7FL	268
Figure 7.10: Pull-down interaction assay subjected to 15% SDS-PAGE.....	271
Figure 7.11: Western blot analysis of Co-IP using α -HPV16 E7 antibody with HeLa cells lysate and detected using α -HPV18 E7 monoclonal antibody	274
Figure 7.12: Western blot analysis of pull-down interaction assay.....	274
Figure 7.13: <i>In-vitro</i> CK2 phosphorylation of TS-CTCF-Zn, TS-YB-1 CSD, TS-HPV16 E7FL and TS-HPV18 E7FL immobilised proteins.....	276
Figure 7.14: Analysis of CTCF~YB-1 interaction with FpV (CTCF cognate site) in EMSA with to gradual increase of HPV16 E7FL and HPV18 E7FL.....	280

Figure 7.15: Analysis of CTCF~YB-1 interaction with FpV (CTCF cognate site) in EMSA due to gradual increase of HPV16 E7FL	281
Figure 7.16: Analysis of CTCF~YB-1 interaction with FpV (CTCF cognate site) in EMSA due to gradual increase of HPV18 E7FL	282
Figure 7.17: Analysis of CTCF~YB-1 interaction with FpV (CTCF cognate site) in EMSA due to gradual increase of HPV18 E7FL and maintained the amount of HPV16 E7FL	284
Figure 7.18: Analysis of CTCF~YB-1 interaction with FpV (CTCF cognate site) in EMSA due to gradual increase of HPV16 E7FL and maintained the amount of HPV18 E7FL	285
Figure 7.18: Schematic diagram showing CTCF (A), YB-1 (B) and <i>c-myc</i> gene (C)...	298
Figure 7.19: Schematic diagram indicating the binding of CTCF~YB-1 complex to the promoter of <i>c-myc</i> gene, represses the expression of <i>c-myc</i> gene	298
Figure 7.20: Schematic diagram showing the interaction of HPV16 E7 protein with CTCF transcription factor	299
Figure 7.21: Schematic diagram postulating the formation of CTCF~YB-1~HPV18 E7 complex on the <i>c-myc</i> promoter.....	301
Figure 7.22: Schematic diagram elucidating the physical interaction of CTCF~HPV16 E7~HPV18 E7 and mediates release of YB-1	305

BIOCHEMICAL CHARACTERIZATION OF THE INTERACTION BETWEEN
CTCF/YB-1 TRANSCRIPTION FACTORS WITH HPV 16 AND 18 E7
ONCOPROTEIN AND THEIR INVOLVEMENT DURING C-MYC GENE
REGULATION IN CELL PROLIFERATION PATHWAY

ABSTRACT

Human Papillomaviruses (HPVs) type HPV-16 and HPV-18 are known as high risk HPV, which are cause 95% of cervix cancer. The E7 oncoprotein of HPV is able to interact and inactivate cellular protein pRB which further allow progression of the cell cycle and trigger cancer development. CTCF is a multivalent transcription factor which has been found to interact directly with YB-1. Both are reported to bind synergistically on *c-myc* gene promoter and down regulate the gene. Interaction of E7 oncoprotein of HPV16 and 18 to CTCF and YB-1 can help to understand the biological role of this oncoprotein in *c-myc* gene regulation. In this study, the interactions between HPV16 and 18 E7 with CTCF and YB-1 were characterized biochemically using *in-vivo* and *in-vitro* assays. This study confirms the novel interaction between HPV16 E7 with CTCF at Zn-domain and HPV18 E7 with YB-1 at CSD domain and both interactions are direct. However CTCF and YB-1 do not interact with HPV18 E7 and HPV16 E7, respectively. EMSA has shown that interaction of HPV16 E7 protein towards CTCF~YB-1 complex have release YB-1 from the complex that binds to CTCF cognate site FpV. Meanwhile the interaction of HPV18 E7 does not disrupt the CTCF~YB-1 complex. However the introduction of HPV16 E7 to the complex of CTCF~YB-1~HPV18 E7 disassociate YB-1 from the complex but forms a new complex CTCF~HPV16 E7~HPV18 E7, this was confirmed *via* a pull-down assay. Therefore, we suggest interaction of E7 protein with

CTCF and YB-1 significantly reduces the formation of CTCF~YB-1 complex. Thus, enhances the up-regulation of *c-myc* promoter and promotes tumourogenesis of cervix.

PENCIRIAN BIOKIMIA INTERAKSI ANTARA FAKTOR TRANSKRIPSI CTCF/YB-1 DENGAN ONKOPROTEIN HPV 16 DAN 18 E7 DAN KETERLIBATAN KEDUADUANYA SEMASA PENGAWALATURAN GEN C-MYC DALAM LALUAN PROLIFERASI SEL

ABSTRAK

Human Papillomaviruses (HPVs) jenis HPV-16 dan HPV-18 digolongkan sebagai HPV risiko tinggi yang merupakan 95% punca kanser serviks. Protein E7 HPV boleh berinteraksi dan menyahaktifkan protein sellular pRb dan menggalakkan peningkatan kitar sel sambil mencetuskan perkembangan kanser. CTCF merupakan faktor transkripsi ‘multivalent’ yang didapati berinteraksi secara langsung dengan YB-1. Kedua-duanya berinteraksi satu sama lain pada promoter gen *c-myc* dan menindas regulasi transkripsi gen tersebut. Maklumat interaksi onkoprotein E7 HPV16 dan 18 terhadap CTCF dan YB-1 bakal membantu untuk lebih memahami peranan biologi onkoprotein tersebut. Kajian ini menaksirkan interaksi biokimia antara onkoprotein E7 HPV16 dan 18 dengan CTCF dan YB-1 secara *in-vitro* dan *in-vivo*. Kajian ini mendapati wujudnya interaksi antara HPV16 E7 dengan CTCF pada Zn-domain, dan HPV18 E7 dengan YB-1 pada CSD, serta kedua-dua interaksi berlaku secara langsung. Walau bagaimanapun, CTCF tidak menunjukkan interaksi dengan HPV18 E7 dan YB-1 tiada interaksi dengan HPV16 E7. EMSA menunjukkan bahawa interaksi onkoprotein HPV16 E7 terhadap kompleks CTCF~YB-1 telah membebaskan YB-1 daripada kompleks yang terikat pada tapak ‘cognate’ CTCF (FpV). Selain itu, interaksi HPV18 E7 tidak mengganggu kompleks CTCF~YB-1. Seterusnya, penambahan HPV16 E7 pada kompleks CTCF~YB-1~HPV 18 E7 dapat menyingkirkan YB-1 dan membentuk kompleks baru CTCF~HPV16 E7~HPV18 E7, hal ini dipastikan dengan kaedah ‘pull-down’. Kami mencadangkan

bahawa interaksi onkoprotein E7 dengan CTCF dan YB-1 dapat mengurangi pembentukan kompleks CTCF~YB-1 dengan jelas. Seterusnya, meningkatkan regulasi gen *c-myc* dan menggalakkan penularan kanser serviks.

Chapter 1: Introduction

1.1 Research Background and Hypothesis

1.1.1 Human Papillomavirus and cervical cancer

Human Papillomaviruses (HPVs) are circular double-stranded DNA approximately 8000 base pairs that replicates in the nucleus and have icosahedral capsule that form non-enveloped virions. There are more than 130 subtypes of HPV and about 70 subtypes infect human and 35 of the subtypes infect genital tracts (de Villiers, 1994; Stanley, 2001). HPV is a risk factor of cervical cancer, the second most common cancer in women worldwide after breast cancer. HPV infection has been implicated in the aetiology of cervical cancer and more than 90% of cervical cancers contain HPV DNA (Bosch *et al.*, 1995). Low-risk HPVs such as HPV-6 and HPV-11 causes benign genital warts and whereas high-risk types such as HPV 16 and 18 are associated with the development of high risk intra-epithelia squamous lesion (HSIL) and cervical cancer (Walboomers, *et al.*, 1999). HPV 16 and 18, represent 58% and 12% in prevalence of cervical cancer, respectively (Bosch *et al.*, 2002).

The genome of HPV contains three regions. The two encoding regions called the early and late regions encodes for six early and two late genetic open reading frames (OFR). The third region, which is both non-coding and small (1000 base pairs), is often designated as the non-coding region (NCR), long control region (LCR), or upstream regulatory region (URR) (Robboy *et al.*, 2000). The early region covers some 69% of the genome and encodes a series of proteins E1, E2, E3, E4, E5, E6 and E7. The viral DNA

which integrates into the genome of cancer cells is truncated to various degrees. However, E6 and E7 open reading frame are consistently retained and expressed as mRNA or protein (Takabe, 1987). Protein E6 is known to bind with protein p53 that encodes oncogene *p53* that suppress the cell growth. This process deactivate the function of *p53* gene, thus develops cancer cells (Scheffner *et al.*, 1990; Boyer *et al.*, 1996).

Interaction of E7 protein with retinoblastoma protein pRb leads to disassociation of pRb-E2F complex, and stimulates the transcription of cellular genes involved in S-phase entry (Dyson, 1998; Whyte *et al.*, 1988; Chan *et al.*, 2001). The binding affinity of high risk E7 to pRb is 10 fold higher than the low risk (zur Hausen, 1996). E7 open reading frame is the most abundant viral protein in cells from cervical cancer and E7 is sufficient to immortalize human epithelial cell, and therefore considered as potential tumour specific antigen that could be a target of immunotherapy for cervical and precancerous (Tindle and Frazer, 1995). Besides that, few studies have indicated that an enhanced level of c-Myc was observed in cells expressing HPV E7 (Gewin and Galloway, 2001; Oh *et al.*, 2001; DeFilippis *et al.*, 2003). Meanwhile, Abba *et al.*, (2004) showed *c-myc* gene copy number increased according to the grade lesion of cervical carcinoma and the results indicate that infection with HPV 16 tightly associated with *c-myc* gene amplification. On the other hand, Lui *et al.*, (2007) reported that c-Myc expression in the cells transfected with E7 protein showed significant increases the level of expression. These findings have confirmed that E7 protein is essential for the activation of *c-myc* gene, but the mechanism is yet to be discovered.

1.1.2 CTCF transcription factor

CTCF is a transcription factor which was first identified as the protein interacting with three repeats of the CCCTC sequence that regularly spaced at 12-13 base pairs interval in the chicken *c-myc* promoter (Lobanenkov *et al.*, 1990). Therefore it was named CCCTC Binding Factor or CTCF. CTCF protein was later shown to bind to a number of different sequence in the human, mouse and avian *c-myc* promoters (Lobanenkov *et al.*, 1990; Klenova *et al.*, 1993; Fillipova *et al.*, 1996). It has been found that the human CTCF gene is localized at the chromosome 16q22.1 locus, a region commonly detected in breast and prostate cancer and suggested CTCF gene is a tumour suppressor gene (Fillipova *et al.*, 1998). CTCF is a multivalent transcriptional factor with 11 zinc-fingers which participate in the binding of DNA elements, promoters, silencer and insulator (Fillipova *et al.*, 1996). A number of different target genes regulated by CTCF are implicated in a variety of regulatory functions, ranging from promoter repression and activation, to the criterion of hormone responsive silencers and enhancer-blocking and/or boundary elements between *Igf2* and *H19* genes (Ohlsson *et al.*, 2001). Transcription of the two imprinted genes *H19* and *Igf2* is controlled in part by CTCF binding insulators located between them (Bell and Felsenfeld, 2000; Hark *et al.*, 2000).

On the other hand, Loss of Imprinting (LOI) defines loss of normal pattern of expression of a specific parental allele. In cancer it can lead to activation of growth promoting imprinted genes such as *H19* and *Igf2*. Douc *et al.*, (1996) have reported the methylation status of *H19* and *Igf2* genes in 29 invasive cervical carcinomas in different clinical stages. Fourteen (48%) and 13 (45%) tumours were heterozygous for *H19* and

Igf2, respectively. LOI of *H19* and *Igf2* was detected in 2 of 12 (17%) and 5 of 10 (15%) tumours, respectively. Therefore, suggested that *H19* and *Igf2* genes, *via* deletion and/or abnormal imprinting, could play a crucial role in a large proportion (58%) of cervical cancers where they may be associated with disease progression (Douc *et al.*, 1996). However, until now there have been no study has been reported of CTCF interaction with HPV oncogenes correlating with the tumourogenesis of cervical cancer.

1.1.3 YB-1 transcription factor

The Y-box protein (YB-1) is the highly conserved 70 amino acid DNA domain, the so-called ‘cold shock domain’ (CSD) was defined initially in bacteria as a characteristic feature of this family (Wolffe *et al.*, 1992). The name Y-box protein comes from the ability of the CSD to bind to the Y-box sequence [5’- CTGATTGG – 3’] of DNA, which is an inverted CCAAT box, in the promoter region of many genes (Wolffe *et al.*, 1992 and Wolffe, 1994). It can bind to double and single stranded DNA in a sequence-specific manner (Wolffe, 1994), but shows preference for duplex DNA enriched with pyrimidines and purines on opposite strands (Ozer *et al.*, 1990 and Sakura *et al.*, 1988). Diverse functional roles have attributed the Y-box ranging from prokaryotic cold shock response to eukaryotic transcription factors, chromatin modification proteins, DNA repair proteins, RNA packaging proteins and translational repressors (Wolffe, 1994).

A correlation has been developed between YB-1 expression and the development of malignant phenotype in several tumours such as breast cancer (Bargou *et al.*, 1997), osteosarcoma (Oda *et al.*, 1999), colorectal carcinoma (Shibao *et al.*, 1999) and ovarian scrous adenocarcinoma (Kamura *et al.*, 1999). In addition, the presence of YB-1 has been detected in HeLa cells (cervical cancer cells) by Shamsuddin (2002), and HPV 18 enhancer oligonucleotide was used as the binding material to detect the presence of YB-1 (Spitkovsky *et al.*, 1992). Besides that, Chernukhin *et al.*, (2000) have revealed the interaction YB-1 with CTCF towards the suppression of *c-myc* gene. However, there were no reports indicating the mechanism of *c-Myc* expression in cervical carcinoma samples correlating with HPV oncogenes.

1.1.4 Synergistic effect of CTCF and YB-1 towards *c-myc* oncogene

CTCF was known to bind to the CCCTC nucleotide sequence of *c-myc* promoter and YB-1 was known bind to CTCF and synergistically represses the *c-myc* expression (Lobanenkov *et al.*, 1990; Chernukhin *et al.*, 2000) (Figure 1.1). This repression was able to control the cell cycle progression and allow the cells to mature in natural condition before dividing. However, in cervical carcinoma cells which have been infected with HPV 16 and 18 the expression of *c-myc* was known to be high (Abba *et al.*, 2004). Furthermore, the presence of E7 oncoprotein was tightly associated with increased level of *c-myc* expression compare to normal cells (Gewin and Galloway, 2001; Oh *et al.*, 2001; DeFilippis *et al.*, 2003; Lui *et al.*, 2007). Therefore, this research

was carried out to study the effect of E7 oncoprotein of HPV 16 and 18 with CTCF~YB-1 complex correlating with the expression of *c-myc*.

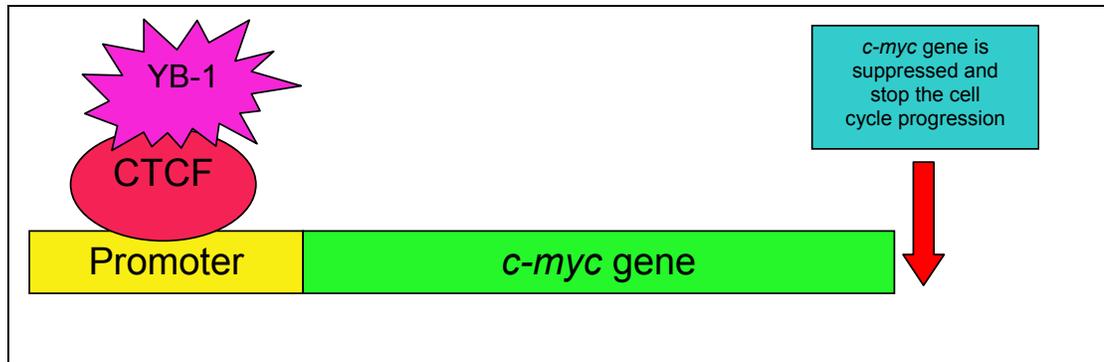


Figure 1.1: The binding of CTCF~YB-1 complex to the promoter of *c-myc* gene and down-regulates the expression of *c-myc* gene (Chernukhin *et al.*, 2000).

1.2 Cervical Cancer

Cervical cancer affects the epithelial cell of the cervix. Cervical cancer is the second most common cancer of women worldwide after breast cancer (Parkin *et al.*, 2001). It is estimated that about 470 000 of new cervical cancer cases occur per annum, which contributes towards 1.4 million of invasive cancer of cervix. Cervical cancer is the leading cause of the death among women in the developing countries with approximately 233,000 deaths per annum (Ferlay, 2000). Meanwhile, in Malaysia cancer of the cervix is ranked as the second major cause of mortality among women after breast cancer. The annual incidence rate of cervical cancer in Malaysia is estimated at 19.7 per 100,000 population and the incidence rate increases after the age of 30 years, when a peak incidence rate at the age of 60-69 years (National Cancer Registry, 2003). For the past 20 years, the Ministry of Health, Malaysia, has recorded an average of 2,200 new cases per year (Ministry of Health Annual Reports 1980-2000).

The etiology of cervical cancer explains how normal columnar epithelial cells of the cervix changes to carcinoma. Cervical cancer develops in the lining of the cervix on the lower part of uterus that elongate to vagina. Normally the exterior of the cervix appears smooth, shiny and moist with a thin layer of mucus coating the surface. However, in appearance it looks like florid exophytic cauliflower-like growth during cancerous stage (Othman, 2003). Cervical cancer is known to progress through multiple processes by increasing severe premalignant dysplastic lesion called cervical intraepithelial neoplasia (CIN) I, II, III and carcinoma *in-situ*, thus progresses to invasive carcinoma. The low grade CIN and high grade CIN resembles minimal and greater

degree of abnormality (Iwasaka *et al.*, 1998). In nature, columnar epithelial cells transform to normal squamous epithelial cells (mature metaplastic) by induction of estrogen hormone and acidic vaginal condition. During this transformation stage, exposure to the carcinogens and human papillomavirus (HPV) infection contributes towards CIN. The carcinogens are chemicals, smoking, alcohol, contraceptive, radiation and infection of human papillomavirus (HPV) (Coker *et al.*, 1992; Parazzini *et al.*, 1998; Franco *et al.*, 1999). A PCR-based study showed that 99% of invasive cervical cancer contains high risk HPV DNA (Munoz, 2000). High risk HPV type 16 and 18 are known as high risk HPV because they are strongly associated with cervical carcinoma (zur Hausen, 2002).

The high risk HPVs exerts their oncogenicity by constitutively expressing two major oncoproteins E6 and E7 (Finzer *et al.*, 2002). The interaction of E6 with p53 causes rapid p53 degradation in a ubiquitin-dependent manner resulting in cell resistance to apoptosis and hence chromosomal instability (Scheffner *et al.*, 1993; Rapp and Chen, 1998; Finzer *et al.*, 2002; zur Hausen, 2002). Meanwhile, E7 binds to pRB and causes the release of E2F from E2F/pRB complex, subsequently promotes cell cycle progression (Morris *et al.*, 1993; Boyer *et al.*, 1996; Morozov *et al.*, 1997). It has been demonstrated that high-risk HPV E6/E7 can efficiently immortalize human primary cells (Shiga *et al.*, 1997; Thonemann and Schmalz, 2000). Besides that, the high risk HPV E7 proteins is able to induce DNA synthesis in quiescent or differentiated cells, thus transforms primary baby rat kidney cells (Phelps *et al.*, 1988; Crook *et al.*, 1989; Morozov *et al.*, 1997).

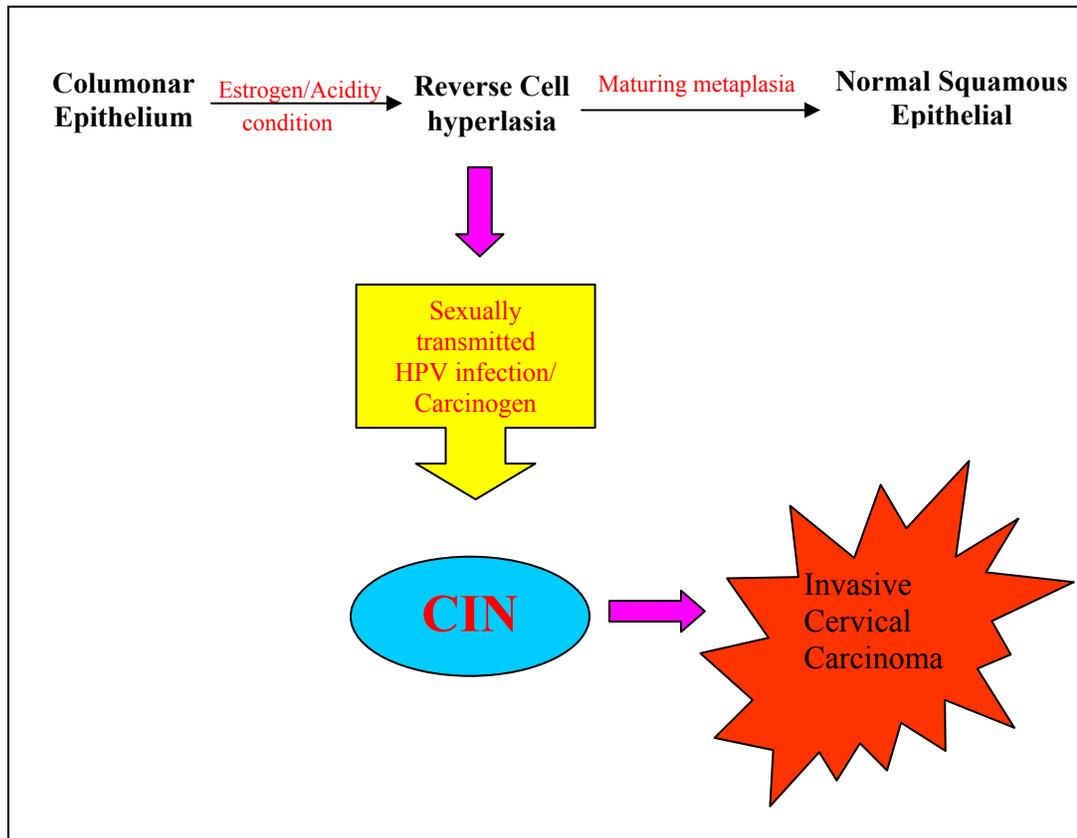


Figure 1.2: Pathophysiology of squamous cell carcinoma of the cervix. Hormonally induced eversion of the cervix and acidic vaginal environment encourage the development of the transformation zone. In physiological conditions, benign squamous metaplasia is the eventual outcome. In the presence HPV 16 and 18, the benign metaplasia process is diverted into a malignant transformation, resulting first in increasingly severe grades of cervical intraepithelial neoplasia (CIN) and then, progress to invasive squamous cell carcinoma. (Modified from Robboy *et al.*, 2000).

1.2.1 Cervical cancer and cellular mechanism

The development and progression of cervical carcinoma has been shown to be dependent on various cellular genetic and epigenetic events, especially alterations of the cell cycle machinery at various checkpoints. The precise control of the cell cycle in mammalian cells is regulated by the activity of cyclin-dependent kinases (CDK1, CDK2, CDK4, CDK6) and their essential activating co-enzymes, the cyclins (cyclins A, B, D, E). The kinase activities of these CDKs are regulated by the abundance of their partner cyclins, phosphorylation by various kinases, dephosphorylation by cell cycle phosphatases, and interaction with CDK-inhibitory proteins (CDKIs) [Funk, 1999; Clarke and Chetty, 2001; Milde-Langosch and Riethordf, 2003].

The CDK family is an important group of molecules that regulate cell proliferation. In addition, two classes of mammalian cyclin-dependent kinase inhibitors (CDKIs) have been described: the CIP/KIP family, comprised of p21, p27, and p57, and the INK4 family, comprised of p15, p16, p18, and p19 (Sherr and Roberts, 1999). The INK4 molecules specifically inhibit cyclin D complexes by interaction with the CDK4 and CDK6 components. The KIP family is promiscuous, affecting cyclin E, cyclin A/CDK2, and cyclin B/CDK1 by binding to both cyclin and CDK subunit (Clarke and Chetty, 2001). CDKs, cyclins, and CDKIs generally function within several defined pathways, including the p16^{INK4A}-Cyclin D1-CDK4/6-pRb-E2F pathway, the p21^{WAF1}-p27^{KIP1}-cyclinE-CDK2 pathway, and the

p14^{ARF}-MDM2-p53 pathway (Semczuk and Jakowicki, 2004). Each of these components plays either a positive or a negative role in cell-cycle control mechanisms in cervical carcinogenesis. Besides that, alterations in CDKs, CDKIs, and cyclins can lead to uncontrolled proliferation and might contribute to malignant transformation of the uterine cervix (Kim and Zhao, 2005).

1.2.1.1 The p16^{INK4A}-cyclin D1-CDK4/6- pRb-E2F pathway

1.2.1.1.1 p16^{INK4A}

The p16^{INK4A} gene maps to 9p21 and contains three exons, which encodes a nuclear phosphoprotein with a molecular weight of 16 kDa. The p16 protein functions in the negative regulation of the cell cycle through the inhibition of cyclin-dependent kinases (CDK) 4 and 6, and interactions with cyclin D1 (Murphy *et al.*, 2004). In the absence of p16, CDKs bind to cyclin D1, and the Retinoblastoma protein (pRb) is phosphorylated. Phosphorylation of pRb leads to its deregulation at the G₁/S checkpoint, thus cell proliferation is switched on. In a variety of human malignant tumors and cell lines, the p16^{INK4A} gene is inactivated by various genetic mechanisms, including point mutations, homozygous deletions, and hypermethylation of CpG islands in the p16^{INK4A} promoter (Kim and Zhao, 2005). Kim *et al.*, (1998) found a high percentage of p16 exon 2 mutations in cervical cancer specimens. Meanwhile, Dong *et al.*, (2001) and Nuovo *et al.*, (1999) described the hypermethylation of p16 promoter and

documented inactivation of the gene as a frequent epigenetic event in cervical carcinoma.

The portion of p16-positive samples increases as the tumor progresses from the CIN I to the invasive carcinoma stage (Klaes *et al.*, 2001). This indicates over-expression of the p16 protein is a characteristic of dysplastic and neoplastic alterations of the cervical epithelium. Some reports have shown that p16 expression is detectable by immunohistochemistry in cervical neoplasia and this expression may be a direct result of HPV infection with inactivation of pRb, which is known to bind with p16 (Nuovo *et al.*, 1999; Klaes *et al.*, 2001). Squamous cell carcinomas (SCCs), high-grade squamous intraepithelial lesions (HSILs), and adenocarcinomas (ACs) of the cervix have shown increased p16 immunostaining (Marjoniemi, 2004). Klaes *et al.*, (2001) postulated p16 expression was restricted to cervical cancer, CIN 2-3, and those cases of CIN 1 associated with high-risk HPV types only.

The interaction of p16 with pRb is thought to be central role of p16 in controlling cell cycle progression. It has been suggested that HPV infection leads to HPV-E7 binding to pRb, which in turn results in increased p16 expression. Some studies have suggested that p16 expression is induced by E2F factor, which disassociates from pRb due to the interaction of pRb with high risk HPV E7 oncoprotein (Giarre *et al.*, 2001). The p16 has been used as a biomarker for dysplasia in the diagnosis of cervical squamous lesions and has the

potential to be used as an additional screening tool (Von Knebel, 2001; Murphy *et al.*, 2003; Murphy *et al.*, 2004). The overexpression of p16 is closely associated with high-risk HPV infection and high-grade CIN (Guo *et al.*, 2004). A recent report showed that in cervical biopsy specimens, the staining pattern of p16 and a high percentage of p16-positive cells are closely related to infection with high-risk HPV types 16 and 18, and with CIN 2/3 (Saqi *et al.*, 2003; Hu *et al.*, 2005).

1.2.1.1.2 Cyclin D1

The cyclin D1 (PRAD-1, CCND-1) gene maps to 11q13 and shows the characteristics of a cellular oncogene. Expression of cyclin D1 moderately oscillates throughout the cell-cycle, reaching peak levels in G-phase (Semczuk and Jakowicki, 2004). Cyclin D1 serves as a key sensor and integrator of extracellular signals in early to mid-G₁ phase, mediating its function through binding with CDKs, histone acetylase, and histone deacetylases to modulate local chromatin structure around the genes that are involved in regulation of cell proliferation and differentiation. Genetic aberrations in the regulatory circuits that govern transit through the G₁ phase of the cell cycle occur frequently in human cancer, and overexpression of cyclin D1 is one of the most commonly observed alterations (Fu *et al.*, 2004).

The role of cyclin D1 in cervical carcinogenesis is not clearly understood, and controversial results have been described. Cho *et al.*, (1997) found that cyclin D1 levels were significantly lower in HPV-positive HSIL, invasive SCC, or adenocarcinoma compared to HPV-negative cases and normal cervical epithelium, consistent with previous findings (Southern and Herrington, 1998; Bae *et al.*, 2001). Contrary to this, the results of Nichols *et al.*, (1996) described elevated cyclin D1 mRNA levels in invasive cervical cancer that were not associated with increased protein amounts. In addition, there was no significant increase in cyclin D1 protein levels were detected in cervical carcinoma, despite overexpression of cyclin D1 mRNA demonstrated by *in-situ* hybridization (Cho *et al.*, 1997; Bae *et al.*, 2001).

Few studies showed that the level of cyclin D1 was significantly lower in HPV-positive LSIL, HSIL, invasive SCC and AC compared to HPV-negative cases and normal cervical epithelium (Cho *et al.*, 1997; Southern and Herrington, 1998; Psyrris *et al.*, 2004). Cyclin D1 and HPV E7 possess similar binding regions for pRb and pRb-related pocket proteins, and inactivation of pRb either by the cyclin/CDK complexes in G₁ phase or by interaction with the high-risk HPV E7 oncoprotein may result in a decreased of cyclin D1 expression (Kim and Zhao, 2005).

1.2.1.1.3 CDK4

The D-type cyclins (D1, D2, and D3) and their catalytic partners CDK4 and CDK6 act in the early G₁ phase of the cell cycle (Clarke and Chetty, 2001). Mitogen-induced signal transduction pathways promote the activation of cyclin D/CDK complexes at different levels such as gene transcription, cyclin D translation and stability, assembly of D cyclins with their CDK partners, and import of the holoenzymes into the nucleus (where they ultimately phosphorylate their substrates). Besides that, cyclin D-dependent kinases (CDK4 and CDK6) can phosphorylate pRb family members (pRb, p107, and p130), thus inactivate their transcriptional corepressor activities (Kim and Zhao, 2005).

Aberrantly expressed CDK4 could play an important role in cervical tumorigenesis. It is postulated that CDK4 oscillates between the INK4 and KIP inhibitors by blocking their suppressor activity. In cervical cancer, it was demonstrated that the level of INK4 is low compared to CDK4, which would favor the binding of more abundant KIP inhibitors to these kinases, and their ability to inhibit of cyclin E is disrupted (Kim and Zhao, 2005). The high risk E7 oncoprotein would deregulate pRb initially and unleashing E2F-induced cyclin E expression, then the over-expressed CDK4 binds to KIP molecules, thus allowing cyclin E to become sufficiently active to phosphorylate and inactivate pRb and p27^{KIP1} (Grana and Reddy, 1995; Milde-Langosch and Riethdorf, 2003; Sheer and Roberts, 2004).

Yoshinouchi *et al.*, (2000) have found overexpression of CDK4 in 72.6% of cervical cancer specimens and correlates with previous studies of CDK4 expression in cervical carcinoma (Skomedal *et al.*, 1999; Cheung *et al.*, 2001). Phosphorylation of pRb by CDK4 was found to be non-critical in the carcinogenesis or establishment of HPV-positive cervical cancer cell lines, since the HPV oncoproteins E6 and E7 inactivate p53 and pRb tumor suppressor functions, respectively, thus resulting in deregulated progression of the cell cycle (Yoshinouchi *et al.*, 2000). Interestingly, very recent work has indicated that cyclin D/CDK4 complexes phosphorylate Smad3, thus negatively regulates the functions of transcriptional complexes that mediate cell growth inhibition by TGF family proteins (Matsuura *et al.*, 2004). Importantly, several lines of evidence indicate that cyclin D/CDK complexes play a second non-catalytic role in G₁ progression by sequestering proteins of the Cip/Kip family, including p27^{KIP1} and p21^{WAF1/CIP1}, two potent inhibitors of CDK2 (Toyoshima and Hunter, 1994; Hall *et al.*, 1995).

1.2.1.1.4 pRb/E2F

The retinoblastoma tumor suppressor gene (Rb) encodes a nuclear phosphoprotein known as pRb, which has been found mutated or deleted in several types of human cancer. The pRb and other pRb family members such as p107 and p130 regulate the activity of E2F transcription factors (Lukas *et al.*, 1994). Complexes consisting of E2F and hypophosphorylated pRb repress the transcription of genes that are required for cell cycle progression, and repression is relieved by CDK-mediated phosphorylation of pRb (Salcedo *et al.*, 2002). The pRb protein is subjected to regulation by many

factors, including E2F and cyclin D1. The hypophosphorylated pRb, complexed with a transcription factor, serves as a transcriptional activator of cyclin D1 by binding to its promoter (Muller *et al.*, 1994). On the other hand, inactivation of pRb by phosphorylation via the cyclin D/CDK complex in late G₁ would not only unleash E2F transcription factors, but would also decrease cyclin D1 expression (Muller *et al.*, 1994; Clarke and Chetty, 2001). High risk HPV E7 oncoprotein was found to interact with hypo- or hyper-phosphorylated pRb (Milde-Langosch and Riethdorf, 2003). Therefore, this oncoprotein occupies the pRb pocket and displace E2F factors, thus preventing pRb/E2F from inducing cyclin D1 transcription and undermining its normal growth-suppressive function (Salcedo *et al.*, 2002; Fiedler *et al.*, 2004).

Both binding and degradation of the Rb proteins by the HPV E7 protein are essential for sustained proliferation of HeLa cervical carcinoma cells, and E7 repression triggers senescence at least in part by activating the Rb pathway in both HeLa and HT-3 cells (Lukas *et al.*, 1994). Besides that, pRb immunostaining using invasive cervical lesions was frequently lower than in SIL. This low level of Rb expression might be resulted from *Rb* gene mutations or down-regulation mechanisms, but may also be related to pRb inactivation resulting from complex formation with high-risk HPV E7 oncoproteins (Salcedo *et al.*, 2002). On the other hand, mutations in the Rb gene seem to be rare events in cervical cancer (Choo and Chong, 1993; Sano *et al.*, 1998). In most studies, *Rb* gene expression did not strictly correlate with the HPV status (Chetty *et al.*, 1997).

1.2.1.2 The p21^{WAF1/CIP1}-p27^{KIP1}-cyclin E-CDK2 pathway

1.2.1.2.1 p21^{WAF1/CIP1}

The p21^{WAF1/CIP1} is a cyclin-dependent kinase inhibitor that associates with a class of CDKs and inhibits their kinase activities, leading to cell cycle arrest and the dephosphorylation of pRb. A large body of evidence suggests that p21^{WAF1/CIP1} plays an important role in cell fate decisions during growth and differentiation (Kim and Zhao, 2005). The p21^{WAF1/CIP1} protein is a p53-inducible protein that inactivates the cyclin/CDK complexes, blocking the cell cycle progression in the G₁-S transition. However, p21^{WAF1/CIP1} is expressed in cells undergoing either G₁ arrest or apoptosis by p53-dependent or -independent mechanisms (El-Deiry *et al.*, 1994; Michieli *et al.*, 1994).

The p21^{WAF1/CIP1} expression usually correlates with favorable prognosis in ovarian, gastric, colorectal, and superficial bladder cancers and in esophageal squamous cell carcinoma (Kim and Zhao, 2005). However, in cervical cancer, the conclusions about p21 expression and its prognostic importance vary considerably. Many authors found increased p21 expression in invasive carcinomas (Skomedal *et al.*, 1999) and elevated number of p21-expressing cases during the progression from normal epithelia through precancerous lesions to invasive cervical cancer (Lie *et al.*, 1999; Bae *et al.*, 2001). However, others have detected under-expression of p21 in micro-invasive and invasive cervical cancer compared to normal cervical epithelium (Kim *et al.*, 1998; Huang *et al.*, 2001).

Meanwhile, Van de Putte *et al.*, (2004) did not find any expression of p21 in normal squamous cervical epithelium, in agreement with Giannoudis and Herrington (2000) and Skomedal *et al.*, (1999) but in contrast with other reports (Kim *et al.*, 1998; Bae *et al.*, 2001). However, the p21 level might be increased in a futile attempt to overcome its impaired or bypassed function. In squamous cell carcinoma, its function could be impaired through the inactivation of p21 by the HPV-16 E7 oncoprotein (Bae *et al.*, 2001). On the other hand, (Lu *et al.*, 1998) reported that expression of p21^{WAF1/CIP1} was correlated with a favorable prognosis in adenocarcinoma of the uterine cervix.

1.2.1.2.2 p27^{KIP1}

The p27^{KIP1} is a negative regulator of the G₁ phase of the cell cycle. The p27^{KIP1} gene is a tumor suppressor gene and is frequently lost in tumor cells. It has been implicated in the negative regulation of cell proliferation in response to extracellular signals and is induced upon serum deprivation. In normal epithelial cells, increased expression of P27^{KIP1} mediates the arrest of cells in the G₁ phase of the cell cycle when induced by TGF- β (Sgambato *et al.*, 2000). It was found that p27^{KIP1} associates mainly with the cyclin E/CDK2 and this complex inhibits pRb phosphorylation, thus, p27^{KIP1} blocks the cell from entering S phase (Shiozawa *et al.*, 2001). Although p27^{KIP1} is a putative tumor-suppressor gene, mutation or homozygous deletion of this gene is rarely found in human cancer (Huang *et al.*, 2002). Expression of p27^{KIP1} protein correlates with human tumor progression and Huang *et al.*, (2002) demonstrated that decrease or

absences of p27^{KIP1} protein expression was associated with more aggressive clinical behavior in a variety of human tumors, including breast, lung, colon, gastric, and ovarian carcinoma (Huang *et al.*, 2002)

Goff *et al.*, (2003) found that p27^{KIP1} was strongly expressed in both the epithelium and stroma of normal cervix, which correlate with other investigators. Shiozawa *et al.*, (2001) revealed that the expression of p27^{KIP1} in the normal squamous epithelium of the uterine cervix was observed mainly in the intermediate and superficial layers cells, but enlighten weaker expression in the parabasal cells, which replicate most actively. This is consistent with a previous study conducted by Troncone *et al.*, (1999). The trend of reduced p27^{KIP1} expression in microinvasive and invasive carcinomas further supports the notion that p27^{KIP1} plays a tumor suppressor function during neoplastic transformation in cervical epithelium. Sgambato *et al.*, (2004) demonstrated that a decrease in p27^{KIP1} expression is associated with the development of cervical cancer and may play an important role in the early stage of cervical tumorigenesis (Sgambato *et al.*, 2004). In fact, p27^{KIP1} expression has been reduced in pre-invasive lesions of the cervix and became progressively more evident during the progression from low- to high-grade SIL and from SIL to carcinoma. This result of p27^{KIP1} expression in cervical carcinomas is in agreement with similar findings in several other types of human malignancies (Sgambato, *et al.*, 2000) and with previous reports of cervical cancers (Troncone *et al.*, 1999; Shiozawa, 2001).

1.2.1.2.3 Cyclin E

Cyclin E exists in two isoforms with high homology, designated cyclin E1 and E2. There are no major differences in expression or functions between both have been found, and their expression has been assumed to be governed by the same molecular circuitry (Geng *et al.*, 2001; Erlandsson *et al.*, 2003). Cyclin E, whose catalytic partner is CDK2, is another rate-limiting regulator of the G₁ phase of the cell cycle (Dirks and Rutka, 1997). Appropriate regulation of this cyclin is essential for S-phase transition and numerous processes that determine the accuracy of chromosome replication. It can play a role similar to that of cyclin D1, driving the cell cycle by phosphorylation of Rb, p107, and p130 and the subsequent release of E2F and transcription of key proteins. Cyclin E appears in late G₁ after passage through the restriction point and disappears again in early S phase (Kim and Zhao, 2005).

Even though gene amplification and post-transcriptional modification are the common causes of aberrant cyclin E expression in different malignancies, high-risk-HPV oncoprotein-associated mechanisms might contribute to the aberrant cyclin E expression in cervical lesions. Releasing of E2F factors from pRb by the action of the HPV E7 protein could result in the over-expression of cyclin E. This connection, however, was not enough to explain the highly expressed cyclin E in cervical epithelium. The HPV E7 oncoprotein was demonstrated in complexes with cyclin E and cyclin A, thus eliminating phase-dependent variation in activity. Also, the HPV E7 was found to interact with p21 and thereby block p21-mediated inhibition of cyclin E-associated kinase activity (Zerfass-Thome *et al.*, 1996).

In normal cervical squamous epithelium, cyclin E expression was not immunohistochemically detectable or was very weak (Cho *et al.*, 1997; Quade *et al.*, 1988; Kim *et al.*, 2000; Shiozawa *et al.*, 2001; Southern *et al.*, 2001). However, cyclin E expression was increased in both low- and high-risk HPV-infected squamous and glandular lesions (Cho *et al.*, 1997; Dellas *et al.*, 1998). Cyclin E expression correlated strongly with morphologic features of the HPV-infected lesions, and has been observed in intermediate, and partly in superficial, cells from LSIL and HSIL, implying sequential dysregulation (Southern *et al.*, 2000). Cyclin E over-expression or over-activity may be the direct result of the presence of the HPV E6 and E7 proteins in invasive disease. The HPV E7 oncoprotein inactivates p27 and leads to decrease inhibition of cyclin E, thus promotes its expression (Southern *et al.*, 2001).

1.2.1.2.4 CDK2

The CDK2 functions as an effector through phosphorylation of key substrate such as the transcription factor p53, the cell cycle regulating phosphatase cdc25A/cdc25C, MDM2, BRCA1 and the transcription factor E2F1 (Castedo *et al.*, 2004). E-type cyclins (E1 and E2) govern the activity of the single catalytic subunit of CDK2. Unlike various combinations of D-type cyclins that are expressed in different cell types, cyclin E-CDK2 complexes are periodic and maximal at the G₁ to S phase transition (Dulic *et al.*, 1992). Cyclin E-CDK2 also preferentially phosphorylates pRb at different sites from the cyclin D-dependent kinases, and these modifications may differentially impact on the interactions of pRb with E2Fs, histone deacetylases,

and other chromatin-remodeling proteins (Harbour and Dean, 2000). One of the most significant consequences of pRb inactivation is activation of cyclin E/CDK2 subunits, often as a result of increased cyclin E expression (Kim and Zhao, 2005).

The HPV oncoprotein E7 may abrogate p21-mediated sequestration of cyclin E and render its catalytic partner CDK2 resistant to p27 (Jones *et al.*, 1997). Shiozawa *et al.*, (2001) revealed that no p27 expression was observed in atypical epithelial cells of SIL with elevated expression of Ki-67, cyclin E, and CDK2. The expression of cyclin-dependent kinases (CDK2 and cdc2) was also increased in a considerable number of invasive cervical carcinomas (Kanai *et al.*, 1998; Shiozawa *et al.*, 2001). In those cases, p27 expression was also high and retained the ability to bind to CDK2, but the p27~cyclin E/CDK2 complex still possessed phosphorylating activity (Shiozawa *et al.*, 2001). CDK2 also regulates the cell cycle through its interactions with cyclin A2, thus its functions extend beyond G₁ regulation to govern events in S and G₂/M as well. Whereas maximal periodic cyclin E-CDK2 activity is detected at G₁/S, low levels of cyclin A-CDK2 activity are first detected in late G₁ phase. Consistent with crucial roles for cyclin E/CDK2 downstream of pRb, many tumor cells are extremely sensitive to inactivation of cyclin E/CDK2 whether or not they express pRb. This conclusion has been drawn from a multitude of studies demonstrating antiproliferative effects of overexpression of p27^{KIP1}, a protein inhibitor of CDK2, or of dominant-negative CDK2 subunits (Knockaert *et al.*, 2002; Tetsu and McCormick, 2003).

1.2.1.3 The p14^{ARF}-MDM2-p53 pathway

1.2.1.3.1 P14^{ARF}

The CDK^{N2A} gene on human chromosome 9p21 encodes two distinct proteins, p14^{ARF} and p16^{INK4A}, which arise from the same gene by alternative mRNA splicing (Kanao *et al.*, 2004). The p14^{ARF} has a mass of 15 kDa and is translated from mRNAs bearing a unique first exon, called 1-beta, and located 15 kb upstream of the exon 1-alpha of p16^{INK4A}. Both transcripts share common exons 2 and 3. The p14^{ARF} functions as a cell cycle regulator, stopping cell growth at the G₁-S border and also at G₂-M (Quelle *et al.*, 1995). The p14^{ARF} interaction inhibits the MDM2 oncoprotein, thereby blocking formation of the MDM2-p53 complex and preventing MDM2-induced p53 degradation (Zhang *et al.*, 1998). Kanao *et al.*, (2004) reported that the over-expression of p14^{ARF} and p16^{INK4A} is strongly associated with HPV-positive cervical cancers and that reduced expression of p14^{ARF} and p16^{INK4A} is correlated with HPV-negative cervical cancers. These findings may indicate that impaired p14^{ARF} and p16^{INK4A} mRNA expression contribute to tumor development in HPV-negative cervical cancers by failure to support p53 and Rb instead of their inactivation by HPV E6 and E7.

In human fibroblasts, it has been shown that p14^{ARF}-induced cell cycle arrest is p53-dependent and is abrogated by the co-expression of the HPV E6 protein (Stott *et al.*, 1998; Middlelangosch *et al.*, 2001). Therefore, p14^{ARF} up-regulation was a consequence of inactivation of p53 rather than a sign of cell cycle inhibition. Brooks *et al.*, (2002) demonstrated that the expression levels of