

# New Approaches to Functional Process Discovery in HPV 16-Associated Cervical Cancer Cells by Gene Ontology

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**Purpose:** This study utilized both mRNA differential display and the Gene Ontology (GO) analysis to characterize the multiple interactions of a number of genes with gene expression profiles involved in the HPV-16-induced cervical carcinogenesis.

**Materials and Methods:** mRNA differential displays, with HPV-16 positive cervical cancer cell line (SiHa), and normal human keratinocyte cell line (HaCaT) as a control, were used. Each human gene has several biological functions in the Gene Ontology; therefore, several functions of each gene were chosen to establish a powerful cervical carcinogenesis pathway. The specific functions assigned to these genes were then correlated with the gene expression patterns.

**Results:** The results showed that 157 genes were up- or down-regulated at least 2-fold and organized into reciprocally dependent sub-function sets, depending on their cervical cancer pathway, suggesting the potentially

significant genes of unknown function affected by the HPV-16-derived pathway. The GO analysis suggested that the cervical cancer cells underwent repression of the cancer-specific cell adhesive properties. Also, genes belonging to DNA metabolism, such as DNA repair and replication, were strongly down-regulated, whereas significant increases were shown in the protein degradation and synthesis.

**Conclusion:** The GO analysis can overcome the complexity of the gene expression profile of the HPV-16-associated pathway, identify several cancer-specific cellular processes and genes of unknown function. It could also become a major competing platform for the genome-wide characterization of carcinogenesis. (*Cancer Research and Treatment 2003;35:304-313*)

**Key Words:** Cervix neoplasia, mRNA differential display, Gene ontology

## INTRODUCTION

Human papillomaviruses (HPV-16 and -18) infection has been commonly identified in cervical carcinomas (1). Generally, after a high-risk HPV infection, the E6 and E7 oncoproteins are consistently expressed, and essential for the immortalization and transformation of human squamous epithelial cells (2,3). Although a number of cervical cancer-related genes and cellular processes have been studied, many of the molecular events involved in the cervical cancer pathway are still unclear, as a gene can be involved in multiple independently regulated cancer-specific pathways. Moreover, many studies on cervical cancer lack physiological relevance because those were per-

formed by using the small number of genes and established only in cell lines.

While it is becoming increasingly clear that there are wide variations in the efficiency of cancer therapy among different cell types, there is still relatively little known regarding the mechanism by which genes or gene complexes are directly cancer-specific. Here, to quantitatively understand the possible multiple relationships between differentially expressed profiles of a gene and the cervical cancer-specific pathway, the annotation project, directed by the Gene Ontology (GO) Consortium (<http://www.geneontology.org>), was used (4). Despite the significance of functional analysis in cancer research, the GO analysis has not been widely used in carcinogenesis, mainly due to its complexity and rapidly evolving property. With the GO analysis, the regulated genes are organized into three separated ontologies comprised of; biological process, cellular component and molecular function, and defines a set of well-defined terms and relationships by which the role of a particular gene, gene product or gene-product group can be interpreted. Thus, an advanced strategy for the identification of preferential tumor-specific pathways would be needed by using the GO analysis. As of March 12, 2003, GO contained 6959 processes, 5339 functions and 1199 component terms, with a

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total of 9791 GO term definitions.

In this study, mRNA differential displays were used to investigate the expression patterns of genes related to the cervical cancer pathway, where the biological functions were primarily affected by the HPV-16-derived gene regulation. These functional profiles are cervical cancer-dependent, clearly differentiated and resulted in finding a large subset of cellular functional changes that could be described as tending to increase, or decrease, with the number of cervical cancer cells. The GO analysis was identified as being descriptive of cervical carcinogenesis, suggesting that several previously unreported functions were cervical cancer-dependent. This report has shown that the GO analysis is a valuable tool for diagnostic and therapeutic interventions in cancer research.

## MATERIALS AND METHODS

### 1) Cell lines and cell culture

The human cervical cancer cell line, HPV-16 positive SiHa, and human keratinocyte cell line, HaCaT (5), which has similar properties to normal keratinocytes, were used for the experiment. The cells were cultured in a 5% CO<sub>2</sub> incubator at 37°C.

### 2) mRNA differential display

The total RNA was isolated from cultured cells using TRIzol (Gibco-BRL). The differential display was carried out as previously described (6). Briefly, RT-PCR was performed using RNImage kits (GenHunter, Brookline, MA), according to the manufacturer's instructions. 0.2 µg of total RNA was used for the PCR amplification, and the amplified cDNA fragments were cloned into the pGEM-T easy vector (Promega, Madison, WI). Plasmid DNA was prepared using the Wizard Miniprep Purification System (Promega). The sequencing reactions were performed with an Applied Biosystems sequencer, model 3,100, with T7 and SP6 primers. The homology of the cDNA sequences to other nucleic acids was determined using the National Center for Biotechnology Information BLAST database search algorithms.

### 3) Northern blotting

20 µg of total RNA was electrophoresed, and transferred to nylon membranes (Hybond-N+; Amersham, Uppsala, Sweden). The insert into the pGEM-T easy vector was amplified using T7 and SP6 primers, and then gel-eluted. The probes were then [ $\alpha$ -<sup>32</sup>P]-dCTP-labeled with a Random primer labeling kit (TaKaRa, Shiga, Japan). The hybridization was carried out overnight, at 68°C, in Rapid Hyb-buffer (Amersham). After hybridization, the membrane was radiophotographed at -70°C for 24~48 hr.

### 3) Analysis

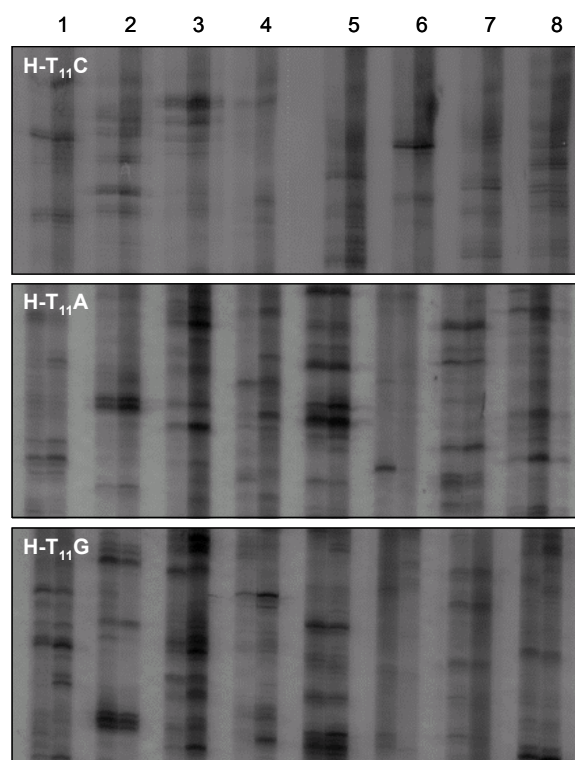
The ImaGene v5.0 (Biodiscovery, Marina Del Rey, CA) was used to analyze the image data. Genes were excluded from the analyses if their expressions were negative or too smeared, with those showing differences of at least 2 fold in their expression levels being selected for the function analysis. To classify the gene expression profiles, functional analyses were carried out as previously described (7). Each gene was annotated by

integrating the information (as of March 12, 2003) on the Gene Ontology website (<http://www.geneontology.org>). All the files, including the BLAST database search results for the transcripts from differential displays, and those of the Gene Ontology analysis were downloaded from our anonymous FTP site: <ftp://160.1.9.42/work/ddpcr>.

## RESULTS

As shown in Fig. 1, after the mRNA differential display and gel electrophoresis, approximately 640 bands were identified. After the exclusion of the poor bands, 349 of the 640 bands remained. From the sequencing and BLAST database search, 157 transcripts showing at least a 2-fold increased change were identified.

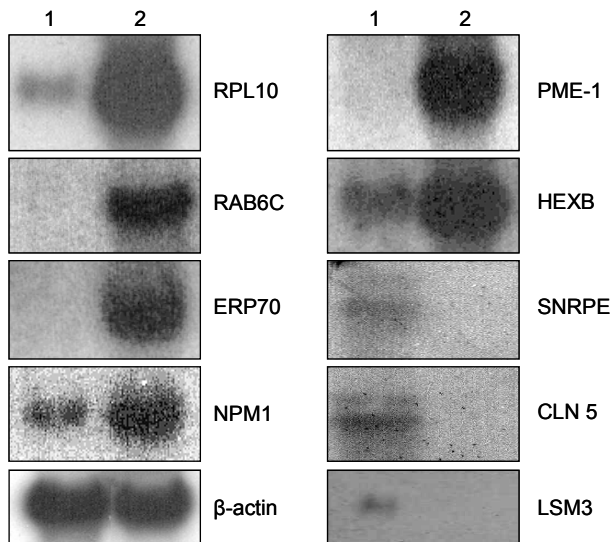
Table 1 shows a description of the transcripts, with new cervical cancer-related genes differentially up- or down-regulated in SiHa cells. The expression profiling for the down-regulation was different from that for the up-regulation, indicating that repression of the gene expression may have an important impact on the HPV-16-derived cellular processes. Northern



**Fig. 1.** Differential display of mRNA expression between HaCaT and SiHa cells. Representative mRNA DD results were obtained using different H-T primers for reverse transcription and H-AP random primer pairs for PCR. 1. H-AP17; 2. H-AP18; 3. H-AP19; 4. H-AP20; 5. H-AP21; 6. H-AP22; 7. H-AP24. Each number consists of two lanes of HaCaT and SiHa cell lines.

**Table 1.** Summary of gene expression changes. The genes are adversely ranked by fold change, i.e., from down-regulation to upregulation

| Increase       |                 |             |                |                     |             |                |                     |             |
|----------------|-----------------|-------------|----------------|---------------------|-------------|----------------|---------------------|-------------|
| Unigene number | Gene symbol     | Fold change | Unigene number | Gene symbol         | Fold change | Unigene number | Gene symbol         | Fold change |
| Hs.83916       | NDUFA5          | -8.9        | Hs.301434      | <b>KIAA1387</b>     | -2.8        | Hs.2726        | HMGA2               | 2.9         |
| Hs.181369      | UFD1L           | -8.9        | Hs.211577      | KTN1                | -2.8        | Hs.40342       | HRIHFB2122          | 3.4         |
| Hs.77171       | MCM5            | -8.7        | Hs.285976      | <b>LASS2</b>        | -2.6        | Hs.127376      | <b>KIAA0266</b>     | 3.7         |
| Hs.75355       | UBE2N           | -8.6        | Hs.326766      | <b>LOC220070</b>    | -2.6        | Hs.32511       | KIAA0441            | 3.9         |
| Hs.227730      | ITGA6           | -6.9        | Hs.75639       | LOC91137            | -2.6        | Hs.6834        | <b>KIAA1014</b>     | 3.9         |
| Hs.151134      | OXA1L           | -6.8        | Hs.3688        | LUC7A               | -2.6        | Hs.288697      | <b>MGC11349</b>     | 4.0         |
| Hs.153834      | PUM1            | -6.8        | Hs.45105       | <b>MCJ</b>          | -2.6        | Hs.250705      | MGC17921            | 4.3         |
| Hs.184014      | RPL31           | -6.8        | Hs.179565      | MCM3                | -2.6        | Hs.66521       | MJD                 | 4.3         |
| Hs.334612      | SNRPE           | -6.7        | Hs.39132       | <b>MGC11115</b>     | -2.5        | Hs.111039      | NMT1                | 4.3         |
| Hs.25197       | STUB1           | -6.7        | Hs.40094       | <b>MGC8974</b>      | -2.5        | Hs.89545       | PSMB4               | 4.5         |
| Hs.30213       | CLN5            | -6.7        | Hs.108931      | MPP6                | -2.5        | Hs.196209      | RAE1                | 4.5         |
| Hs.306237      | <b>KIAA1680</b> | -6.5        | Hs.107474      | NAB1                | -2.5        | Hs.241567      | RBMS1               | 4.5         |
| Hs.111632      | LSM3            | -6.5        | Hs.5273        | NDUFS3              | -2.5        | Hs.356593      | RPL7                | 4.7         |
| Hs.184352      | <b>PCNT1</b>    | -5.8        | Hs.64056       | PAK1                | -2.5        | Hs.182740      | RPS11               | 4.8         |
| Mm.6332        | ALOX15B         | -5.7        | Hs.267289      | POLA                | -2.5        | Hs.151604      | RPS8                | 4.8         |
| Hs.72160       | AND-1           | -5.6        | Hs.110857      | POLR3K              | -2.5        | Hs.1027        | RRAD                | 4.8         |
| Hs.155172      | AP3B1           | -5.6        | Hs.184011      | PP                  | -2.5        | Hs.271926      | SDCCAG16            | 4.9         |
| Hs.154149      | APEX2           | -5.4        | Hs.106415      | PPARD               | -2.5        | Hs.75231       | SLC16A1             | 5.0         |
| Hs.227913      | API5            | -5.4        | Hs.279554      | PSMD13              | -2.3        | Hs.77496       | SNRPG               | 5.0         |
| Hs.7351        | ARPP-19         | -5.1        | Hs.366         | PTS                 | -2.3        | Hs.128645      | SNX16               | 5.4         |
| Hs.81886       | AUH             | -4.9        | Hs.168095      | <b>RNF20</b>        | -2.3        | Hs.7766        | UBE2E1              | 5.4         |
| Hs.75415       | B2M             | -4.9        | Hs.278526      | <b>RNTRE</b>        | -2.3        | Hs.78563       | UBE2G1              | 5.4         |
| Hs.15259       | BAG3            | -4.7        | Hs.110165      | RPL26L1             | -2.3        | Hs.75875       | UBE2V1              | 5.5         |
| Hs.202         | BZRP            | -4.7        | Hs.301547      | RPS7                | -2.2        | Hs.78902       | VDAC2               | 5.5         |
| Hs.3402        | C17orf26        | -4.7        | Hs.21858       | SERPINE2            | -2.2        | Hs.206594      | VPS33A              | 5.5         |
| Hs.155560      | CANX            | -4.8        | Hs.78713       | SLC25A3             | -2.2        | Hs.149923      | XBP1                | 5.9         |
| Hs.1592        | CDC16           | -4.5        | Hs.2043        | SLC25A4             | -2.2        | Hs.173518      | <b>ZFR</b>          | 6.1         |
| Hs.179902      | <b>CDW92</b>    | -4.5        | Hs.878         | SORD                | -2.2        | Hs.172673      | AHCY                | 6.2         |
| Hs.278562      | CLDN7           | -4.1        | Hs.75975       | SRP9                | -2.1        | Hs.7041        | <b>MGC4170</b>      | 6.4         |
| Hs.106552      | CNTNAP2         | -4.0        | Hs.380938      | STX8                | -2.1        | Hs.159322      | SLC35A3             | 6.4         |
| Hs.79194       | CREB1           | -3.9        | Hs.380364      | TARS                | -2.1        | Hs.11411       | <b>DKFZp566O084</b> | 6.7         |
| Hs.330208      | CRYZL1          | -3.8        | Hs.173824      | TDG                 | -2.1        | Hs.75334       | EXT2                | 6.7         |
| Hs.349699      | DSP             | -3.8        | Hs.1287        | TRIM26              | -2.1        | Hs.63304       | PME-1               | 6.8         |
| Hs.177534      | DUSP10          | -3.8        | Hs.100090      | TSPAN-3             | -2.1        | Hs.164915      | SNAPC3              | 6.8         |
| Hs.346868      | EBNA1BP2        | -3.7        | Hs.28423       | UBP1                | -2.1        | Hs.12163       | EIF2S2              | 7.3         |
| Hs.275959      | EEF1B2          | -3.7        | Hs.234282      | VPS11               | -2.0        | Hs.51043       | HEXB                | 7.3         |
| Hs.348389      | SFXN1           | -3.7        | Hs.173081      | ZNF292              | -2.0        | Hs.210850      | KIAA1131            | 7.4         |
| Hs.331318      | <b>FLJ13213</b> | -3.7        | Hs.189829      | <b>AGO3</b>         | 2.0         | Hs.334368      | <b>MGC11257</b>     | 7.4         |
| Hs.47125       | <b>FLJ13912</b> | -3.7        | Hs.1227        | ALAD                | 2.0         | Hs.44143       | <b>PB1</b>          | 7.7         |
| Hs.71616       | <b>FLJ14431</b> | -3.5        | Hs.168625      | AS3                 | 2.0         | Hs.333139      | RAB6C               | 7.7         |
| Hs.79828       | FLJ20333        | -3.5        | Hs.78629       | ATP1B1              | 2.2         | Hs.178391      | <b>RPL36A</b>       | 7.8         |
| Hs.349887      | <b>FLJ30656</b> | -3.5        | Hs.73851       | ATP5J               | 2.2         | Hs.113029      | RPS25               | 9.0         |
| Hs.374897      | <b>FLJ36874</b> | -3.3        | Hs.76572       | ATP5O               | 2.2         | Hs.79968       | SPF30               | 9.4         |
| Hs.180296      | FOP             | -3.3        | Hs.77204       | CENPF               | 2.2         | Hs.9614        | NPM1                | 9.5         |
| Hs.191356      | GTF2H2          | -3.2        | Hs.289097      | DDX19               | 2.3         | Hs.93659       | ERP70               | 9.8         |
| Hs.55468       | <b>H4F2</b>     | -3.2        | Hs.5364        | <b>DKFZP564I052</b> | 2.4         | Hs.29797       | RPL10               | 10.2        |
| Hs.13340       | HAT1            | -3.2        | Hs.334798      | EEF1D               | 2.4         | Hs.106826      | <b>BHC80</b>        | 10.3        |
| Hs.15318       | HAX1            | -3.2        | Hs.100914      | <b>FLJ10352</b>     | 2.5         |                |                     |             |
| Hs.372673      | <b>HNRPDL</b>   | -3.0        | Hs.168232      | FLJ13855            | 2.5         |                |                     |             |
| Hs.282804      | <b>HPS3</b>     | -3.0        | Hs.300700      | <b>FLJ20727</b>     | 2.5         |                |                     |             |
| Hs.154762      | <b>HRB2</b>     | -2.9        | Hs.42194       | <b>FLJ22649</b>     | 2.7         |                |                     |             |
| Hs.31323       | IKBKAP          | -2.9        | Hs.78915       | GABPB1              | 2.7         |                |                     |             |
| Hs.141296      | <b>KIAA0226</b> | -2.9        | Hs.111676      | H11                 | 2.7         |                |                     |             |
| Hs.71109       | <b>KIAA1229</b> | -2.9        | Hs.180877      | <b>H3F3B</b>        | 2.7         |                |                     |             |
| Hs.106204      | KIAA1327        | -2.9        | Hs.343261      | HM13                | 2.8         |                |                     |             |



**Fig. 2.** Results of Northern blot. Total RNA obtained from HaCAT cells (lane 1) and SiHa cells (lane 2) was subjected to Northern analysis as described in materials and methods.

blots were performed to confirm the results of the differential displays. As shown in Fig. 2, several up- and down-regulated transcripts confirmed the patterns obtained from the differential displays, showing the consistency of the experimental repeatability.

As shown in Tables 2, 3 and 4, the significantly up- and down-regulated functional activities, as diverse as cell communication, cell growth and maintenance, cell death, development, apoptosis regulation, nucleic acid binding, chaperon and enzymes, etc., were analyzed according to the biological processes, cellular components and molecular function ontologies. Of the HPV-16-derived biological functions, over 50% of the functions were included in the biological processes, with half of these being in the cell growth and maintenance.

As shown in Table 2 and Fig. 3A, the transcripts involved in the cell communication (6/10, i.e., up-regulation of RAB6C, EXT2, XBP1, SNX16, RRAD and MJD, or the down-regulation of ITGA6, OXA1L, B2M, CNTNAP2, CREB1, DUSP10, IKBKAP, PAK1, MPP6 and SORD) were relatively repressed in the SiHa cells as compared to the normal cells. The transcripts in the signal transduction (4/6: RAB6C, EXT2, SNX16, RRAD/ITGA6, OXA1L, CREB1, DUSP10, PAK1, MPP6) showed relatively repressed expression profiles. The cell adhesion function (0/2) was likely to be affected by the HPV 16-associated cellular process. For instance, alpha-6 integrin (ITGA6) and contactin-associated protein 2 (CNTNAP2), another cell-to-matrix attachment-related components, were highly down-regulated.

As shown in Table 2, the transcripts involved in the cell cycle (2/3: UBE2V1, CENPF/MCM5, MCM3, CDC16) were repressed in the SiHa cells. As shown in Fig. 3B, the transcripts in the transport (10/9: RAB6C, SLC35A3, SNX16, SLC16A1, RAE1, ATP5O, ATP5J, VDAC2, ATP1B1, DDX19/LOC91137, STX8, SLC25A4, SLC25A3, SFXN1, AP3B1, C17orf26, KTN1,

VPS11) were relatively balanced.

As shown in the metabolism process, a number of transcripts were repressed in the SiHa cells. The transcripts involved in the DNA metabolism, such as DNA repair (1/4: UBE2V1/GTF2H2, TDG, UBE2N, APEX2) and DNA replication (1/3: RBMS1/POLA, MCM3, MCM5), were down-regulated. In the lipid and carbohydrate metabolism, the HEXB, associated with HPV 16-derived cervical cancer, was highly up-regulated. Also, the NADH dehydrogenase subunit 4, as a mitochondrial enzyme, was the highly over-expressed clone involved in the metabolic pathway.

In the development function, several transcripts involved in the morphogenesis (0/2: TSPAN-3, DSP) were down-regulated, whereas those in the organogenesis (2/2: MJD, EXT2/PTS, UFD1L) were maintained at relatively constant levels, as shown in Table 2 and Fig. 3D.

As shown in Table 3, the apoptosis function, the transcripts involved in heat-shock proteins, such as H11, the eighth known human small Hsp, named HspB8 described as a serine-threonine protein kinase, were up-regulated in the SiHa cells. It is well known that cervical carcinomas produce DNA damage in cancer cells, where tumor suppressor-related genes, such as EXT2, were highly induced, as shown in this study. In contrast, down-regulation of the genes related to DNA repair, such as UBE2N and TDG, were also apparent in the SiHa cells. Conversely, there was a down-regulated change in the antiapoptotic transcripts, BAG3 and API5. The molecular function ontology includes transcripts with an apoptosis regulator and chaperone. The transcripts in chaperone (1/2: H11/BAG3, CANX) were shown in the SiHa cells.

The DNA binding function includes several transcripts, which were down-regulated (6/12: SNAPC3, XBP1, KIAA0441, HMGA2, GABPB1, AS3/MCM5, PPARC, CREB1, GTF2H2, MCM3, NAB1, POLR3K, UBPI, ZNF292, KIAA1327, FLJ20333, AND-1), as shown in Table 3 and Fig. 3E. Note that the GABPB1, involved in the expression of the adenovirus E4 gene, was up-regulated. Several transcripts were involved in protein translation, where the generally down-regulated genes were translation elongation factors, such as EEF1B2 and SRP9, compared to highly up-regulated genes, such as EEF1D, EIF2S2 and RBMS1. The E2F family of transcription factors, which have a central role in the regulation of cell proliferation, were not shown in this study.

As shown in Table 4, the transcripts in the cytoskeleton (4/3: HRIHFB212, RAE1, MGC17921, CENPF/DSP, CLDN7, CDC16) were balanced, whereas a clone on chromosome Xq25-26.3, which is remarkably similar to that of the human molecule beta-tubulin, with 98% homology, was highly down-regulated in the SiHa cells. No nuclear structural proteins were expressed in this assay.

Transcripts in the proteasome and ubiquitin degradation pathways, such as UBE2E1, UBE2G1, KIAA1131, FLJ13855 and PSMB4, were up-regulated 2-fold, but in contrast, the UBE2N, FLJ20333 and UFD1L, involved in the degradation of ubiquitin fusion proteins, were down-regulated. On the other hand, as shown in Table 2 and Fig. 3F, the protein biosynthesis activity in the metabolism was relatively up-regulated (8/6: RPS25, EIF2S2, RPS8, RPS11, RPL7, EEF1D, RBMS1, ATP5O/RPL31 EEF1B2, RPS7, TARS, RPL26L1, SRP9).

**Table 2.** Summarized biological process ontology of up- and down-regulated transcripts. The GO code and the number of up-/down-regulated genes were shown

| Biological process ontology                                    | GO code    | Up | Down |
|--|------------|----|------|
| Cell communication   | GO:0007154 | 6  | 10   |
| Cell adhesion  | GO:0007155 | 0  | 2    |
| Cell-matrix adhesion   | GO:0007160 | 0  | 1    |
| Cell-cell signaling  | GO:0007267 |    |      |
| Transmission of nerve impulse                                  | GO:0019226 | 0  | 1    |
| Synaptic transmission  | GO:0007268 | 1  | 0    |
| Response to external stimulus                                  | GO:0009605 |    |      |
| Vision   | GO:0007601 | 0  | 1    |
| Antigen presentation, endogenous antigen                       | GO:0019833 | 0  | 1    |
| Antigen processing, endogenous antigen via MHC class 1         | GO:0019885 | 0  | 1    |
| Immune response  | GO:0006955 | 1  | 1    |
| Signal transduction  | GO:0007165 | 4  | 6    |
| Integrin-mediated signaling pathway                            | GO:0007229 | 0  | 1    |
| Intracellular signaling cascade                                | GO:0007242 | 1  | 0    |
| JNK cascade  | GO:0007254 | 0  | 2    |
| Small GTPase mediated signal transduction                      | GO:0007264 | 1  | 0    |
| Cell growth and maintenance                                    | GO:0008151 | 3  | 1    |
| Cell cycle   | GO:0007049 | 2  | 3    |
| DNA replication and chromosome cycle                           | GO:0000067 | 1  | 0    |
| Mitosis  | GO:0007067 | 1  | 0    |
| Regulation of cell cycle                                       | GO:0000074 | 0  | 1    |
| Cell motility  | GO:0006928 | 0  | 1    |
| Regulation of cell migration                                   | GO:0030334 | 0  | 1    |
| Cell organization and biogenesis                               | GO:0016043 |    |      |
| Cell-substrate junction assembly                               | GO:0007044 | 0  | 1    |
| Cell proliferation   | GO:0008283 | 0  | 1    |
| Metabolism   | GO:0008152 |    |      |
| Amino acid metabolism  | GO:0006520 | 0  | 1    |
| Protein biosynthesis   | GO:0006412 | 8  | 6    |
| ATP biosynthesis   | GO:0006754 | 1  | 0    |
| Carbohydrate metabolism  | GO:0005975 | 1  | 1    |
| Regulation of proteolysis and peptidolysis                     | GO:0030162 | 0  | 1    |
| Electron transport   | GO:0006118 | 1  | 2    |
| Energy pathways  | GO:0006091 | 1  | 3    |
| Lipid metabolism   | GO:0006629 | 0  | 1    |
| Fatty acid metabolism  | GO:0006631 | 0  | 1    |
| Nucleobase, nucleoside, nucleotide and nucleic acid metabolism | GO:0006139 |    |      |
| DNA metabolism   | GO:0006259 |    |      |
| DNA repair   | GO:0006281 | 1  | 4    |
| DNA replication  | GO:0006260 | 1  | 3    |
| RNA metabolism   | GO:0016070 |    |      |
| RNA processing   | GO:0006396 | 1  | 0    |
| mRNA splicing  | GO:0006371 | 1  | 2    |
| Transcription  | GO:0006350 | 0  | 1    |
| Regulation of transcription, DNA-dependent                     | GO:0006355 | 5  | 7    |
| RNA elongation   | GO:0006354 | 0  | 1    |
| Protein metabolism   | GO:0019538 |    |      |
| Protein modification   | GO:0006464 | 2  | 2    |
| Protein folding  | GO:0006457 | 0  | 1    |
| Protein complex assembly                                       | GO:0006461 | 0  | 2    |
| Response to stress   | GO:0006950 | 0  | 1    |
| Response to heat   | GO:0006951 | 1  | 0    |
| Transport  | GO:0006810 | 10 | 9    |
| Nucleotide-sugar transport                                     | GO:0015780 | 1  | 0    |
| Hydrogen transport   | GO:0006818 | 1  | 0    |
| Protein transport  | GO:0015031 | 1  | 1    |
| Intracellular protein transport                                | GO:0006886 | 1  | 1    |
| Death  | GO:0016265 |    |      |
| Cell death   | GO:0008219 |    |      |
| Apoptosis  | GO:0006915 | 0  | 1    |
| Development  | GO:0007275 | 1  | 0    |
| Morphogenesis  | GO:0009653 | 0  | 2    |
| Epidermal differentiation                                      | GO:0008544 | 0  | 1    |
| Organogenesis  | GO:0009887 | 2  | 2    |
| Neurogenesis   | GO:0007399 | 1  | 0    |
| Skeletal development   | GO:0001501 | 1  | 1    |

**Table 3.** Summarized molecular function ontology of up- and down-regulated transcripts

| Molecular function ontology              | GO code    | Up | Down |
|--|------------|----|------|
| Apoptosis regulator                      | GO:0016329 | 0  | 1    |
| Apoptosis inhibitor                      | GO:0008189 | 0  | 1    |
| Binding                                  | GO:0005488 | 0  | 2    |
| Calcium ion binding                      | GO:0005509 | 0  | 1    |
| Calcium ion storage                      | GO:0005514 | 0  | 1    |
| Glycosaminoglycan binding                | GO:0005539 |    |      |
| Zinc binding                             | GO:0008270 | 0  | 3    |
| Magnesium binding                        | GO:0000287 | 0  | 2    |
| Nucleic acid binding                     | GO:0003676 | 2  | 0    |
| DNA binding                              | GO:0003677 | 6  | 12   |
| Damaged DNA binding                      | GO:0003684 | 0  | 1    |
| Single-stranded DNA binding              | GO:0003697 | 1  | 0    |
| AT DNA binding                           | GO:0003680 | 1  | 0    |
| Exonuclease                              | GO:0004527 | 0  | 1    |
| RNA binding                              | GO:0003723 | 10 | 5    |
| mRNA binding                             | GO:0003729 | 1  | 2    |
| rRNA binding                             | GO:0019843 | 1  | 0    |
| Translation elongation factor            | GO:0003746 | 1  | 1    |
| Translation initiation factor            | GO:0003743 | 1  | 0    |
| Nucleotide binding                       | GO:0000166 |    |      |
| ATP binding                              | GO:0005524 | 0  | 4    |
| GTP binding                              | GO:0005525 | 1  | 0    |
| Protein binding                          | GO:0005515 | 2  | 4    |
| Transcription cofactor                   | GO:0003712 | 0  | 1    |
| Chaperone                                | GO:0003754 | 1  | 2    |
| Heat shock protein                       | GO:0003773 | 1  | 0    |
| Hsp70/Hsc70 protein regulator            | GO:0030192 | 0  | 2    |
| Enzyme                                   | GO:0003824 | 2  | 0    |
| Hydrolase                                | GO:0016787 | 4  | 3    |
| Isomerase                                | GO:0016853 | 1  | 0    |
| Ligase                                   | GO:0016874 | 2  | 2    |
| Lyase                                    | GO:0016829 | 1  | 1    |
| Oxidoreductase                           | GO:0016491 | 0  | 5    |
| Ubiquitin conjugating enzyme             | GO:0004840 | 5  | 3    |
| Transferase                              | GO:0016740 | 3  | 4    |
| Protein phosphatase                      | GO:0004721 | 0  | 1    |
| Enzyme regulator                         | GO:0030234 |    |      |
| Serine protease inhibitor                | GO:0004867 | 0  | 1    |
| Signal transducer                        | GO:0004871 | 0  | 1    |
| Receptor                                 | GO:0004872 | 0  | 3    |
| Ligand-dependent nuclear receptor        | GO:0004879 | 0  | 1    |
| Benzodiazepine receptor                  | GO:0008503 | 0  | 1    |
| Cell adhesion receptor                   | GO:0004895 | 0  | 1    |
| MHC class 1 receptor                     | GO:0030106 | 0  | 1    |
| Receptor signaling protein               | GO:0005057 | 1  | 0    |
| Structural molecule                      | GO:0005198 | 0  | 2    |
| Structural constituent of cytoskeleton   | GO:0005200 | 0  | 1    |
| Structural constituent of ribosome       | GO:0003735 | 5  | 2    |
| Transcription regulator                  | GO:0030528 |    |      |
| Transcriptional repressor                | GO:0016564 | 0  | 1    |
| Translation regulator                    | GO:0045182 |    |      |
| Translation factor, nucleic acid binding | GO:0008135 | 2  | 0    |
| Translation initiation factor            | GO:0003743 | 1  | 0    |
| Transporter                              | GO:0005215 | 2  | 0    |
| Auxiliary transport protein              | GO:0015457 | 2  | 1    |
| Carrier                                  | GO:0005386 | 0  | 1    |
| Channel/pore class transporter           | GO:0015267 | 2  | 1    |
| Electron transporter                     | GO:0005489 | 1  | 1    |
| Ion transporter                          | GO:0015075 | 4  | 1    |
| Protein transporter                      | GO:0008565 | 1  | 1    |

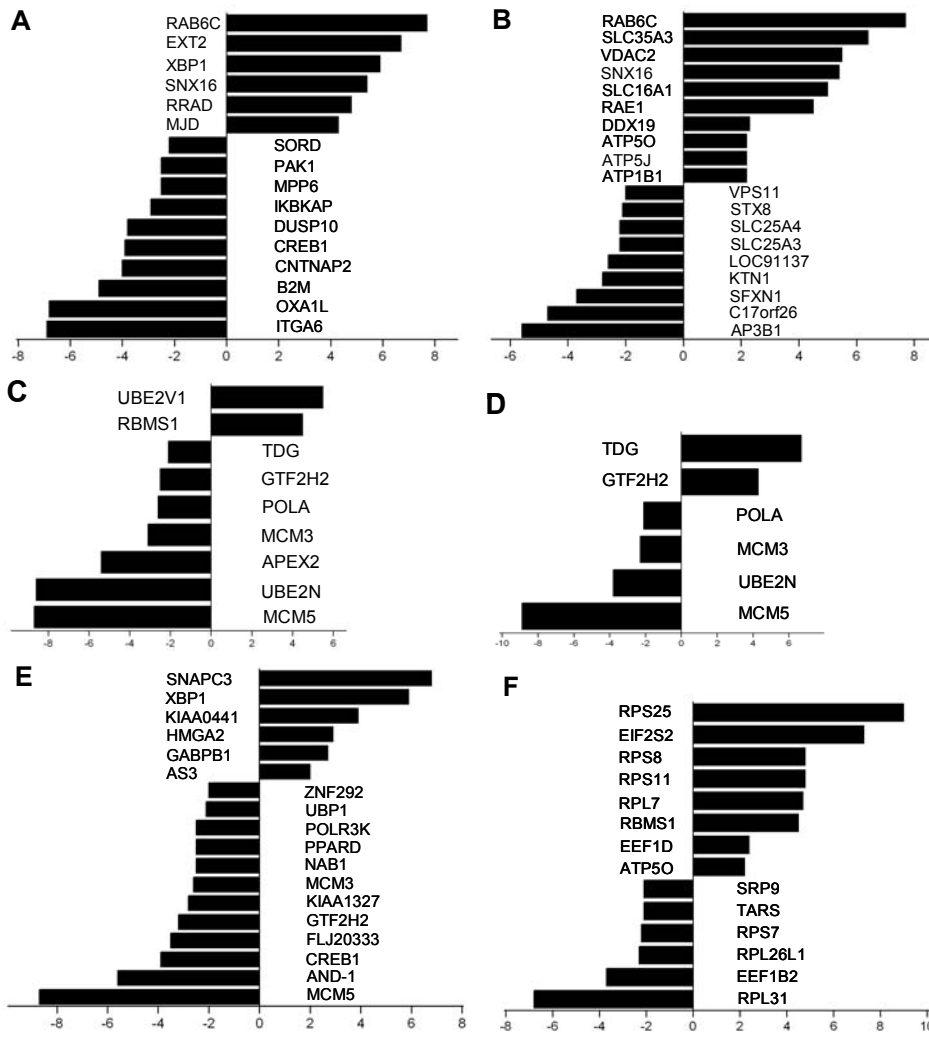
**Table 4.** Summarized cellular component ontology of up- and down-regulated transcripts

| Cellular component ontology                    | GO code    | Up | Down |
|--|------------|----|------|
| Cell fraction                                  | GO:0000267 |    |      |
| Membrane fraction                              | GO:0005624 | 3  | 2    |
| Soluble fraction                               | GO:0005625 | 0  | 2    |
| Intracellular                                  | GO:0005622 | 4  | 3    |
| Chromosome                                     | GO:0005694 | 2  | 0    |
| Centromere                                     | GO:0005698 | 1  | 0    |
| Chromatin                                      | GO:0005717 | 1  | 0    |
| Kinetochores                                   | GO:0005699 | 1  | 0    |
| Cytoplasm                                      | GO:0005737 | 5  | 4    |
| Cytoskeleton                                   | GO:0005856 | 4  | 3    |
| Intermediate filament                          | GO:0005882 | 0  | 1    |
| Spindle  | GO:0005819 | 1  | 0    |
| 26S proteasome                                 | GO:0005837 | 1  | 0    |
| Endoplasmic reticulum                          | GO:0005783 | 2  | 3    |
| Endoplasmic reticulum lumen                    | GO:0005788 | 1  | 0    |
| Endoplasmic reticulum membrane                 | GO:0005789 | 1  | 1    |
| Golgi apparatus                                | GO:0005794 | 2  | 1    |
| Mitochondrion                                  | GO:0005739 | 3  | 8    |
| Mitochondrial outer membrane                   | GO:0005741 | 3  | 4    |
| Ribosome                                       | GO:0005840 | 5  | 2    |
| Lysosome                                       | GO:0005764 | 11 | 0    |
| Nucleus  | GO:0005634 | 0  | 13   |
| Nuclear membrane                               | GO:0005635 | 0  | 1    |
| Nuclear outer membrane                         | GO:0005640 | 1  | 0    |
| Nuclear pore                                   | GO:0005643 | 1  | 1    |
| Nucleolus                                      | GO:0005730 | 1  | 0    |
| Small nucleolar ribonucleoprotein complex      | GO:0005732 | 1  | 2    |
| Nucleoplasm                                    | GO:0005654 | 1  | 1    |
| DNA-directed RNA polymerase III complex        | GO:0005666 | 0  | 1    |
| Alpha DNA polymerase:primase complex           | GO:0005658 | 0  | 1    |
| Spliceosome complex                            | GO:0005681 | 1  | 1    |
| Integral to plasma membrane                    | GO:0005887 | 0  | 4    |
| Integrin complex                               | GO:0008305 | 0  | 1    |
| Sodium/potassium-exchanging ATPase complex     | GO:0005890 | 1  | 0    |
| Respiratory chain complex I                    | GO:0045271 | 0  | 2    |
| Membrane                                       | GO:0016020 | 2  | 3    |
| Inner membrane                                 | GO:0019866 | 0  | 2    |
| Mitochondrial inner membrane                   | GO:0005743 | 1  | 2    |
| Mitochondrial electron transport chain complex | GO:0005746 | 0  | 1    |
| Integral membrane protein                      | GO:0016021 | 5  | 12   |
| Outer membrane                                 | GO:0019867 | 1  | 0    |
| Plasma membrane                                | GO:0005886 |    |      |
| Cell-cell adherens junction                    | GO:0005913 | 0  | 1    |
| Tight junction                                 | GO:0005923 | 0  | 1    |
| Extracellular                                  | GO:0005576 | 0  | 1    |

## DISCUSSION

Most of clones and transcripts with less than a 2-fold change were not used in this assay, indicating that the transcriptional regulation of many transcripts was actively maintained at certain levels, regardless of the HPV-16-derived cellular pro-

cess. Several of the up-regulated transcripts, such as 16S ribosomal RNA, casein kinase 2 subunit beta and NADH dehydrogenase subunit 4, were previously reported to be over-expressed in squamous cell cervical carcinomas (8). Also, a number of genes, known as being differentially up- or down-regulated compared to normal, such as XBP1, CENPF, RPS25, HEXB, ITGA6, CNTNAP2, MCM5, MCM3 and IKBKAP, were identified. Reliable gene expression data shows the



**Fig. 3.** Relative expression levels for genes with function-dependent transcript representation; (A) cell communication, (B) transport, (C) DNA metabolism, (D) development, (E) DNA binding, (F) protein biosynthesis.

consistency of the above-regulated transcripts to those of the previously reported results, and validates the profiling method for the study of the cervical cancer-specific pathway.

As usual, the gene expression profiles have been classified into reasonable groups using statistical data mining methods, such as the hierarchical clustering and K-Means clustering. These are, however, based on the statistical relationships between the genes, resulting in a biological description limit, as well as an analytical reproducibility problem. Thus, in order to obtain the biological interconnectivity between the gene expression patterns, the differentially regulated genes should be categorized using the Gene Ontology. Correlations are sought between the genes in cellular process groups and their expression patterns. The changes observed in this analysis provide important insights into the more specific cancer-related gene activity and HPV-16-derived functional change patterns. Closer examination of the 157 genes resulted in a number of reciprocally dependent cellular processes, revealing that the expression patterns are not randomly distributed with respect to their function.

On the other hand, as shown in Table 1, the potentially significant genes with unknown functions are shown in bold

type. The reported genes can be noted as new putative cervical cancer-related genes, as there were no matches between the genes and any other known functional activities in the GO database search. Of these genes, for instance, the down-regulation of LASS2 in hepatocellular carcinoma cell lines was only reported to lead to the progression of cancer cell growth. Also, MPP6, a member of the p55-like membrane-associated guanylate kinase (MAGUK) subfamily, which functions in tumor suppression and receptor clustering by forming multi-protein complexes, contains distinct sets of transmembrane, cytoskeletal and cytoplasmic signaling proteins (9). The MPP6 was down-regulated in this study. Future studies will be required to clarify these genes regulatory mechanisms and their role in cervical carcinogenesis.

With cell communication, the down-regulated cell adhesion activity was likely to be affected by the HPV 16-associated cellular pathway, indicating a decrease in the adhesive properties of cervical carcinogenesis. For instance, the alpha-6 integrin (ITGA6) and contactin-associated protein 2 (CNTNAP2), two other cell-to-matrix attachment-related components, were highly down-regulated. It was also reported that mice deficient in alpha-6 integrin, through a targeted disruption, died with



severe blistering of the skin and other epithelia (10). The phenotype was reminiscent of human epidermolysis bullosa. Transcripts in the immune response (1/1: XBP1/IKBKAP) contained only two genes, but a previous study reported that inflammatory and chemotactic-related transcripts were not detected in cervical cancer cells compared to normal cells (11). Especially, the up-regulated XBP1, involved in B-cell biology, was reported to be able to differentiate myeloma from non myeloma cell lines (12). Recent studies have shown that two mutations in the IKBKAP gene are responsible for a developmental disorder of the sensory and autonomic nervous system (13). One of the highly up-regulated genes in normal uterus tissue (14), CREB1, was down-regulated in the cell communication function.

With cell growth and maintenance, many cell cycle checkpoints are deregulated in oncogenesis, which results from interference with the cell cycle regulation of the oncoproteins by destruction of the p53 and pRb tumor suppressors. Their inactivation leads to alteration in the cellular gene expression. It was reported that CENPF, which is involved in DNA replication and mitosis, was induced by E6 and/or E7 retroviruses, after immortalization, thus confirming the consistency of our study. Also, the MCM3 protein is down-regulated in cells that have not ceased to proliferate. As shown in our study, the transcription level of VDAC2 in the malignant tumor cell line was significantly higher than that in normal cells (15). There are several notable gene expression patterns in response to stress function. Nearly all the stress response genes, including those involved in the response to pathogens and bacteria, were not expressed, regardless of HPV 16. Only H11, the eighth known human small Hsp, described as a serine-threonine protein kinase (16), was up-regulated, whereas the DUSP10 in the stress response was down-regulated.

With the metabolism, HEXB has not been fully described in the context of cervical cancer. Recently, it was represented, in the GenBank (accession number: AF378118.1), to be up-regulated in human cervical cancer, as a proto-oncogene. Its role in cervical carcinogenesis is being investigated further. For example, the question is to test if the HEXB gene maps to the cervical cancer susceptibility locus, which would support a potential link in the malfunctioned metabolism related to disease development, and could be regarded as being responsible for an increased susceptibility to the HPV 16 in cervical cancer. A highly over-expressed clone, NADH dehydrogenase subunit 4, as a mitochondrial enzyme, is involved in the metabolic pathway. It has been suggested that metabolic transformation is likely to occur during the early malignant stage of carcinogenesis (17). Also, the level of NADH dehydrogenase subunits expression in pre-malignant colon adenomas was up regulated, indicating that the tumorigenicity of cells was associated with the overexpression of the NADH dehydrogenase subunit 4 (18). All these evidence suggest the NADH dehydrogenase subunit 4 serves an important role in cervical carcinogenesis.

With the development, an overexpressed clone, the *Mus musculus* casein kinase 2 beta subunit, is highly homologous to that of the human casein kinase 2 subunit beta, an enzyme that is involved in the transcription, signaling and proliferation, and in various stages of the development. More significantly,

it has been reported that the phosphorylation of HPV E7, by casein kinase 2, enhances the disruption of the G1/S transition by HPV E7, suggesting that the human casein kinase 2 subunit beta plays an important role in human cervical neoplasia (19).

With apoptosis, a gene involved with the Hsp70/Hsc70 protein regulator, BAG3, which binds with high affinity to the ATPase domain of the Hsc70, inhibiting its chaperone activity in a Hip-repressible manner, allows opportunities for the specification and diversification of the Hsp70/Hsc70 chaperone functions (20). CANX (calnexin) functions as a chaperone, regulating the transit of proteins through the ER. Comparisons of the sequences from cDNA clones of human, mouse and rat CANXs have demonstrated a high level of conservation in the sequence identity, suggesting that CANX performs important cellular functions (21). The gene expression profiles showed that SiHa cells do not induce significant levels of apoptosis, as the rate of protein synthesis (nearly up-regulated) was promoted at the polypeptide chain initiation level.

Transcription is a complex category that can lead to global alterations in the whole network of gene expressions. It has been reported that transcriptional modification was accomplished by SNAPC3 proteins, which acted at a specific promoter, as well as by regional activation (CENPF, HMGA2) of the chromosomal domain. Some of these transcriptional regulators are expected to be highly relevant in the cervical cancer pathway, and their regulation may affect different cellular functions. The HMGA2 gene has been considered, for several reasons, to be an especially good candidate for involvement in lipomas. Firstly, its location, 12q15, is involved in a variety of malignant and benign solid tumors, such as lipomas, pleomorphic salivary adenomas and uterine leiomyomas. Secondly, the chromosomal abnormalities of the 12q13-q15 region, frequently found in human benign tumors, are the critical events in the genesis of the tumors (22). Conversely, the transcriptional repressor, NAB1, was down-regulated, and whose aberrant expression may serve to activate other downstream pathways that further contribute to the development of a tumor. One of the highly expressed clones is homologous to the human 16S ribosomal RNA required for the translation of mitochondrial subunits.

Several genes, coding for cellular structure proteins, changed their expression in the pathway to that of the HPV-16-dependent transcription. Transcripts in this category can be subdivided into two groups: cytoskeletal and nuclear-related genes. The cytoskeleton integrity is known to play an important role in cell cycle progression, cell death and cell differentiation. An abnormal cytoskeleton is often observed in cancer cells. In this study, however, the gene expression profiles involved in the cytoskeleton were balanced, whereas a clone, similar to the human molecule, showing 98% homology on beta-tubulin, was highly down-regulated in the SiHa cells, which was consistent with a previous study (23).

Genes involved in the proteasome and ubiquitin degradation pathways form another important group. For protein degradation arrest, the repression of the ubiquitin-conjugating enzyme, UBE2E1, which is involved in the selective protein destruction by a specific protease complex, and the 26S proteasome, PSMB4, may be required in a dominant negative form for these proteins to prevent the recognition into

degradation (24). However, the transcripts in this category were up-regulated 2-fold, whereas, the degradation of ubiquitin fusion proteins were down-regulated. On the other hand, the protein biosynthesis function in the metabolism was relatively up-regulated. The over-expression of RPS25 especially, can result in significant effects on protein synthesis and SRP9, which leads to an inhibition of polypeptide elongation, known as translation arrest, were down-regulated, indicating that they are essential for overcoming the translation arrest.

Taken together, the expression behaviors of genes in protein degradation suggests that SiHa cells may increase the turnover of many proteins, which could be due to either the replacement of damaged molecules, or to the need to significantly change the proteome of the cell, probably to obtain new molecules that are involved in the cervical carcinogenesis or the replacement of damaged proteins.

## CONCLUSIONS

Our results have shown that GO analysis can describe the cellular processes that occur in HPV 16-positive cervical cancer cells, and overcomes the complexity of gene expression profiles, and can be an alternative to the hierarchical or K-Means clustering. Further systematic approaches, including cervical cancer lesions at various stages and genome-wide analyses, using the GO, can certainly elucidate new connections between gene expression profiles and the cellular pathways of cervical carcinogenesis, in order to identify valuable prognostic candidate genes.

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