

Deregulation of the G1/S phase transition in cancer and squamous intraepithelial lesions of the uterine cervix: A case control study

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Abstract. High-risk types of HPV express the oncoproteins, E6 and E7, that can inactivate TP53 and RB1, respectively, and thus take control of both cell cycle and apoptosis. Herein, the mRNA expression profiles of 24 G1/S checkpoint genes were analysed in cancer and squamous intraepithelial lesions (SIL) of the uterine cervix. In total 35 squamous cervical carcinomas, 26 high-grade SIL (HSIL), 33 low-grade SIL (LSIL) tissues, and 28 normal uterine cervix specimens as controls were assessed by RT-PCR. Five genes were found to be upregulated only in tumours, RBL2, E2F2, CDK6, CCNE1 and MYC; eight in tumours and HSILs, E2F1, E2F3, E2F5, CCND1, CDK2, CDKN1B, PCNA and POLA, and five in tumours, HSILs and LSILs, TP53, E2F4, CDKN1A, CDKN2A and DHFR. MDM2 was found to be upregulated in SIL, while RBL1 was found to be downregulated in all three groups of cases. TP73 exhibited lower levels in carcinomas; however, its exon 13-containing isoforms were increased and exon 2-containing isoforms were reduced in both cancer and HSIL. Three genes, RB1, CDK4 and CDKN2D, did not exhibit any significant alteration in gene expression. Hierarchical clustering revealed that this set of G1/S checkpoint genes was able to discriminate the total 122 samples into groups of disease and non-disease with only 8 exceptions (6.6%). Our data suggest that deregulation of G1/S phase transition in cervical carcinogenesis is a progressive process. Certain clusters of genes are activated very early in pre-cancerous SILs while others are activated later, during malignant transformation. The ability of this array of markers to identify disease status suggests that it could be used for diagnostic purposes.

Introduction

Cervical cancer is the second most common gynaecological malignancy worldwide, with ~500,000 new cases and 274,000 deaths per annum. It is much more common in the developing countries, where 83% of cases occur (1). Chronic infection of keratinocytes of the uterine cervix by certain types of human papillomavirus (HPV), classified as high-risk, is well established as the causative agent of both invasive cervical cancer and cervical intraepithelial neoplasia (2,3). Despite the development of an HPV vaccine for certain high-risk types (4-6) and the development of national HPV vaccination policies, a large number of the female population, remains at risk (7).

Carcinogenesis of the uterine cervix, from HPV infection to viral persistence, to the development of precancerous lesions, and finally invasive cancer, is a complex process that remains to be elucidated. Cervical carcinoma develops infrequently, even after infection with high-risk types of HPV, and it typically occurs years to decades after the initial infection (8). The cancer precursors, squamous intraepithelial lesions (SILs) are also infected by high-risk HPV types but do not exhibit such a proliferative or invasive phenotype as tumours (9). Thus, the development of new biomarkers that could accurately characterize each step of this process is important for the better clinical management of women suffering HPV infection.

Unlike the HPV-associated benign lesions, where the viral genome is maintained episomally, the majority of HPV-associated cancers contain viral DNA integrated into the genome of the host cells (10,11). While most of the open reading frames of the integrated viral DNA are lost or interrupted, the HPV oncoproteins, E6 and E7, continue to be expressed (10). Furthermore, the expression of both proteins is upregulated upon integration, because the gene encoding the viral transcriptional repressor E2 is generally lost and the mRNA encoding E6 and E7 is stabilized (12,13). E6 and E7 proteins encoded by high-risk types of HPV interact with TP53 and the retinoblastoma protein (RB1) respectively to inactivate these tumour suppressors (14-16).

Progression through the cell cycle depends on the coordinated synthesis, activation, and degradation of a family of cyclins (CCNs) that act as catalytic subunits of

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cyclin-dependent kinases (CDKs). Different CCN/CDK holoenzymes are activated at specific phases of the cell cycle and exert their regulatory control by phosphorylating key proteins, such as transcription factors, involved in cell cycle progression (17). The activity of these kinases is directed in part by inhibitors of CDKs (CKNs). Two classes of CKN exist; the Cip/Kip family, which includes CDKN1A (p21), CDKN1B (p27) and CDKN1C (p57), CKNs that regulate cell proliferation throughout the cell cycle; and the INK4 proteins, CDKN2B (p15), CDKN2A (p16), CDKN2C (p18) and CDKN2D (p19), which are inhibitors of CDK4 and CDK6 and are active only at G1 phase (18,19).

The RB1 protein is also an important negative regulator of proliferation that, during the resting G0 state, is unphosphorylated and inhibits cell cycle progression. During G1 to S phase transition, when it is hypophosphorylated, it binds to the transcription factor, E2F, and prevents the transcription of genes involved in the transition, such as polymerase A (POLA), dihydrofolate reductase (DHFR), MYC and proliferating cell nuclear antigen (PCNA); and, when it is phosphorylated by CCND1/CDK4/6, it permits cells to traverse the G1/S checkpoint and proliferate (20). The Rb-like RBL1 (p107) and RBL2 (p130), also known as pocket proteins, display similar and distinct functions to RB1 in G1/S transition control, but they contain a Cip/Kip like motif which enables binding and inhibition of CDKs (20).

The E2F family of transcription factors plays an important role in the control of the cell cycle and action of tumour suppressor proteins. The E2F proteins contain conserved domains, including a DNA-binding domain, a dimerization domain which determines interaction with the differentiation-regulated transcription factor proteins (DP), a transactivation domain enriched in acidic amino acids, and a tumour suppressor protein association domain which is embedded within the transactivation domain. E2F1, E2F2 and E2F3 have an additional cyclin-binding domain, while E2F4 binds preferentially to RBL1/RBL2 and E2F5 to RBL2 (21,22).

The TP53 protein regulates, as transcriptional factor, the expression of CDKN1A, which acts as a universal inhibitor, able to bind to and inhibit CDKs at all stages of the cell cycle. When it is overexpressed, CDKN1A inhibits CCND1/CDK4/6 kinase activity and arrests cells in G1 (23). TP53 can be induced in response to DNA damage and could be degraded by the action of the MDM2 protein, while CDKN2D counteracts this effect (24). TP73, one of the two TP53 homologues, can be divided similarly to p53 functional domains, into amino-terminal transactivation, central DNA-binding domain and homo-oligomerization domain. However, it harbours an extended, compared with TP53, carboxy-terminal where two additional domains exist, a secondary transactivation and a sterile alpha motif (SAM) domain (25). It shares overlapping functions with TP53 to some extent, such as CDKN1A and MDM2 transcriptional activation, but has a unique role in TP53-independent apoptosis through E2F1 (26). The various TP73 isoforms, those with truncated carboxy-terminal (exon 13 lacking) and others with truncated amino-terminal (exon 2 lacking) have different transactivation efficacies and are able to hetero-oligomerize with TP53 mutants to form tetramers with variable ability to activate and antagonise for TP53-responsive promoters (25).

Table I. Oligonucleotide primer sequences.

Primers	Sequence (5'-3')	Fragment (bp)
E2F1-F	GTGCAGATTGGAGGGTGG	154
E2F1-R	GATGGATATGAGATGGGAGAGG	
E2F2-F	CCTTGGAGGCTACTGACAGC	143
E2F2-R	CCACAGGTAGTCGTCTGGT	
E2F3-F	CACGTACCCCGTAGGAAAAG	120
E2F3-R	TCAGTGTGTGCATGACAACCTC	
E2F4-F	TTTTATTCACTTGAACACTGTAC	113
E2F4-R	CAGAGATTTAGAAAGATTACAG	
E2F5-F	TTTAATATCCACAAACGTCCCC	134
E2F5-R	GGCCACTGTTTTGATGACCT	
RB1-F	TGGGTGATTCTTAAGCCACTTGA	165
RB1-R	GGACTCCCCTCTAGGGCCATT	
RBL1-F	TGTGGTGAGCCAAGGTTG	148
RBL1-R	TGGGGTTTCTCCTTATTCA	
RBL2-F	AGCAGCAGCCTTTAATGCAT	249
RBL2-R	CTTGAGTATTTCTAAAAGCTTTGC	
POLA-F	TTGCCCTTAAATAGAAAGTGC	215
POLA-R	TTTTGCTGCTGCCAAAGAC	
B2M-F	TCCAACATCAACATCTTGGT	122
B2M-R	TCCCCAAATTCTAAGCAGA	
CCND1-F	AAAGACAGTTTTTGGGTAATCTTTT	126
CCND1-R	CCGGAGCATTTTGATACCAG	
CCNE1-F	ACAAAACAGGTTTCATCAAAGGC	225
CCNE1-R	GGTGCTACTTGACCCTAAGGG	
CDK2-F	TTCTTGAAGCCCCCAGC	152
CDK2-R	CCCCTGTATTCCAGAGTTG	
CDK4-F	CTTCTGGACACTGAGAGGGC	110
CDK4-R	TGGGAGGGGAATGTCATTAA	
CDK6-F	CGGAGAACACCCTTGGTG	105
CDK6-R	GAGCCTGTCCAGAAGACAGC	
PCNA-F	ACTCGCATTTAATGATGGTG	167
PCNA-R	CTTCTTTCATGAAGCAGTGG	
MYC-F	GGAAAAGTAAGGAAAACGATTCC	333
MYC-R	TAGGATTGAAATTCTGTGTAACCTGC	
DHFR-F	ACCAACATGTGAAAAGCCCCG	87
DHFR-R	ACCTGCTACAGTGAGCTGCC	
MDM2-F	TGAAGGTTTCTTCTCCTGAAGC	180
MDM2-R	AAGGTGGGAGTGATCAAAGG	
TP53-F	TTTGGGTCTTTGAACCCTTG	117
TP53-R	CCACAACAAAACACCAGTGC	
CDKN1A-F	ATTCAGCATTGTGGGAGGAG	131
CDKN1A-R	TGGACTGTTTTCTCTCGGCT	
CDKN1B-F	GGATAAGTGAAATGGATACTACATC	228
CDKN1B-R	AAAAAGAGGGGAAAACCTATTCTAC	

Primers	Sequence (5'-3')	Fragment (bp)
CDKN2A-F	CATCAGTCACCGAAGGTCCT	150
CDKN2A-R	AATGGACATTTACGGTAGTGGG	
CDKN2D-F	GATCTGGGGTCCACCCTCTC	207
CDKN2D-R	CCAACACACAAAAGGAGTG	
TP73-exon2-F	CACCACGTTTGAGCACCTC	94
TP73-exon2-R	CGCCACCACCTCATTATT	
TP73-F	GGACGTCTCCACCTGGAG	216
TP73-R	GTAGTCGGTGTGGAGGGG	
TP73-exon13-F	TTTTAACAGGATTGGGGTG	91
TP73-exon13-R	CTCAATGGTCAGGTTCTGC	

The transition of the cell cycle from G1 to S phase requires both cyclin D-dependent Cdk4 and Cdk6, and cyclin E/Cdk2 holoenzymes, and is regulated by the TP53/MDM2/CDKN1A/CDKN2D and RB1/E2F/CCND1/CDK4/CDK6/CDKN2A pathways (23,27). Inactivation of TP53 and pocket proteins, RB1, RBL1 and RBL2, by the viral oncoproteins of high-risk HPV types, E6 and E7, is a prerequisite for oncogenesis but is not sufficient to convert normal cells into an immortalized or malignant state (2). A number of secondary factors, such as smoking, multiparity and oral contraceptives, are thought to influence the likelihood that a high-risk type HPV infection will persist and progress to cervical cancer, possibly through genetic and epigenetic alterations, activation of proto-oncogenes and loss of tumour suppressors as well as immunological responses (28,29).

In order to investigate the potential contribution to human cervical carcinogenesis of the genes implicated in G1/S phase transition, and to identify possibly useful biomarkers for HPV infection progression, the aim of this study was to determine and analyse the transcriptional levels of RB1, RBL1, RBL2, TP53, TP73 and its isoforms, MDM2, E2F1, E2F2, E2F3, E2F4, E2F5, CCND1, CCNE1, CDK2, CDK4, CDK6, CDKN1A, CDKN1B, CDKN2A, CDKN2D, DHFR, MYC, PCNA, and POLA in cervical cancer, SIL and the normal uterine cervix.

Materials and methods

Specimen collection. Thirty-five squamous cervical carcinomas, staged between IA and IIB according to the International Federation of Gynaecology and Obstetrics (FIGO) and World Health Organization (WHO) systems; 26 high-grade SIL (HSIL) and 33 low-grade SIL (LSIL) tissues, classified according to the Bethesda system; and 28 normal uterine cervix specimens, were obtained from 122 women aged 25-60 (40.6±7.9) years, at the Department of Obstetrics and Gynaecology of the University Hospital of Heraklion, Crete. Disease status was determined by cytological, colposcopy and biopsy examination. All tissue samples were snap

frozen, stored at -80°C and tested for HPV DNA, using the GP5+/6+ primer set (30). The positive samples were further investigated using specific primers, previously described (31), for HPV-16, -18, -33 and -11, in multiplex PCR reactions with β 2-microglobulin (B2M) as internal control. The University of Crete ethics committee approved this study and all the participants gave written informed consent.

RNA preparation and RT-PCR. Total RNA was extracted from cervical epithelium tissues that were separated from adjacent stromal tissue by microdissection, using the Trizol reagent (Invitrogen Corp, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA quality was estimated by agarose electrophoresis, while its concentration and purity was determined by UV spectrophotometry. Using Thermo-script reverse transcriptase (Invitrogen Corp.) cDNA was prepared from 3 μ g of total RNA followed by multiplex PCR amplification with Platinum Taq DNA polymerase (Invitrogen Corp.) and the appropriate primer sets (Table I). In each multiplex PCR assay, the β 2-microglobulin (B2M) target cDNA was amplified as reference. The RT-PCR products were analyzed on 8% polyacrylamide gels (29:1 ratio acrylamide/bis-acrylamide), in 0.5X Tris-Boric-EDTA (TBE) buffer and silver-stained. The analysis was repeated 3 times for each sample and marker, and the results were reproducible. Integrated density (ID) of the bands was calculated by digital imaging using the ImageJ 1.32j software (Wayne Rasband, National Institutes of Health, USA, <http://rsb.info.nih.gov/ij/>), as $ID = [\text{mean density} - \text{background}] * \text{pixels}$. These measurements were used as a quantitative parameter for gene expression.

Statistics. Descriptive statistics, calculations of mean, standard deviation (SD), standard error (SE), Kolmogorov-Smirnov goodness of fit and Levene median test, for the examination of normality and equivalency of variances respectively, were performed for each marker and group examined. Evaluation of the power of this experimental protocol to identify differences of gene expression was tested by multiple comparison analysis of the reference gene B2M expression from independent RT-PCR reactions of the same sample. Student's t-test, 2-tailed, was executed and a 99% confidence interval was determined between 0.82 and 1.20 ($p < 0.01$). Thus, a ratio of average gene expression levels between case and control group < 0.82 was considered as downregulation, while a ratio > 1.20 was considered as upregulation. Kruskal-Wallis one-way analysis of variance on ranks, a nonparametric test, was used to compare the expression levels of each marker between different groups. Another nonparametric test, the Spearman rank order correlation, was performed to investigate correlations of expression among genes. Classification of samples according to gene expression was performed by hierarchical clustering using calculation of Euclidean distance. The 99% reference range for the mean was constructed conservatively as mean $\pm 3 \times \text{SD}$. A p-value < 0.05 was considered statistically significant. All statistical calculations were carried out by the SigmaStat 3.00 (SPSS Inc., USA) software except hierarchical clustering which was performed by SYSTAT 10 (SPSS Inc.).

C)	E2F5	E2F1	POLA	TP53	TP73	E2F3	TP73 exon13	CCND1	TP73 exon2
E2F4	0.647	0.747	0.698	0.676	-0.475	0.602	0.677	0.723	-0.726
CDK2	0.576	0.619	0.613	0.678	-0.440	0.568	0.625	0.702	-0.657
CDKN2D									
MYC	0.511	0.552	0.526	0.529	-0.463	0.535	0.485	0.542	-0.568
CDKN1A	0.505	0.551	0.563	0.551	-0.353	0.532	0.487	0.533	-0.540
CDKN2A	0.528	0.658	0.559	0.620	-0.406	0.557	0.501	0.572	-0.598
MDM2	0.228								
RBL2	0.404	0.450	0.410	0.476	-0.261	0.458	0.337	0.418	-0.405
CDK4	0.387	0.289	0.321	0.430	-0.334	0.213	0.366	0.381	-0.364
RBL1	-0.660	-0.767	-0.703	-0.751	0.549	-0.662	-0.677	-0.767	0.782
PCNA	0.604	0.600	0.667	0.668	-0.515	0.604	0.680	0.683	-0.687
CDKN1B	0.615	0.667	0.682	0.647	-0.509	0.613	0.664	0.647	-0.674
CDK6	0.412	0.552	0.418	0.471	-0.353	0.461	0.478	0.485	-0.469
E2F2	0.495	0.548	0.537	0.525	-0.428	0.422	0.538	0.484	-0.460
RB1	0.234	0.378	0.324	0.379	-0.383	0.317	0.339	0.290	-0.367
CCNE1	0.391	0.377	0.471	0.521	-0.422	0.422	0.447	0.407	-0.464
DHFR	0.712	0.784	0.684	0.716	-0.534	0.555	0.687	0.743	-0.682
E2F5		0.674	0.653	0.650	-0.498	0.483	0.546	0.667	-0.603
E2F1			0.666	0.578	-0.475	0.499	0.583	0.719	-0.644
POLA				0.675	-0.509	0.506	0.706	0.709	-0.646
TP53					-0.650	0.590	0.622	0.700	-0.670
TP73						-0.512	-0.497	-0.504	0.630
E2F3							0.508	0.510	-0.593
TP73 exon13								0.592	-0.620
CCND1									-0.721

Results

Five genes, RBL2, E2F2, CDK6, CCNE1 and MYC, were found to be upregulated only in cervical cancer, in 80, 80, 97, 74 and 74% of cases, respectively. Eight genes were upregulated in both cancer and HSIL: E2F1 in 84 and 71% of cervical carcinomas and HSILs, respectively; E2F3 in 81 and 74%, E2F5 in 86 and 81%, CCND1 in 77 and 52%, CDK2 in 88 and 62%, CDKN1B in 86 and 77%, PCNA in 89 and 69%, and POLA in 62 and 52%. Only 5 out of the 24 tested genes were found to be upregulated compared to the controls in all three groups of cases, cervical cancer, HSIL and LSIL. TP53 was upregulated in 80% of tumours, 69% of HSILs and 64% of LSILs; E2F4 in 89, 81 and 76%; CDKN1A in 82, 73 and 70%; CDKN2A in 86, 82 and 77%; and DHFR in 75, 70 and 62%, respectively. MDM2 exhibits a unique pattern of upregulation in 88 and 73% of HSILs and LSILs, respectively.

RBL1 was found to be downregulated in 97, 88 and 73% of tumours, HSILs and LSILs. TP73 exhibited lower levels in 60% of cervical cancers. However, TP73 exon 13-containing isoforms were substantially increased in 73 and 69% of cancers and HSILs, while exon 2-containing isoforms were reduced in 97 and 73% of tumours and HSILs, respectively. Three genes, RB1, CDK4 and CDKN2D, exhibited approximately the same expression levels between cases and controls. All the observed differences of gene expression were statistically

significant ($p < 0.05$) as tested by Kruskal-Wallis one-way analysis of variance on ranks. Representative examples of the RT-PCR analysis mean ID with SD per gene and group are shown in Fig. 1 and graphically in Fig. 2.

The Spearman correlation coefficient (CC) that is calculated with this test quantifies the strength of the association between the variables and varies between -1 and +1. A CC near +1 indicates there is a strong positive relationship between the two variables, with both increasing together. A CC near -1 indicates there is a strong negative relationship between the two variables, with one always decreasing as the other increases. A CC of 0 indicates no relationship between the two variables. Spearman rank order correlation test was performed and significant correlations ($p < 0.0001$) between pairs of tested genes were observed (Table II). The highest positive CC significant values were calculated for the pairs; PCNA-CDKN1B (CC: 0.812), DHFR-E2F1 (CC: 0.784), RBL1-TP73 exon 2 (CC: 0.782), E2F4-DHFR (CC: 0.765), E2F4-CDKN1B (CC: 0.759), and E2F4-PCNA (CC: 0.757). The lowest significant negative CC values were calculated for the pairs; RBL1-DHFR (CC: -0.800), E2F4-RBL1 (CC: -0.793), RBL1-E2F1 (CC: -0.767), RBL1-CCND1 (CC: -0.767), RBL1-CDKN1B (CC: -0.755), and RBL1-TP53 (CC: -0.751).

Nearly all the cervical carcinomas (94.3%) were found to be HPV positive, while the vast majority of HSILs and LSILs

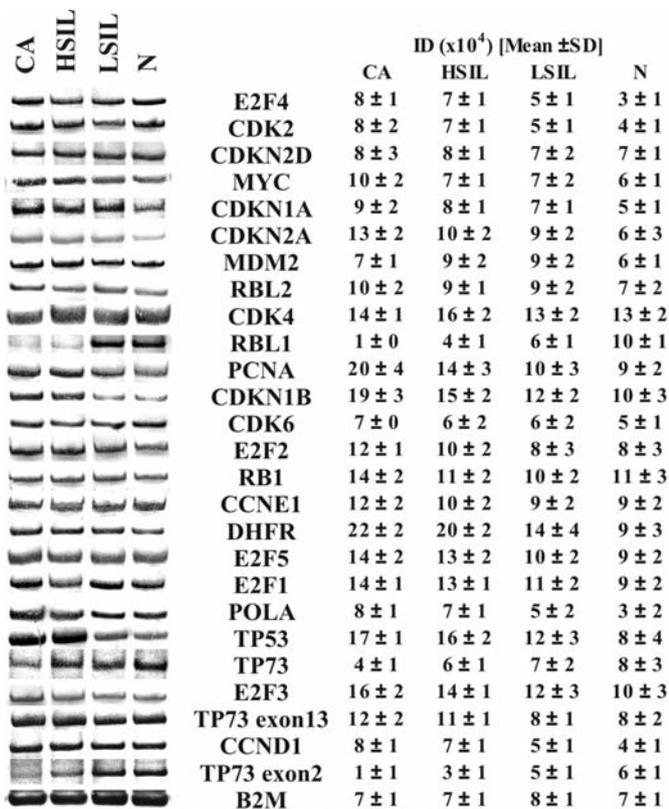


Figure 1. Gene expression analysis. Representative examples of the electrophoresis analysis of RT-PCR products (left panel) and mean ID \pm SD observed in each group. CA, cervical carcinoma; N, normal cervix.

(88.5 and 84.5%, respectively) also harbour the virus. In contrast, all the normal uterine cervix specimens tested were found to be HPV free. The results of genotyping of the HPV positive cases (Table III) shows the predominant presence of HPV-16 in the affected patients. No association were found between gene expression and HPV types.

Hierarchical clustering was performed using calculation of Euclidean distance (Fig. 3). The 122 examined cervical tissue samples were separated into three major clusters (Fig. 3, right panel, sample clustering), normal cervix, LSIL, and cervical cancer together with HSIL. However, the latter cluster

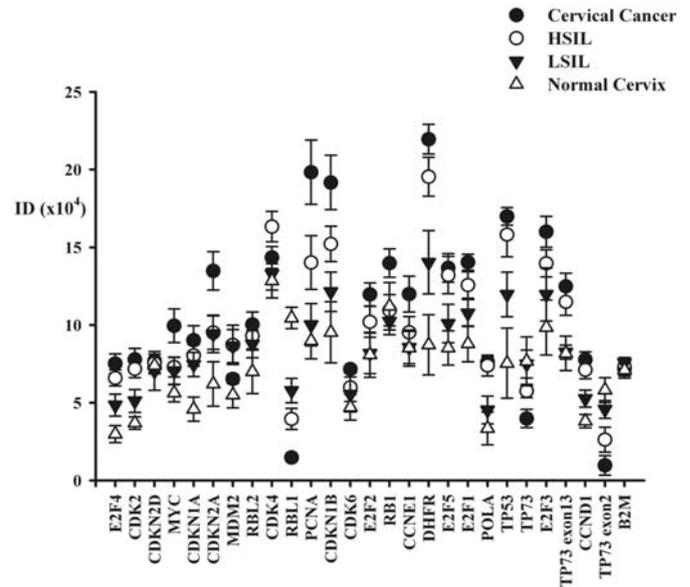


Figure 2. Multiple scatter plot with error bars. Mean ID observed per gene and group is presented. The error bars represent \pm 3SD

is clearly separated into two sub-clusters, discriminating HSIL from cancer cases. Only 8 out of 122 samples were incorrectly clustered (6.6%). The 24 tested genes were also separated into three clusters (Fig. 3, lower panel, gene clustering). Classification of samples according to gene expression was performed by hierarchical clustering using calculation of Euclidean distance, and a permuted data matrix was designed (Fig. 3). The first contains TP73 exon 2-containing transcripts, RBL1 and TP73 that are expressed in low levels. The second contains POLA, CCND1, CDK2, E2F4, CDK6, MDM2, B2M (reference), CDKN2D, MYC, CDKN1A, RBL2, E2F2, TP73 exon 13-containing transcripts, CCNE1, and CDKN2A, genes with moderate expression. Finally, the third cluster contains CDK4, E2F3, E2F5, E2F1, RB1 PCNA, CDKN1B, TP53 and DHFR that are expressed in high levels.

Discussion

Deregulation of G1 to S phase transition in cervical carcinogenesis appears to be a progressive process. Initially, after

Table III. Detection of HPV DNA and integration status.

Specimens	HPV-16 (%)	HPV-18 (%)	HPV-33 (%)	HPV-11 (%)	Other HPV type (%)	Total HPV positive (%)
Cervical cancer	19/35 (54.3)	5/35 (14.3)	3/35 (8.6)	0/35 (0.0)	6/35 (17.1)	33/35 (94.3)
HSIL	13/26 (50.0)	3/26 (11.5)	1/26 (3.9)	0/26 (0.0)	6/26 (23.1)	23/26 (88.5)
LSIL	15/33 (45.5)	4/33 (12.1)	2/33 (6.1)	0/33 (0.0)	7/33 (21.2)	28/33 (84.5)

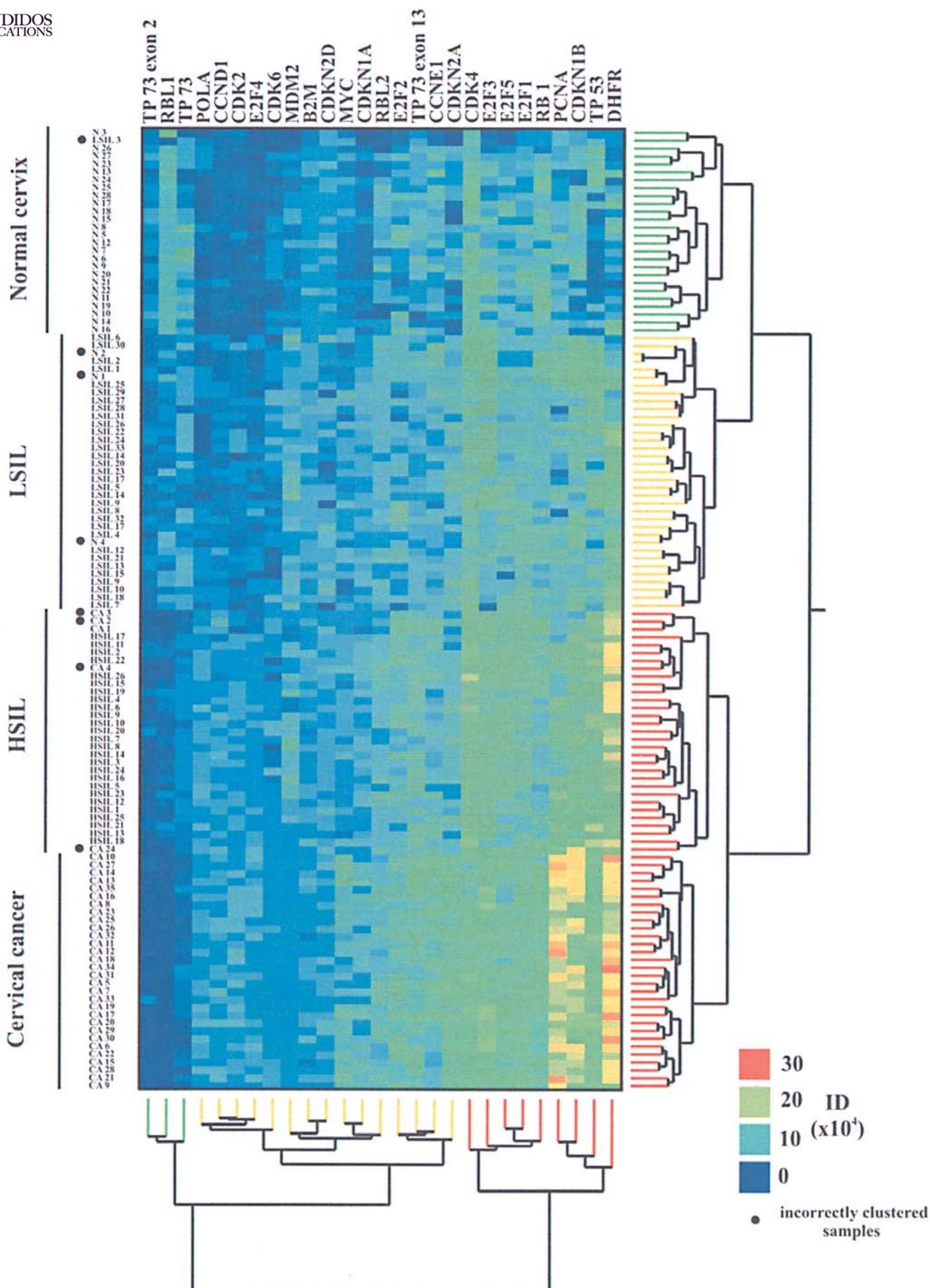


Figure 3. Permuted data matrix. Hierarchical clustering of tested samples and genes. Right panel, sample clustering; lower panel, gene clustering.

high-risk HPV infection, expression of TP53, MDM2, E2F4, CDKN1A, CDKN2A and DHFR is markedly elevated in LSIL. For RBL1 a decrease in transcription levels was observed in the same set of samples. These alterations that were observed in the majority of LSIL cervical biopsies, when compared with normal cervical tissues, with limited inter-individual variation, affect both TP53 and RB1 pathways.

The TP53 upregulation is probably triggered by episomal HPV presence and then both MDM2 and CDKN1A are also upregulated under the transcriptional activity of TP53 (23). CDKN1A has a major inhibitory effect on the G1/S checkpoint of the cell cycle. The CDKN2A promoter responds to stress signals, limiting cell proliferation and modulating oncogene-induced apoptosis (24). The increase in DHFR levels, which converts dihydrofolate into tetrahydrofolate, a methyl group shuttle required for the *de novo* synthesis of purines, necessary for DNA replication, in LSIL suggests deregulation of E2F transcriptional activity (32). RBL1 regulates cell proliferation via phosphorylation-sensitive interactions with E2F transcription factors, and predominantly with E2F4. The combination of RBL1 downregulation and E2F4 upregulation deregulates the ratio of RBL1-E2F4 to RBL2-E2F4 complexes and may alter the transcriptional activity of E2F4 (33,34). Furthermore, the E2F4 and E2F1 transcription factors have opposing roles in cell cycle progression. While E2F1 is an activator of cell cycle progression, E2F4 functions as a transcriptional repressor (35). Thus, overexpression of E2F4 alone deregulates the balance between different activities of E2F factors and might compensate for aggressive cell proliferation.

The findings on gene expression profiles of HSIL cervical biopsies introduce a second group of upregulated genes together with the previously described. This group of genes comprises of E2F1, E2F3, E2F5, CCND1, CDK2, CDKN1B, PCNA and POLA. The TP73 expression remains at the same levels with LSIL and normal cervix. However, its exon 13 containing isoforms are increased while its exon 2 containing isoforms are reduced in HSIL.

E2F1, E2F3, and E2F5 overexpression counteracts the E2F4 upregulation effects in LSIL, and promotes transcriptional activation of the cell cycle progression S phase genes, such as PCNA and POLA (35,36). CCND1 is one of the main components of G1/S checkpoint and its upregulation contributes to malignancy (37). Furthermore, CCND1 transcription is regulated by E2F (38). CDK2 is a component of the CCNE1/CDK2 complex that along with the CCND1/CDK4/6 complex are master regulators of progression through G1 phase of the cell cycle (39). CDK2 upregulation promotes G1 to S phase transition (40). CDKN1B, which is another major inhibitor of the G1/S checkpoint, is possibly overexpressed as a compensatory cellular mechanism to regain normal cell cycle control. Even though TP73 expression is unaltered, the increase of exon 13-containing isoforms together with the decrease of exon 2-containing isoforms suggest that the majority of TP73 isoforms in HSIL cannot surrogate TP53 functions (23,41,42). Our data show that deregulation of G1/S phase transition is much more severe in HSIL than in LSIL. It is characteristic that, according to gene expression levels, HSIL samples are classified as a subgroup in the same cluster with cervical tumours in hierarchical clustering.

Sequentially, in cervical cancer a third group of genes were found to be upregulated; RBL2, E2F2, CDK6, CCNE1, TP73 and MYC. These alterations in the background of that previously described, with the exception of MDM2, which in turn exhibits normal expression levels, reveal a completely uncontrolled G1/S phase transition. The MDM2 expression levels in cervical cancer might be the outcome of rapid degradation of TP53 through its binding with E6 viral oncoprotein, since MDM2 transcription is controlled by TP53 in a feedback regulation mechanism. The TP73 downregulation effect is further contributing to the disease phenotype together with its isoform equilibrium imbalance.

In interpreting our gene expression data, it is assumed that protein levels in these tissue samples reflect the expression of their corresponding mRNAs. Thus, we generally assume that samples with high mRNA content for a particular gene are likely to have elevated levels for the corresponding protein. We acknowledge that this need not be the case, as post-transcriptional, translational and protein degradation controls probably have a significant influence. However, the limited amounts of biopsy material available have precluded analysis at the protein level.

Spearman rank order correlation test provided additional evidence for the complex relationships between the examined genes. The statistically significant Spearman CC values ($p < 0.0001$) calculated for each pair of genes are depicted in Table II. The positive or negative relationships observed between the tested genes are in agreement with the above described observations.

Hierarchical clustering of gene expression was able to classify the cervical tissue samples according to disease status, with few discrepancies (6.6%). Thus, this array of G1/S checkpoint markers could be used for diagnostic purposes. The examined genes were clustered in three groups of low, TP73 exon 2, RBL1 and TP73; moderate, POLA, CCND1, CDK2, E2F4, CDK6, MDM2, B2M, CDKN2D, MYC, CDKN1A, RBL2, E2F2, TP73 exon 13, CCNE1, and CDKN2A; and high, CDK4, E2F3, E2F5, E2F1, RB1 PCNA, CDKN1B, TP53 and DHFR, expression.

Previous studies have recognized the diagnostic significance of immunohistochemical analysis for CDKN2A alone (43-46) or in combination with other proteins, such as CDKN1A, CDKN1B, CCNE, CDKN2D and TP53 (47-50), for the early detection of reactive changes from LSIL to HSIL and cervical cancer. In this study, we identified CDKN1A as an early responsive gene to neoplasia.

In conclusion, our data outline important steps in carcinogenesis of the uterine cervix. Deregulation of G1/S phase transition takes place sequentially. Different genes are transcriptionally activated in low- and high-grade pre-cancerous lesions, and cancer. The first group of early responsive genes, comprised TP53, MDM2, E2F4, CDKN1A, CDKN2A and DHFR, are activated in LSIL. The second group, comprised E2F1, E2F3, E2F5, CCND1, CDK2, CDKN1B, PCNA and POLA, are activated in HSIL. The third group of late responsive or cancer-related genes comprised RBL2, E2F2, CDK6, CCNE1, TP73 and MYC, which are upregulated only in squamous cervical carcinomas. RBL1 was found to be downregulated in all three groups of cases and TP73 exhibited deregulation of isoform equilibrium. Hierarchical clustering

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 immunate the cervical tissue biopsies according to status, which suggests a possible diagnostic importance.

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