Proteomic Analysis of Progressive Factors in Uterine Cervical Cancer

Yoon Pyo CHOI

Department of Medical Science

The Graduate School, Yonsei University

Proteomic Analysis of Progressive Factors in Uterine Cervical Cancer

Directed by Professor Nam Hoon CHO

The Master's Thesis
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements
for the degree of Master of Medical Science

Yoon Pyo CHOI

August 2005

This certifies that the Master's Thesis of Yoon Pyo CHOI is approved.

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Thesis Supervisor : Nam Hoon CHO

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Young Tae KIM

_____

Hye Young KIM

The Graduate School
Yonsei University

August 2005

ACKNOWLEDGEMENTS

It completes the dissertation that is prudent to Professor Nam Hoon CHO long time to lead in deep concern thanks it gives with sincerity. Also Professor Young Tae KIM and Professor Hye Young KIM who convey it give thanks with good advice. And in always care that it does as a favor, Suki KANG and Mi Kyung LEE, gives the speech of thanks. Also even to the fence, all of who are a department of pathology thanks it wants giving. Although the period of 2 years as a graduate school student never is easy, it gives me the possibility and the chance that can challenge what is something new. And it is same last and conspicuously believes and with me loves this dissertation to my parents whom it does as a favor to put in the mind of thanks, it gives.

Sincerely,

Yoon Pyo CHOI

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ABSTRACT

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Yoon Pyo CHOI

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Human papillomavirus (HPV) infections play a crucial role in the progress of cervical cancer. The high-risk HPV types are frequently associated with the development of malignant lesions. Some of the latest studies have demonstrated that the high-risk HPV 16 and 18 are predominantly detected in the more aggressive cancers. In the present study, we aimed to establish the proteomic profiles and characterization of the tumor related proteins by using 2-DE and MALDI-TOF MS. For proteomic analysis, patients infected by HPV 16 or 18 were included in this study. We compared nuclear protein and cytoplasmic protein, separately by using the subcellular fraction. Differential protein spots between cervical cancer with high-risk HPV, HPV

16 or HPV 18 and HaCaT Cell lines were characterized by 2-DE. Those proteins analyzed by peptide mass fingerprinting based on MALDI-TOF MS and database searching were the products of oncogenes or proto-oncogenes, and the others were involved in the regulation of cell cycle, for general genomic stability, telomerase activation and cell immortalization. However, there was no difference of protein characterization for cervical cancer between HPV 16 and HPV 18 infection. Nonetheless, these data are valuable for the mass identification of differentially expressed proteins involved in human uterine cervical cancer. Moreover, the data has enormous value for, establishing the human uterine cervical cancer proteome database that can be used in screening a molecular marker for the further study of human uterine cervical cancer, and also for studying any correlation among the cancers induced by HPV.

Key words : Human uterine cervical cancer; 2-DE; MALDI-TOF MS; Proteome; Differential expression protein

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I. INTRODUCTION

Human papillomaviruses (HPVs) are small DNA viruses with a genome of approximately 8 kb. A small subgroup, types 16, 18, 31, 33 and 45, has been designated high-risk HPVs, and they are found to be associated with more than 90% of cervical cancers. [1] The development and progression of cervical cancers infected by HPVs is likely to be associated with alterations in the cell cycle, apoptosis (programmed cell death), and disturbances in immune surveillance, increased cell growth and uncontrolled proliferation. [2] Lately, some studies have demonstrated that the high-risk types HPV 16 and 18 are predominantly detected in more aggressive cancer. [3] The high-risk HPV E6/E7 oncoproteins interact and interfere with the functions of tumor suppressor proteins, p53 [4] and retinoblastoma protein (RB) [5], respectively. Thus, it is thought that these viral oncoproteins contribute to HPV-induced

cancer formation.

At present, the molecular pathogenesis of human uterine cervical cancer has been partially revealed at the levels of genomic alteration and transcriptional regulation (mRNA). [6] However, this pathogenesis is not dictated solely by these processes. Although, some tumor-related markers have already been identified and are being applied in clinical diagnosis [7], the lack of study on the overall proteomic profiles of human uterine cervical cancer hinders our effort to understand and cure this cancer. Thus, the proteomics approach is essential to understand the pathogenesis and development of human uterine cervical cancer.

Therefore, in this study, we separated the total proteins from human uterine cervical cancer and the control cells with 2-DE, which is the principal step of proteomics and is widely used in comparative studies of protein expression levels between healthy and diseased states, and we analyzed these proteins. In addition, by using the subcellular fractions of the nucleus and cytoplasm, we considered the correlation of protein localization and functional information together. Differential expression of certain proteins in the tumor and the corresponding control was revealed by MALDI-TOF MS and database analysis. The results presented here will no doubt provide clues for further study of the carcinogenic mechanisms, diagnosis and therapy of human uterine cervical cancer. Moreover, the results may be valuable for studying extragenital neoplasm induced by HPV, such as laryngopharyngeal cancer, head and neck cancer, esophageal cancer and oral cancer.

II. MATERIALS AND METHODS

1. HPV genotyping

HPVDNAChip.

HPV detection and genotyping was performed with HPVDNAChip (Biomedlab Co., Seoul, Korea). HPVDNAChip contains 22 typespecific probes that consist of 15 high-risk groups (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 69) and 7 low-risk groups (6, 11, 34, 40, 42, 43, and 44). [8]

2. Proteomic analysis

2.1 Cell culture.

HaCaT (Immortalized human keratinocyte cell line) was obtained from the Korean Cell Line Bank (KCLB). The cells were cultivated in Dulbecco's modified Eagle's medium (DMEM, GIBCO, Invitrogen Corp.) supplemented with 10% heat-inactivated bovine serum albumin (BSA, GIBCO, Invitrogen Corp.), 100 U/mL penicillin and 100 mg/mL streptomycin. [9]

2.2 Tissue specimens and sample preparation for 2-DE.

6 cervical cancer tissue samples were obtained from the Yonsei University College of Medicine in Korea (Table 1), and the HaCaT cell lines as a control were also prepared. All the patients donating tissue samples were diagnosed by histopathology and examined by HPVDNAChip. The patients' medical records were reviewed. All of samples were divided into nuclear and

cytoplasmic fractions, respectively. To collect molecular proportions in nuclei and cytoplasm, we used a nuclear and cytoplasmic fractional lysis kit (Activemotif, #40010, CA, USA). Briefly, the cells were collected in ice-cold PBS in the presence of phosphatase inhibitors to limit further protein modification, such as dephosphorylation or proteolysis. They were then resuspended in 0.5ml 1X Hypotonic buffer to swell the cell membrane, and 25 $\mu\ell$ of detergent was added to cause leakage of cytoplasmic proteins into the supernatant. After collecting the supernatant as the cytoplasmic fraction, pellets were resuspended in 50$\mu\ell$ Lysis Buffer in the presence of Protease Inhibitor Cocktail. They were then incubated on a rocking platform set at 150 rpm for 30 min at 4^{o}C, and centrifuged at 14,000g for 10 min at 4^{o}C. The supernatant so obtained was used as the nuclear fraction. And protein concentration was then determined by the Bio-Rad Protein Assay.

2.3 IPG-2D PAGE.

IPG dry strips were equilibrated for 12-16 hours with 7M urea, 2M thiourea containing 2% 3-[(3-cholamidopropy) dimethyammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol (DTT), and 1% pharmalyte, and the strips were loaded with 200ug of sample. Isoelectric focusing (IEF) was performed at 20°C using a Multiphor II electrophoresis unit and EPS 3500 X L power supply (Amersham Biosciences) following the manufacturer's instruction. For IEF, the voltage was linearly increased from 150 to 3,500V during 3 hours for sample entry followed by a constant 3,500V, with the focusing complete after 96kVh. Prior to the second dimension, strips were incubated for 10 minutes in equilibration buffer (50mM Tris-Cl, pH6.8 containing 6M urea,

2% SDS and 30% glycerol), first with 1% DTT and second with 2.5% iodoacetamide. The equilibrated strips were inserted onto SDS-PAGE gels (20-24cm, 10-16%). SDS-PAGE was performed using a Hoefer DALT 2D system (Amersham Biosciences) and by following the manufacturer's instruction. The 2D gels were run at 20°C for 1,700Vh. The 2D gels were next silver stained as described by Oakley et al (Anal. Biochem. 1980, 105:361-363), but the fixing and sensitization step with glutaraldehyde was omitted.

2.4 Image analysis.

Quantitative analysis of the digitized images was carried out using the PDQuest software (version 7.0, BioRad) according to the protocols provided by the manufacturer. The quantity of each spot was normalized by the total valid spot intensity. Protein spots were selected for the significant expression of variation that deviated over around two fold in its expression level compared with the normal or control sample.

2.5 MALDI-TOF-MS.

A total of 64 gel-spots (nucleus: 31, cytoplasm: 33) were excised from preparative gels using biopsy punches, and they transferred to a 1.5ml siliconized Eppendorf tube. The gel-spots were destained in the destaining solution consisting of 100mmol/L $Na_2S_2O_3$ (Sigma) and 30mmol/L $K_3Fe(CN)_6$ (Sigma) (V/V, 1:1). Gel pieces were pre-reduced with 100% acetonitrile (HPLC grade). The protein-containing gel-spots were reduced in reduction buffer (100mmol/L NH_4HCO_3 (Sigma) and 10mmol/L DTT (Sigma)) for

30min at 56°C and then alkylated in alkylation buffer (100mmol/L NH_4HCO_3 and 55mmol/L iodoacetamide (Sigma)) in the dark for 25 min at room temperature. The gel pieces were dried in a Speed-Vac. The dried gel-pieces were incubated in ABC buffer (50mM, pH 8.0) containing 0.1ug/ul trypsin for 12-16h at 37°C. After concentration the peptide mixture was desalted using C_{18}ZipTips (Millipore), and the peptides were eluted in 1-5$\mu\ell$ 50% ACN/0.1% TFA. An aliquot of this solution was mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in 50% ACN/0.1% TFA, and 1$\mu\ell$ of the mixture was spotted onto a target plate. The tryptic peptide mixture was extracted and concentrated with a C_{18}ZipTips (Millipore). The peptide samples were cocrystallized with matrix on the Voyager 96×2 Samples Plate P/N V700813 using 0.5$\mu\ell$ matrix (a-cyano-4-hydroxytranscinnamic acid) and 0.5$\mu\ell$ sample. The samples were then analyzed with an Applied Biosystems Voyager System 4307 MALDI-TOF Mass Spectrometer (ABI), and des-Arg^1-Bradykinin, Glu^1-Fibrinopeptide B, and ACTH (clip 18-39) served as external standard for mass calibration.

2.6 Database analysis.

The MS-Fit(the UCSF Mass Spectrometry, ProteinProspector v 4.0.5) was used for protein identification by peptide mass fingerprinting. Spectra were calibrated with trypsin auto-digestion ion peak *m/z*(842.510, 2211.1046) as internal standards. The search parameters were set up as follows: the

database is SwissProt. 10.30.2003 and NCBInr. 10.21.2003; the mass tolerance was ±50 ppm; the number of missed cleavage sites was allowed up to 1; the minimum number of matched-peptides was 4; species was selected as *Homo sapiens*(human); the monoisotope masses were used; and the searching range was within the experimental pI value ±2 pH unit and experimental M_r ±20%.

III. RESULTS AND DISCUSSION

1. Detection of the infection with the high-risk HPV types 16 and 18 by using HPVDNAChip

In order to investigate whether patients were infected by the high-risk HPV types 16/18, we performed HPV genotyping with HPVDNAChip (Biomedlab. Co., Seoul, Korea). A total of 6 human uterine cervical cancer tissues were selected from these results: HPV 16 (3) and HPV 18 (3). (Table 1) In our previous study, we had used this to investigate the infection aspect for the high-risk HPV types in Korean women and the paper related to this has already been published.[8] At present, we have used this for the diagnosis of HPV infection.

Table 1. Patient information and pathological diagnosis for the tissue samples used.

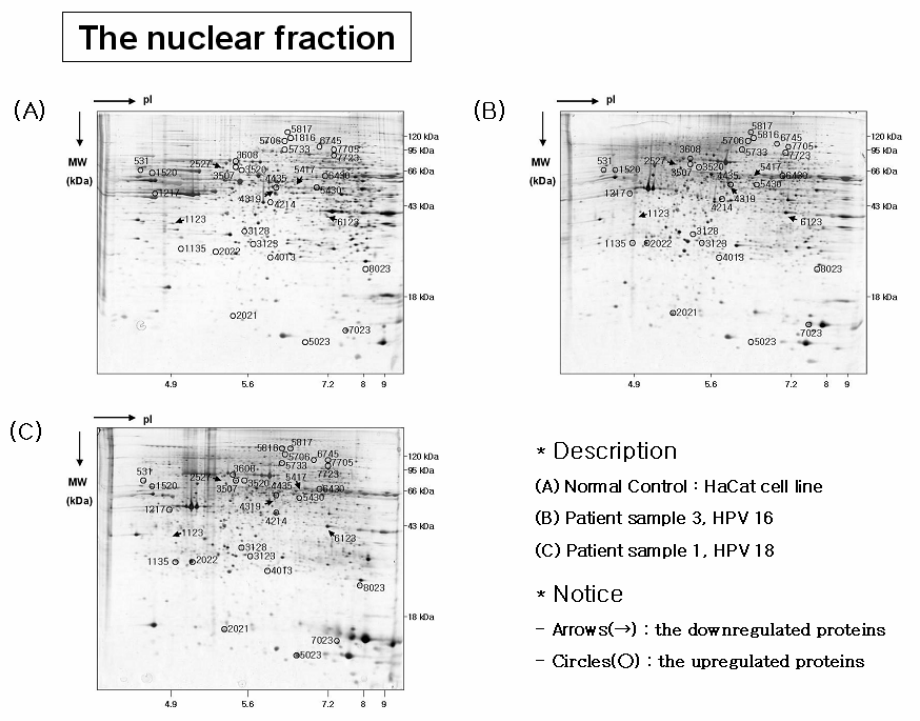
HPV type	Sample #	Receipt number	Gender	Age	Name of operation	Pathological diagnosis
16	1	SS02-24866	Female	45	Uterus, cervix, punch biopsy	Invasive squamous cell carcinoma, large cell, nonkeratinizing type
16	2	SS02-23859	Female	73	Uterus, cervix, punch biopsy	Invasive squamous cell carcinoma, large cell, nonkeratinizing type
16	3	SS02-26096	Female	50	Uterus, cervix, punch biopsy	Invasive squamous cell carcinoma, large cell, nonkeratinizing type

HPV type	Sample #	Receipt number	Gender	Age	Name of operation	Pathological diagnosis
18	1	SR02-00507	Female	43	Uterus, cervix, punch biopsy	Invasive squamous cell carcinoma, large cell, nonkeratinizing type
18	2	SS02-24360	Female	55	Uterus, cervix, punch biopsy	Invasive squamous cell carcinoma, large cell, nonkeratinizing type
18	3	SS02-27180	Female	60	Uterus, cervix, punch biopsy	Invasive squamous cell carcinoma, large cell, nonkeratinizing type

2. Proteome analysis of human uterine cervical cancer tissues infected by the high-risk HPV types 16/18 by using 2-DE and MALDI-TOF MS

We quantified the 2-DE protein patterns and mutually matched them. In order to select protein variations, the protein patterns of tumor and control were set into two classes and quantities of all the detected spots in both classes were compared by the PDQuest software (version 7.0, BioRad). The 2-DE profiles were very similar among the samples. For comparing the protein expression differences of the tumor and the paired normal samples, we selected a total of 64 differential protein spots (Nuclear fraction: 31, Cytoplasmic fraction: 33). (Figure 1,2) The PMF maps were then obtained by MALDI-TOF MS (Figure 3) The PMF data were used to search the MS-Fit (the UCSF Mass Spectrometry, ProteinProspector v 4.0.5). (Table 2) And the resulting protein was then determined by comprehensively considering the corresponding experimental MOWSE score, the number of matched-peptides, Mr, pI, and the sequence coverage. (Table 3 and 4)

(a) The nuclear fraction's 2-DE images



(b) The cytoplasmic fraction's 2-DE images

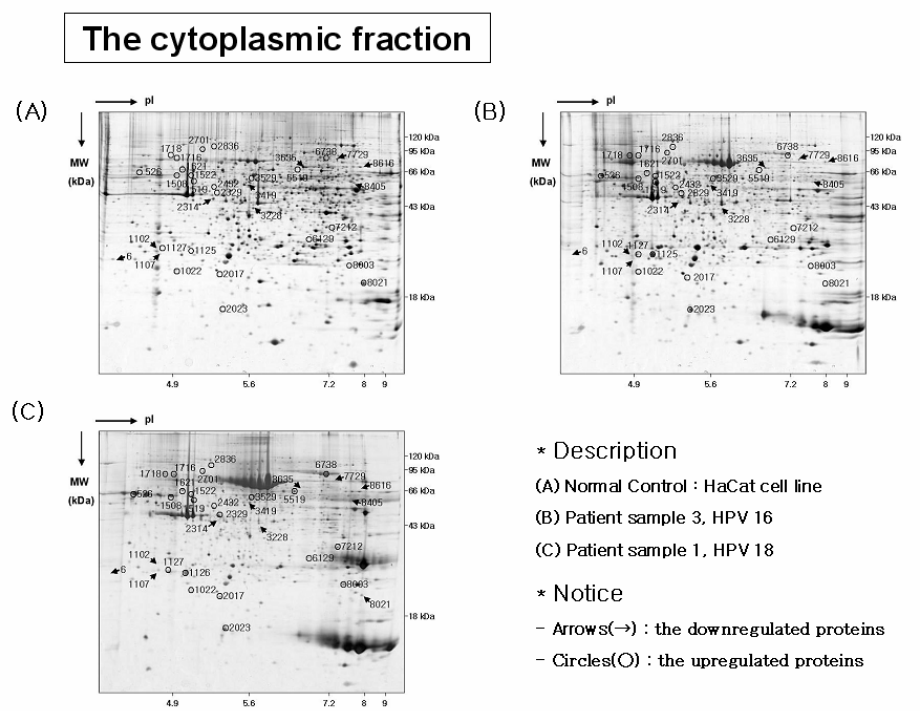


Figure 1. Two dimensional electrophoretic map of normal HaCat cell lines and human cervical cancer tissues. In order to select protein variations, the protein patterns of tumor and control were set into two classes. The 2-DE profiles were very similar among the samples. For comparing the protein expression differences of the tumor and the paired normal samples, we selected a total of 64 differential protein spots (Nuclear fraction: 31, Cytoplasmic fraction: 33). Although we analyzed a total of 6 cervical cancer samples infected by the high-risk HPVs (HPV 16: 3 samples, HPV 18: 3 samples), we showed just a sample in each type HPV. (HPV 16: sample # 3, HPV 18: sample # 1; Table 1)

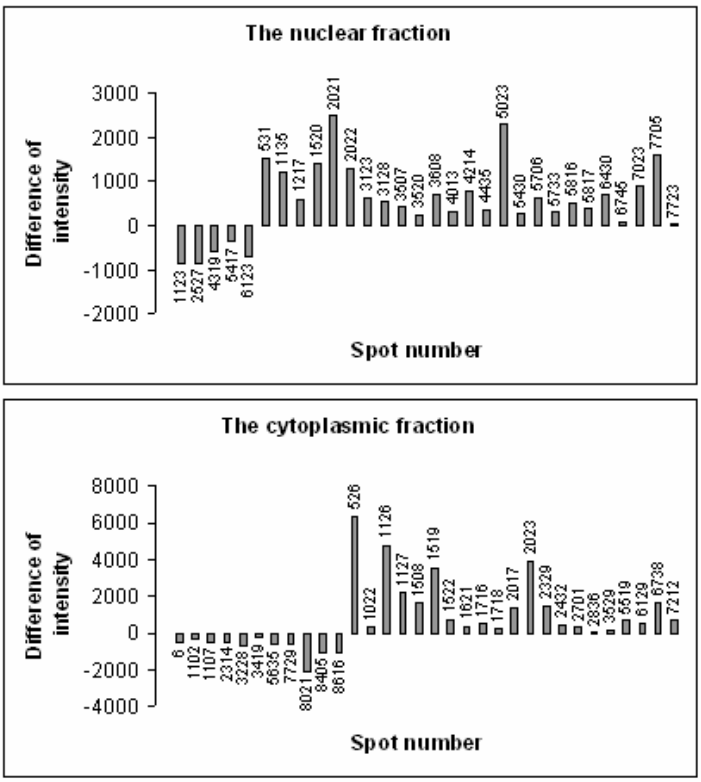


Figure 2. Select the differential expression protein spots of the tumor and the paired normal samples. To select the differential expression protein spots from the 2-DE gels of each fraction, we analyzed those by using the PDQuest software (version 7.0, BioRad). A total of 64 differential protein spots (A; the nuclear fraction: 31, B; the cytoplasmic fraction: 33) is seleted and all of 2-DE gel images went through normalization process. A; 5 one of seleted 31 spots are downregulated proteins and the rest, 26 spots, are upregulated proteins. B; 11 one of seleted 33 spots are downregulated proteins and the rest, 22 spots, are upregulated proteins.

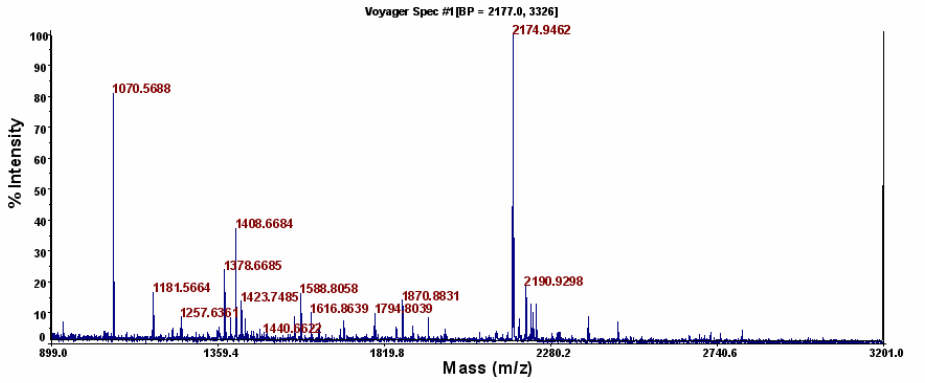


Figure 3. Peptide mass fingerprinting of cytoplasmic fraction spot 7212 in human cervical cancer tissues 2-DE map.

Table 2. Matching of cytoplasmic spot 7212 peptide mass fingerprint data with protein P29508 in database.

18/48 matches (37%).

Acc. # : P29508 Species : HUMAN

Name : Squamous cell carcinoma antigen 1 (SCCA-1) (Protein T4-A)

Index : 72461

MW/pI : 44565(Da)/6.3

m/z Submitted	MH+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1070.5688	1070.5635	5		378	386	0	(K) TNSILFYGR (F)
1181.5664	1181.5816	-13		78	87	0	(R) SGNVHHQFQK (L)
1257.6361	1257.6367	-0.46		287	296	1	(R) FKVEESYDLK (D)
1378.6685	1378.6618	4.9		11	20	0	(K) FMFDLFQQFR (K)
1394.6662	1394.6567	6.8	1Met-ox	11	20	0	(K)FMFDLFQQFR(K)
1408.6684	1408.6717	-2.4		266	276	0	(K) LMEWTSLQNMR (E)
1423.7485	1423.7334	11	pyroGlu	375	386	1	(R)QNKTNSILFYGR(F)
1424.6549	1424.6666	-8.3	1Met-ox	266	276	0	(K)LMEWTSLQNMR(E)
1434.7111	1434.6865	17		147	158	0	(K) INSWVESQTNEK (I)
1440.6622	1440.6615	0.46	2Met-ox	266	276	0	(K)LMEWTSLQNMR(E)
1588.8058	1588.7971	5.5		56	69	0	(K) VLHFDQVTENTTGK (A)
1616.8639	1616.8576	3.9		112	124	0	(K) TYLFLQEYLDAIK (K)
1794.8039	1794.8631	-33		266	279	1	(K) LMEWTSLQNMRETR (V)
1853.8874	1853.871	8.8	pyroGlu	215	230	0	(R)QYTSFHFASLEDVQAK(V)
1870.8831	1870.8976	-7.7		215	230	0	(R) QYTSFHFASLEDVQAK (V)
2174.9462	2174.952	-2.7		301	321	0	(R) TMGMVDIFNGDADLSGMTGSR (G)
2175.9598	2175.9835	-11		126	144	0	(K) FYQTSVESVDFANAPEESR (K)
2190.9298	2190.947	-7.8	1Met-ox	301	321	0	(R)TMGMVDIFNGDADLSGMTGSR(G)

*** The mached peptide sequences are shown in gray.**

1 MNSLSEANTK FMFDLFQQFR KSKENNIFYS PISITSALGM VLLGAKDNTA QQIKKVLHFD QVTENTTGKA ATYHVDRSGN

81 VHHQFQKLLT EFNKSTDAYE LKIANKLFGE KTYLFLQEYL DAIKKFYQTS VESVDFANAP EESRKKINSW VESQTNEKIK

161 NLIPEGNIGS NTTLVLVNAI YFKGQWEKKF NKEDTKEEKF WPNKNTYKSI QMMRQYTSFH FASLEDVQAK VLEIPYKGKD

241 LSMIVLLPNE IDGLQKLEEK LTAEKLMEWT SLQNMRETRV DLHLPRFKVE ESYDLKDTLR TMGMVDIFNG DADLSGMTGS

321 RGLVLSGVLH KAFVEVTEEG AEAAAATAVV GFGSSPTSTN EEFHCNHPFL FFIRQNKTNS ILFYGRFSSP

Table 3. Charaterized diferentially expressed proteins in the nuclear fraction of human cervical cancer tissues compared to the normal controls. The peptide profiles of the protein spots treated with trypsin were analyzed by MALDI-TOF MS. And MS-Fit (the UCSF Mass Spectrometry, ProteinProspector v 4.0.5) was used for the database search: SwissProt. 10.30.2003 and NCBInr. 10.21.2003.

(a) Downregulated proteins

Spot #	Description	MOWSE Score	Matched Peptides	Cov(%)	Protein MW(Da)/pI	Acession #	Intensity Rate
1123	DNA-repair protein XRCC2	281	6	27	31957/5.7	O43543	-7.28
2527	Dual specificity phosphatase Cdc25B	181	9	16	64988/6.0	P30305	-5.82
4319	Hypothetical protein DJ845O24.1	50.7	4	10	53386/8.6	O60809	-3.06
5417	Caspase-2 precursor (CASP-2) (ICH-1 protease)	1.59E+07	20	43	54040/6.2	Q16877	-5.16
6123	DNA-repair protein XRCC3	1.78E+04	12	35	37850/8.8	O43542	-10.00

*** Minus(-) :the rate of decrease of intensity (normal samples' average intensity/tissue samples' average intensity)**

*** The MW and pI values specified are theoretically matched by database search.**

(b) Upregulated proteins

Spot #	Description	MOWSE Score	Matched Peptides	Cov(%)	Protein MW(Da)/pI	Acession #	Intensity Rate
531	Rho guanine nucleotide exchange factor 5 (Oncogene TIM)	1482	8	16	60057/7.6	Q12774	+75.49
1135	Proliferating cell nuclear antigen (PCNA)	453	8	37	28769/4.6	P12004	+428.76
1217	Hsp90 co-chaperone Cdc37 (p50Cdc37)	101	7	10	44469/5.2	Q16543	+2.41
1520	Proto-oncogene tyrosine-protein kinase FGR (P55-FGR)	162	6	9	59479/5.4	P09769	+11.27
2021	Chromatin accessibility complex protein 1 (CHRAC-1)	101	4	26	14711/5.0	Q9NRG0	+54.24
2022	GTP-binding nuclear protein RAN	535	6	28	24423/7.0	P17080	+10.50
3123	Heat shock 70 kDa protein 7 (Heat shock 70 kDa protein B)	331	4	17	26907/7.0	P48741	+4.45
3128	Protein C21orf59	115	8	30	33224/7.0	P57076	+42.53
3507	Hsp70/Hsp90-organizing protein	273	11	19	62640/6.4	P31948	+2.65
3520	Proto-oncogene tyrosine-protein kinase FYN (P59-FYN)	69.8	5	13	60762/6.2	P06241	+24.80

3608	TRIO and F-actin binding protein (Protein Tara)	449	16	28	68042/5.8	Q9H2D6	+4.54
4013	Tumor protein D53 (hD53) (D52-like 1)	846	9	40	22449/5.5	Q16890	+7.86
4214	Telomeric repeat binding factor 2 interacting protein 1	256	5	15	44260/4.6	Q9NYB0	+10.39
4435	Vimentin (Breast carcinoma protein)	3.44E+04	15	33	53686/5.1	P08670	+8.70
5023	C-Myc binding protein (Associate of Myc 1)	53.5	5	38	11967/5.7	Q99417	+86.28
5430	Mdm4 protein (p53-binding protein Mdm4)	937	9	17	54864/4.9	O15151	+106.27
5706	Nuclear factor NF-kappa-B p105 subunit	785	8	14	105357/5.2	P19838	+166.13
5733	DNA replication licensing factor MCM8	1.05E+04	12	16	93698/7.8	Q9UJA3	+14.78
5816	Microtubule-associated protein 4 (MAP 4)	428	8	11	121020/5.3	P27816	+386.65
5817	Kinesin-like protein KIF11	3690	19	17	119275/5.5	P52732	+10819.76
6430	Dual specificity phosphatase Cdc25A	2.57E+04	12	24	58797/6.3	P30304	+2.22
6745	DNA replication licensing factor MCM4 (CDC21 homolog)	402	13	12	96612/6.3	P33991	+2.48
7023	Hypothetical protein KIAA1649	139	4	30	11914/8.1	Q9BY77	+80.72
7705	DNA replication licensing factor MCM3	519	12	17	90982/5.5	P25205	+66.04
7723	Zinc finger protein 151 (Myc-interacting zinc finger protein)	1256	6	12	87960/6.0	Q13105	+3.56
8023	Zinc-finger protein DPF3 (cer-d4)	5251	10	43	25826/7.7	Q92784	+75.16

*** Plus(+) : the rate of increase of intensity (tissue samples' average intensity/normal samples' average intensity)**

*** The MW and pI values specified are theoretically matched by database search.**

Table 4. Charaterized diferentially expressed proteins in the cytoplasmic fraction of human cervical cancer tissues compared to the normal controls. The peptide profiles of the protein spots treated with trypsin were analyzed by MALDI-TOF MS. And MS-Fit (the UCSF Mass Spectrometry, ProteinProspector v 4.0.5) was used for the database search: SwissProt. 10.30.2003 and NCBInr. 10.21.2003.

(a) Downregulated proteins

Spot #	Description	MOWSE Score	Matched Peptides	Cov(%)	Protein MW(Da)/pI	Acession #	Intensity Rate
6	Caspase-14 precursor (CASP-14)	364	6	19	27680/5.4	P31944	-3.54
1102	14-3-3 protein sigma (Epithelial cell marker protein 1)	33.7	5	19	27774/4.7	P31947	-9.87
1107	Glutathione S-transferase Mu 1	2961	10	31	25712/6.2	P09488	-44.05
2314	Zinc-finger protein ubi-d4	122	5	13	44156/5.9	Q92785	-6.88
3228	DNA repair protein RAD51 homolog 3	97.6	4	15	42190/6.3	O43502	-6.21
3419	TNF receptor associated factor 2	55.1	6	12	55860/7.7	Q12933	-4.59
5635	Protein C20orf129	97.6	6	9	64425/6.1	Q9H4H8	-2.51
7729	Hypothetical protein KIAA0053	150	9	17	72432/5.8	P42331	-4.62
8021	Undetect				19.5 KDa/ 8.4		-6.93
8405	Growth-arrest-specific protein 8 (Growth arrest-specific 11)	86.5	8	15	56356/7.7	O95995	-4.00
8616	MAGUK p55 subfamily member 2 (MPP2 protein)	145	8	11	64587/6.3	Q14168	-18.58

*** Minus(-) : the rate of decrease of intensity (normal samples' average intensity/tissue samples' average intensity)**

***The MW and pI values specified are theoretically matched by database search.**

(b) Upregulated proteins

Spot #	Description	MOWSE Score	Matched Peptides	Cov(%)	Protein MW(Da)/pI	Acession #	Intensity Rate
526	Tyrosine-protein kinase HCK (p59-HCK/p60-HCK)	132	4	7	59600/6.3	P08631	+509.85
1022	Cell division control protein 42 homolog	77.5	4	25	21311/5.8	P21181	+8.61
1126	Matrilysin precursor (Matrix metalloproteinase-7)	50.1	6	25	29677/7.7	P09237	+1874.77
1127	Spindlin homolog (Protein DXF34)	236	6	29	26537/6.3	Q99865	+100.59
1508	Ubiquitin-protein ligase E3 Mdm2 (p53-binding protein Mdm2)	151	6	11	55233/4.6	Q00987	+9.43
1519	G1 to S phase transition protein 1 homolog	207	7	10	55756/5.4	P15170	+9.46

1522	T-complex protein 1, eta subunit	163	8	11	59367/7.6	Q99832	+10.42
1621	Dual specificity phosphatase Cdc25B	294	7	12	64988/6.0	P30305	+2.08
1716	RAS-responsive element binding protein 1 (RREB-1)	672	9	10	79876/5.8	Q92766	+35.78
1718	MLN 51 protein	150	9	10	76279/6.1	O15234	+40.11
2017	Wilms' tumor 1-associating protein (WT1-associated protein)	100	8	42	17801/5.4	Q15007	+23.72
2023	Calmodulin	126	4	17	16838/4.1	124734	+2219.73
2329	FK506-binding protein 5 (HSP90-binding immunophilin)	114	5	14	51213/5.7	Q13451	+29.37
2432	Mitogen-activated protein kinase kinase kinase 8	124	6	12	52898/5.5	P41279	+6.21
2701	Acylamino-acid-releasing enzyme (Acyl-peptide hydrolase)	7.17E+04	14	17	81293/5.3	P13798	+2.59
2836	Heat shock protein HSP 90-alpha (HSP 86)	349	7	8	84674/4.9	P07900	+1.57
3529	Tyrosine-protein kinase LYN	102	4	10	58575/6.7	P07948	+4.15
5519	Undetect				63.2 KDa/ 6.5		+49.24
6129	Undetect				30.9 KDa/ 6.8		+215.27
6738	Undetect				77.3 KDa/ 7.1		+12.80
7212	Squamous cell carcinoma antigen 1 (SCCA-1)	5.35E+09	18	38	44565/6.3	P29508	+2.47
8003	Undetect				23.5 KDa/ 7.8		+4.47

*** Plus(+) : the rate of increase of intensity (tissue samples' average intensity/normal samples' average intensity)**

*** The MW and pI values specified are theoretically matched by database search.**

3. Malignant transformation by HPV infections

The ability of HPV infections to progress onward to malignancies has been attributed to the actions of the E6 and E7 proteins and their ability to manipulate cell cycle regulators. Recent efforts in the study of HPVs have identified the principle cellular targets of E6 and E7 oncoproteins from the high-risk HPV types. E6 forms a complex with p53 and, in combination with the cellular ubiquitin ligase E6AP [10], induces the degradation of p53 through MDM2 and 4 [11, 12]. E7 binds to and functionally inactivates pRB. Both p53 and pRB play critical roles as regulators of the cell cycle and apoptosis. By inhibiting these and other regulatory mechanisms, HPV E6 and E7 allow for the accumulation of genetic mutations and the survival of mutated cells. [2] E6 and E7 expression also contributes to the immortalization of infected cells. [13] E6 can enhance telomerase activity whereas E7 inhibits a p16*ink4A*-dependent pathway that limits cellular proliferation in epithelial cells. [14] It is generally believed that these functions of the viral oncoproteins contribute to cancer. In addition, the relatively low incidence of malignant progression of high-risk HPV-positive lesions indicates that additional mutations of cellular genes may also be necessary for malignant progression.

4. Classifications of data according to SEBs (Surrogate Endopoint Biomarkers)

As we mentioned above, HPV is critically important in the carcinogenesis of cervical carcinoma. Specific viral transforming genes, E6 and E7 from HPV types 16 and 18 act as oncogenes. [3] However, their expression is not still sufficient to explain an overall influence for malignant conversion. Therefore, in this study, by using proteome analysis, we identified the overall

protein profiles by HPV infections, and then we classified them according to SEBs (Surrogate Endopoint Biomarkers): proliferation markers, regulation markers, differentiation markers, general genomic instability markers, tissue maintenance markers, cancer-related proteins, stress-inducible proteins, hypothetical proteins, and unknown-function proteins. [15] (Table 5) However, in this study, we'll lay emphasis on proliferation markers, regulation markers, and general genomic instability markers. Because these are closely connected with oncogenes E6/E7 and the expression levels of proteins, such as PCNA (proliferating cell nuclear antigen) [16, 17], Mdm 2 and 4 [18, 19], Cdc25 A and B [20-22], 14-3-3 sigma [23, 24], and MCM 3, 4, and 8 [25-28], etc., actually were changed in each fraction. (Figure 4) Therefore, in this section, we'll elicit more clear relationship between the high-risk HPV types 16/18 and the carcinogenesis by reclassifying data according to the SEBs (Surrogate Endopoint Biomarkers).

5. Proliferation markers and human uterine cervical cancer

Proliferation is believed to be an early marker of disordered growth. It is hypothesized that increased proliferation is associated with more advanced lesions and that the distribution of proliferating cells in tissue may be indicative of the regulatory mechanisms that become dysfunctional during the multistep tumor process.

PCNA (proliferating cell nuclear antigen) is a nuclear protein whose expression is associated with late G1-phase, S-phase, and early G2 phase of the cell cycle. [16] Studies of PCNA in invasive cervical carcinoma specimens

have demonstrated its increased activity and up-regulation. [17] In our study, we confirmed that PCNA is up-regulated in the nuclear fraction and it will be important for understanding the neoplastic process by HPV infection in the cervix. (Figure 4, a)

6. Regulation markers and subcellular localization

Oncogens, such as C-myc binding protein (an associate of Myc 1), Mdm4 protein (p53-binding protein Mdm4), Zinc finger protein 151 (Myc-interacting zinc finger protein) and proto-oncogenes in the nuclear fraction, on the one hand, and ubiquitin-protein ligase E3 Mdm2 (p53-binding protein Mdm2), RAS-responsive element binding protein 1 (RREB-1) and Mitogen-activated protein kinase kinase kinase 8 (C-COT) in the cytoplasmic fraction are all up-regulated. In our study, the remarkable point is that Mdm2 and Mdm 4 are up-regulated together. (Figure 4, d) The Mdm2 oncoprotein targets the p53 tumor suppressor protein which results in its ubiquitin-mediated degradation [18], and the Mdm4 oncoprotein prevents MDM2-targeted degradation of p53 while maintaining suppression of p53 transactivation and apoptotic functions. [19] Therefore, it is considered that MDM 2 and 4 up-regulated in each fraction is closely connected with E6 oncoprotein.

Cell cycle-related proteins, such as M-phase inducer phosphatase 2 (Dual specificity phosphatase cdc25B) is down-regulated, whereas GTP-binding nuclear protein Ran (Androgen receptor-associated protein 24), kinesin-like protein KIF11 (kinesin-related motor protein Eg5) and M-phase inducer phosphatase 1 (Dual specificity phosphatase Cdc25A) are all up-regulated in the nuclear fraction. On one hand, in the cytoplasmic fraction, 14-3-3

protein sigma is down-regulated, while Spindin homologue (Protein DXF34), G1 to S phase transition protein 1 homologue (GTP-binding protein GST1-HS), and M-phase inducer phosphatase 2 (Dual specificity phosphatase Cdc25B) are all up-regulated. Three members of the CDC25 family (CDC25A, B and C) are commonly characterized as cell cycle oscillators in different phases of the cell cycle, in which both CDC25B and CDC25C work at the G2/M checkpoint, and CDC25A works at the G1 checkpoint. [20] During carcinogenesis, both CDC25A and CDC25B are over-expressed in various human malignancies. [21] In our result, CDC25A and CDC25B are upregulated in the nuclear and cytoplasmic fraction, respectively. (Figure 4, c) The remarkable point is that CDC25B is downregulated in the nuclear fraction, while it is upregulated in the cytoplasmic fraction. Recent reports have demonstrated that CDC25B is located in the nucleus in G1 and then in the cytoplasm in the S and G2 phase. Moreover, the subcellular localization of CDC25B is controlled by the nuclear localization signal (NLS) and the nuclear export signal (NES). [22] Therefore, these results indicate that it is probably associated with G2 arrest in the carcinogenesis of cervical cancer. The 14-3-3 family plays a key regulatory role in signal transduction, apoptotic, checkpoint control, and nutrient sensing pathway. 14-3-3 proteins may participate in these pathways by alternating the subcellular localization of their binding partners. [23] Recent studies have demonstrated that 14-3-3 promotes the cytoplasmic localization of CDC25B and CDC25C. [24] In our study, 14-3-3 protein sigma is down-regulated in the cytoplasmic fraction. (Figure 4, b) Therefore, it is considered that the degradation of p53 induced by E6 oncoproteins affects the expression of 14-3-3 protein sigma.

7. General genetic instability markers and MCM (Minichromosome Maintenance) series

General genomic instability may be the most important biological marker of all and in tumors, general genomic instability has been demonstrated.

DNA replication-related proteins, such as Chromatin accessibility complex protein 1 (CHRAC-1) and DNA replication licensing factor MCM3, 4, and 8 are all upregulated in the nuclear fraction. (Figure 4, e) The entire Mcm family (Mcm2−7) is essential for eukaryotic DNA replication, and this family plays roles in the initiation and elongation of DNA replication. [25] Recently, several groups have reported that Mcm proteins are more frequently detected in cells from malignant tissues than those from normal tissues. This phenomenon was also observed in dysplastic cells, suggesting that Mcm proteins are a good indicator of proliferative or cancer cells in malignant tissues. [26] At present, MCM7 is known as a highly informative biomarker for cervical cancer [27] and studies for MCM8, a new member of the MCM protein family, have been proceeding. However, the Mcm8 protein is unlike the other Mcm proteins, MCM8 is not structure-bound in cells arrested in the late G1 phase. In other words, MCM8 is structure-bound in the early S phase after the establishment of the replication forks, and not in the G1 phase. [28] At this time, the exact functions of MCM8 are still not apparent. Therefore, we recommend MCM8 as a novel target for cancer researches, including research on human uterine cervical cancer.

Table 5. Classifications of data according to SEBs (Surrogate Endopoint Biomarkers).

(a) The nuclear fraction

SEBs	Descriptions
Proliferation	
Upregulated proteins	1135 Proliferating cell nuclear antigen (PCNA)
Regulation	
Oncogenes	
Upregulated proteins	531 Rho guanine nucleotide exchange factor 5 (Oncogene TIM)
	1520 Proto-oncogene tyrosine-protein kinase FGR (P55-FGR)
	3520 Proto-oncogene tyrosine-protein kinase FYN (P59-FYN)
	5023 C-Myc binding protein (Associate of Myc 1)
	5430 Mdm4 protein (p53-binding protein Mdm4)
	7723 Zinc finger protein 151 (Myc-interacting zinc finger protein)
Cell cyle-related proteins	
Downregulated proteins	2527 Dual specificity phosphatase Cdc25B
Upregulated proteins	2022 GTP-binding nuclear protein RAN
	5817 Kinesin-like protein KIF11
	6430 Dual specificity phosphatase Cdc25A
Differntiation	
Fibrillar proteins	
Upregulated proteins	5816 Microtubule-associated protein 4 (MAP 4)
	3608 TRIO and F-actin binding protein (Protein Tara)
General genomic instability	
DNA replication	
Upregulated proteins	2021 Chromatin accessibility complex protein 1 (CHRAC-1)
	5733 DNA replication licensing factor MCM8
	6745 DNA replication licensing factor MCM4 (CDC21 homolog)
	7705 DNA replication licensing factor MCM3

DNA repair

Downregulated proteins	1123 DNA-repair protein XRCC2
	6123 DNA-repair protein XRCC3

Tissue maintenance

Telomerase-related proteins

Upregulated proteins	4214 Telomeric repeat binding factor 2 interacting protein 1

Apoptosis

Downregulated proteins	5417 Caspase-2 precursor (CASP-2)

Cancer-related proteins

Upregulated proteins	4013 Tumor protein D53 (hD53) (Breast carcinoma protein)
	4435 Vimentin(Breast carcinoma protein)

Stress-inducible proteins

Upregulated proteins	1217 Hsp90 co-chaperone Cdc37 (p50Cdc37)
	3123 Heat shock 70 kDa protein 7 (Heat shock 70 kDa protein B)
	3507 Hsp70/Hsp90-organizing protein
	5706 Nuclear factor NF-kappa-B p105 subunit

Hypothetical proteins

Downregulated proteins	4319 Hypothetical protein DJ845O24.1
Upregulated proteins	7023 Hypothetical protein KIAA1649

Unknown-founction proteins

Upregulated proteins	3128 Protein C21orf59
	8023 Zinc-finger protein DPF3 (cer-d4)

(b) The cytoplasmic fraction

SEBs	Descriptions
Regulation	
Tumor suppressors	
Downregulated proteins	8616 MAGUK p55 subfamily member 2 (MPP2 protein)
Oncogenes	
Upregulated proteins	1508 Ubiquitin-protein ligase E3 Mdm2
	1716 RAS-responsive element binding protein 1 (RREB-1)
	2432 Mitogen-activated protein kinase kinase kinase 8 (C-COT)
Cell cyle-related proteins	
Downregulated proteins	1102 14-3-3 protein sigma (Stratifin)
Upregulated proteins	1127 Spindlin homolog (Protein DXF34) [139]
	1519 G1 to S phase transition protein 1 homolog
	1621 Dual specificity phosphatase Cdc25B
Cell division	
Upregulated proteins	1022 Cell division control protein 42 homolog
Cell growth	
Downregulated proteins	8405 Growth-arrest-specific protein 8
Upregulated proteins	526 Tyrosine-protein kinase HCK (p59-HCK/p60-HCK)
Signal transduction	
Upregulated proteins	2023 Calmodulin
	3529 Tyrosine-protein kinase LYN
Molecular chaperones	
Upregulated proteins	1522 T-complex protein 1, eta subunit (TCP-1-eta) (CCT-eta)
General genomic instability	
DNA repair	
Downregulated proteins	3228 DNA repair protein RAD51 homolog 3

Tissue maintenance	
Metalloproteinases	
Upregulated proteins	1126 Matrilysin precursor (Uterine metalloproteinase) (MMP-7)
Apoptosis	
Downregulated proteins	6 Caspase-14 precursor (CASP-14)
	2314 Zinc-finger protein ubi-d4
Necrosis	
Downregulated proteins	3419 TNF receptor associated factor 2
Metabolism	
Downregulated proteins	1107 Glutathione S-transferase Mu 1 (GSTM1-1)
Cancer-related proteins	
Upregulated proteins	1718 MLN 51 protein
	2017 Wilms' tumor 1-associating protein
	2701 Acylamino-acid-releasing enzyme
	7212 Squamous cell carcinoma antigen 1 (SCCA-1)
Stress-inducible proteins	
Upregulated proteins	2329 FK506-binding protein 5 (HSP90-binding immunophilin)
	2836 Heat shock protein HSP 90-alpha (HSP 86)
Hypothetical proteins	
Downregulated proteins	7729 Hypothetical protein KIAA0053
Unknown-founction proteins	
Downregulated proteins	5635 Protein C20orf129


The nuclear fraction
The cytoplasmic fraction
(a)
1135 Proliferating cell nuclear antigen(PCNA)
(b)
1102 14-3-3 sigma
(c)
6430 Dual specificity phosphatase Cdc25A
2527 Dual specificity phosphatase Cdc25B
1621 Dual specificity phosphatase Cdc25B
(d)
5430 Mdm4 protein (p53-binding protein Mdm4)
1508 Ubiquitin-protein ligase E3 Mdm2
(e)
7705 DNA replication licencing factor MCM3
6745 DNA replication licencing factor MCM4 (CDC21 homolog)
5733 DNA replication licencing factor MCM8
Cervical cancer tissues, HPV 16
Cervical cancer tissues, HPV 18
Spot Intensity
Samples
HaCat 1
HaCat 2
HPV16(1)
HPV16(2)
HPV16(3)
HPV18(1)
HPV18(2)
HPV18(3)

Figure 4. Differential expression of several spots in the nuclear and the cytoplasmic fraction.

(a) The nuclear fraction spot 1135 (proliferating cell nuclear antigen) was upregulated. (b) The cytoplasmic fraction spot 1102 (14-3-3 sigma) was downregulated. (c) The nuclear fraction spot 6430 (CDC25A) upregulated; In the nuclear fraction, spot 2527 (CDC25B) was, whereas in the cytoplasmic fraction, spot 1621(CDC25B) was upregulated. (d) The nuclear fraction spot 5430 (p53-binding Mdm4) and the cytoplasmic spot 1508 (ubiqutin-protein ligase E3 Mdm 2) simultaneously was upregulated in each fraction. (e) Spot 5733 (MCM8), spot 6745 (MCM4) and spot 7704 (MCM3) were upregulated together in the nuclear fraction. (black: normal samples, gray: tumor samples)

IV. CONCLUSION

In conclusion, we have demonstrated that HPVs can cause the unstable regulation of a lot of carcinogenesis-related proteins, such as proliferation factors, cell cycle regulatory factors, cell signaling factors, oncogenes etc. without the significant difference of any specific molecules being found according to the HPV genotypes, in the ongoing process of cervical cancer. Until a marker of such versatility is found, knowing everything possible about the markers that we have is crucial, and this study has shown some new findings about these known markers. Ultimately, the long-term goal of future studies is to establish markers that can be used to detect cancer at an earlier stage than is currently possible, and at the same time, this line of research will almost certainly yield new prognostic and therapeutic information, along with novel treatment modalities.

REFERENCES

1. Furumoto, H., Irahara, M., Human papilloma virus (HPV) and cervical cancer. *J. Med. Invest.* 2002, *49*, 124-133.
2. Frauke Fehrmann and Laimonis A. Laimins, Human papillomaviruses: targeting differentiating epithelial cells for malignant transformation. *Oncogene* 2003, *22*, 5201-5207.
3. Cullen, A. P., Reid, R., Campion, M., Lorincz, A. T., Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm. *J. Virol.* 1991, *65*, 606−612.
4. Scheffner, M., Huibregtse, J. M., Vierstra, R. D., Howley, P. M., The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* 1993, *75*, 495−505.
5. Slebos, R. J., Lee, M. H., Plunkett, B. S., Kessis, T. D., *et al.*, p53-dependent G1 arrest involves pRB-related proteins and is disrupted by the human papillomavirus 16 E7 oncoprotein. *Proc. Natl. Acad. Sci. USA* 1994, *91*, 5320−5324.
6. Lorincz, A. T., Screening for cervical cancer: new alternatives and research. *Salud Publica Mex* 2003, *45 Suppl. 3*, S376-S387.
7. Gadducci, A., Cosio, S., Carpi, A., Nicolini, A., Genazzani, A. R., Serum tumor markers in the management of ovarian, endometrial and cervical cancer. *Biomed. Pharmacother.* 2004, *58*, 24-38.
8. Nam Hoon Cho, Hee Jung An, Jeongmi K. Jeong, *et al.*, Genotyping of 22 human papillomavirus types by DNA chip in Korean women: comparison with cytologic diagnosis. *Am. J. Obstet. Gynecol.* 2003,*188*,

56-62.

9. Rosl F., Achtstatter T., Bauknecht T., Hutter K. J., *et al.*, Extinction of the HPV18 upstream regulatory region in cervical carcinoma cells after fusion with non-tumorigenic human keratinocytes under non-selective conditions. *EMBO J.* 1991, *10*, 1337-1345.
10. Traidej, M., Chen, L., Yu, D., Agrawal, S., Chen, J., The roles of E6-AP and MDM2 in p53 regulation in human papillomavirus-positive cervical cancer cells. *Antisense Nucleic Acid Drug Dev.* 2000, *10*, 17-27.
11. Sharp, D. A., Kratowicz, S. A., Sank, M. J., George, D. L., Stabilization of the MDM2 oncoprotein by interaction with the structurally related MDMX protein. *J. Biol. Chem.* 1999, *274*, 38189-38196.
12. Hoppe-Seyler, F., Butz, K., Repression of endogenous p53 transactivation function in HeLa cervical carcinoma cells by human papillomavirus type 16 E6, human mdm-2, and mutant p53. *J. Virol.* 1993, *67*, 3111-3117.
13. Liu, Y., Chen, J. J., Gao, Q., Dalal, S., Multiple functions of human papillomavirus type 16 E6 contribute to the immortalization of mammary epithelial cells. *J. Virol.* 1999, *73*, 7297−7307.
14. Kiyono, T., S. A. Foster, J. I. Koop, J. K. McDougall, *et al.*, Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature,* 1998, *396*, 84−88.
15. Follen, M., Schottenfeld, D., Surrogate endpoint biomarkers and their modulation in cervical chemoprevention trials. *Cancer* 2001, *91*, 1758−1776.
16. Chuang, L. S., Ian, H. I., Koh, T. W., Ng, H. H., *et al.*, Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1.

Science 1997, *277*, 1996-2000.

17. Mittal K. R, Demopoulos R. I, Goswami S., Proliferating cell nuclear antigen (cyclin) expression in normal and abnormal cervical squamous epithelia. *Am. J. Surg. Pathol.* 1993, *17*, 117−122.
18. Hoppe-Seyler, F., Butz, K., Repression of endogenous p53 transactivation function in HeLa cervical carcinoma cells by human papillomavirus type 16 E6, human mdm-2, and mutant p53. *J. Virol.* 1993, *67*, 3111-3117.
19. Sharp, D. A., Kratowicz, S. A., Sank, M. J., George, D. L., Stabilization of the MDM2 oncoprotein by interaction with the structurally related MDMX protein. *J. Biol. Chem.* 1999,*274*, 38189-38196.
20. Galaktionov, K., Beach, D., Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins. *Cell* 1991, *67*, 1181−1194.
21. Gasparotto, D., Maestro, R., Piccinin, S., Vukosavljevic, T., *et al.*, Overexpression of CDC25A and CDC25B in head and neck cancers. *Cancer Res.* 1997, *57*, 2366−2368.
22. Davezac, N., Baldin, V., Gabrielli, B., Forrest, A., *et al.*, Regulation of CDC25B phosphatases subcellular localization. *Oncogene* 2000, 27, *19*, 2179-2185.
23. Fu, H., Subramanian, R. R., Masters, S. C., 14-3-3 proteins: structure, function, and regulation. *Annu. Rev. Pharmacol. Toxicol.* 2000, *40*, 617-647.
24. Lopez-Girona, A., Furnari, B., Mondesert, O., Russell, P., Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. *Nature* 1999, *397*, 172-175.

25. Labib, K., Tercero, J. A., Diffley, J. F., Uninterrupted MCM2-7 function required for DNA replication fork progression. *Science* 2000, *288*, 1643−1647.
26. Freeman, A., Morris, L. S., Mills, A. D., Stoeber, K., *et al.*, Minichromosome maintenance proteins as biological markers of dysplasia and malignancy. *Clin. Cancer Res.* 1999, *5*, 2121−2132.
27. Brake, T., Connor, J. P., Petereit, D. G., Lambert, P. F., *et al.*, Comparative analysis of cervical cancer in women and in a human papillomavirus-transgenic mouse model: identification of minichromosome maintenance protein 7 as an informative biomarker for human cervical cancer. *Cancer Res.* 2003, *63*, 8173-8180.
28. Gozuacik, D., Chami, M., Lagorce, D., Faivre, J., Identification and functional characterization of a new member of the human Mcm protein family: hMcm8. *Nucleic Acids Res.* 2003,*31*, 570-579.

ABSTRACT (in Korean)

자궁경부암 조직의 단백체 분석

<지도교수 조 남 훈>

연세대학교 대학원 의과학과

최 윤 표

Human papilloma virus(HPV)는 대략 8 kb의 유전자를 가지고 있는 작은 DNA 바이러스이다. 현재까지 연구된 바로는 HPV 감염이 자궁암으로의 진행에 있어서 결정적인 역할을 하는 것으로 알려져 있으며 전체 HPV 아형 중 high-risk type인 16, 18, 31, 33, 45가 90% 이상의 자궁암과 관련된 것으로 보고되어있다. 또한 최근 발표된 몇몇 논문들에서 이들 high-risk type HPV 중 16과 18이 좀 더 예 후가 좋지않은 자궁암에서 우세하게 검출되는 것을 보고함으로써 high-risk type HPV의 감염과 자궁암과의 상관관계를 좀 더 명확히 하게 되었다.

현재 high-risk type HPV의 E6/E7이 P53이나 Rb과 같은 oncogene에

미치는 영향과 함께 HPV 감염에 의한 cell cycle control, chromosomal alteration, telomerase activation, 그리고 proliferation 등에 관계된 연구가 꾸준히 진행 중에 있기는 하지만 생체 내에서 실질적인 기능을 담당하는 단백질이 HPV에 감염되었을 때 어떠한 양상으로 변화하는지에 대한 연구는 부족한 현실에 있고 이것은 자궁암을 이해하고 치료하는데 있어서 상당한 어려움으로 작용하고 있다. 그러므로 이 연구에서는 high-risk type HPV 16, 18의 감염에 의해 야기되는 총체적인 단백질 발현양상의 변화를 밝혀내기 위해서 각각 high-risk type HPV 16, 18에 감염된 것으로 확인된 6명의 환자의 조직(각 아형당 3개의 조직)을 사용하였고 또한 단백질 추출과정에서 핵과 세포질을 나누어 단백질을 분리해내 그것들을 각각을 2-DE와 MALDI-TOF MS라는 proteomics의 대표적인 방법을 사용해 분석함으로서 특정 단백질의 세포 내 위치와 그 작용에 대한 상호관련성에 대한 보다 정확한 결과를 도출해 내고자 하였다.

우선 HPVDNAchip(Biomedlab Co., Seoul, Korea)에 의해서 HPV 감염이 확인된 환자의 조직으로부터 핵과 세포질을 나누어 단백질을 추출해낸 다음 2-DE를 사용해 등전점(Isoelectropoint, pI)과 분자량(Molecular weight, Mr)에 의해서 각각의 단백질을 분리해 내었고 이

것을 바탕으로 하여 cervical cancer와 대조군 사이의 단백질들의 differential expression을 확인한 다음 MALDI-TOF MS와 database analysis에 의해서 단백질의 정체를 확인하였다. 이렇게 정체가 확인되어진 단백질들을 SEB(Surrogate Endopoint Biomarker)에 따라서 재분리 함으로써 단백질의 기능과 세포 내 위치 그리고 자궁암 조기 진단과 치료를 위한 biomarker 발굴을 위한 기반을 마련하고자 하였고 또한 HPV 감염과 관련된 다른 암 종과의 상호연관성도 함께 도출해 내려고 하였다.

이 논문에서 주목할 만한 성과는 위에서 이미 언급한 바와 같이 HPV가 자궁암을 유발하는 핵심인자 이고 이것에 의한 감염이 malignant conversion을 야기하는 것으로 확실시 되었음에도 불구하고 실제 환자의 조직에서 자궁암의 발암과정에 관련된 분자들을 단백질 수준에서 총제적으로 확인하고 체계적으로 분류한 연구가 없던 상황에서 이것에 대한 소기의 목적을 달성했다는데 의의가 있다고 할 수 있다. 또한 이미 발표된 논문들의 결과를 일부분 재확인함으로써 HPV에 의한 발암기전을 이해하고 확고히 하는데 기여했다고 본다.

그리고 이 논문에서 단백질의 분류를 위해서 이용한 Surrogate

Endopoint Biomarker(SEB)는 proliferation marker, regulation marker, differentiation marker, general genomic instability marker, tissue maintenance marker 등으로 세분화 될 수 있으며 MALDI-TOF MS와 database analysis에 의해서 정체가 확인된 분자들은 각각의 기능과 세포 내 위치에 근거해 세부적으로 분류하였다. 이것들 중 PCNA(Proliferating cell nuclear antigen), 14-3-3 sigma, Mdm 2와 4, Cdc25A와 B, 그리고 MCM(Minichromosome maintenance) 3,4,8 등과 같은 발암기전과 밀접한 관계를 맺고있는 중요한 단백질들이 각각의 구획에서 증가 또는 감소되는 것을 확인할 수 있었다. 특히 이중 MCM8이 아직까지도 그것의 정확한 기능과 구조가 밝혀지지 않은 것으로 고려해 볼 때 자궁암을 포함한 HPV 관련 암을 연구하는데 있어서 새로운 target이 될 수 있을 것으로 기대된다.

결론적으로 이전의 연구내용 들과 이 논문의 결과를 종합해 볼 때 HPV가 자궁암의 발암과정에 있어서 명백한 원인자임을 다시 한 번 확인 할 수 있었으며, 더 나아가 이전까지 부족했던 HPV 감염에 의해서 나타나는 총체적인 단백질의 발현양상을 확인 함으로써 자궁암을 진단하고 치료할 수 있는 새로운 관점을 제시했다고 본다.

핵심 되는 말 : 사람자궁경부암; 2차전기영동; MALDI-TOF MS; 단백질체; Differential expression protein