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Proteomic Investigation in A549 Lung Cell Line Stably Infected by HPV16E6/E7 Oncogenes

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Key Words
Papillomaviruses, human · Lung cancer · Proteomics · Molecular networks · Mass spectrometry · Oncogene

Abstract
Background: Data have accumulated implicating the involvement of oncogenic human papillomaviruses (HPVs) in bronchial carcinogenesis. We recently described the presence of oncogenic HPV transcripts in non-small cell lung cancers. ***Objective:*** To investigate the role of oncogenic HPVs in lung carcinogenesis. ***Material and Methods:*** The lung cell line A549 stably infected with HPV16E6, HPV16E7 and HPVE6/E7 constructs was used to investigate the protein profile changes associated with the expression of these oncogenes. Replicated two-dimensional gel electrophoresis gels from uninfected and stably HPV16E6-, E7-, and E6/E7-infected A549 cells were compared for changes in protein profile. Protein identification was achieved by peptide mass fingerprinting by MALDI-TOF-MS and nLC-ESI-Q-TOF-MS/MS peptide ladder sequencing. ***Results:*** We identified 17 different polypeptides whose average normalized spot intensity was statistically significant ($p < 0.05$) and differed by 2-fold. Relationships between differentially expressed proteins and the HPV-induced infection mechanism have been clustered by knowledge-base database functional association network analysis. ***Conclusion:*** The impact of Hsp27, annexin III, annexin IV, Gp96 and TPT1 on the cellular response mechanism to HPV infection is presented and discussed.

Introduction

Lung cancer is one of the leading causes of cancer-related deaths in western countries [1]. Smoking, environmental pollution and exposure to asbestos are considered the main risk factors; however, some 15% of nonsmokers develop bronchial cancer [2]. Therefore, other etiological factors including genetic factors, functional inactivation of tumor suppressor genes such as *p53* or *pRb* and infec-

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tion with oncogenic human papillomaviruses (HPVs) have been implicated [3]. HPV is the etiological agent of cervical cancer, but it has been estimated that about 15–20% of all human malignancies could be related to oncogenic HPVs [4–6]. In two recent studies, we have revealed the presence of oncogenic HPVs (HPV16, 18, 31 and 53) and their transcripts in a series of non-small cell lung cancers [7, 8], reinforcing the relationship between HPV and lung cancer and supporting the hypothesis that oncogenic HPVs might play a role in lung carcinogenesis. The oncogenic action of the virus is exerted through the oncoproteins E6 and E7 by binding to p53 and pRb, respectively, interfering with cell cycle control [9–11]. E6 protein promotes cell proliferation by degradation of p53 protein, which forms a complex with E6 and the cellular ubiquitination enzyme E6-AP. E7 binds to pRb displacing the E2F transcription factor. The release of E2F influences the expression of cellular genes involved in mitosis and cell cycle control [12]. Complex formation between viral oncoproteins and the two oncosuppressor proteins is believed to lead to cell immortalization and transformation [13, 14].

The aim of our study was to identify proteins whose expression is modulated by the HPV16E6 and E7 oncogenes in a lung cancer cell line, A549. We generated stably infected HPV16E6-, HPV16E7- and HPV16E6/E7-A549 cell lines and used a proteomic approach to separate and identify differentially expressed protein.

Materials and Methods

Cell Culture and Retroviral Infection

The human lung adenocarcinoma epithelial cell line A549 was cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, Milan, Italy) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

The retroviral vector pLXSN was obtained from Clontech (Palo Alto, Calif., USA). The empty pLXSN vector, carrying a neomycin resistance gene, was used as a control.

The retroviral vectors pLXSN containing the HPV16E6, E7 and E6/E7 ORFs were kindly provided by Massimo Tommasino (International Agency for Research on Cancer, Lyon, France) and prepared as previously described [15].

High-titer retrovirus-containing supernatants (>5 × 10^6 IU/ml) were generated by transient transfection of second-generation retrovirus producer Phoenix cells (amphotropic viruses) and used to infect the cells as previously described [16]. After infection, A549 cells were selected in 800 μg/ml G418 for 10 days, used as pooled population, and designated A549 pLXSN empty vector, A549 pLXSN-HPV16E6, A549 pLXSN-HPV16E7 and A549 pLXSN-HPV16E6/E7, respectively.

RT-PCR

Total RNA was extracted from 2 × 10^6 A549 cells infected with pLXSN-HPV16E6, pLXSN-HPV16E7, pLXSN-HPV16E6/E7 and mock-infected cells, respectively. One milliliter of Trizol (Invitrogen, SRL, Milan, Italy) was added and total RNA was extracted following the protocol's instructions. The absence of DNA in the extracted RNA samples was checked using primers for the β-actin gene located at the exon 4-intron 5-exon 5 junction [17]. All mRNAs present in 1.5 μg of total RNA extracted were reverse transcribed with the oligo-dT primer using the Omniscript kit (Qiagen, Hilden, Germany) in a final volume of 20 μl. The reaction tubes were incubated at 42°C for 1 h, heat-inactivated at 95°C for 5 min, and then placed on ice. Five microliters of each cDNA were used in the following PCR reaction as previously described [7].

Western Blot Analysis

For crude extract preparation, cells were lysed in 3% sodium dodecyl sulfate (SDS)-lysis buffer containing 125 mM Tris-HCl pH 6.8, 3% SDS, 10 mM dithiothreitol (DTT), 10% glycerol with the addition of protease inhibitors [0.2 mM phenylmethanesulfonyl fluoride (PMSF), 1 mg/ml pepstatin, 0.1 mM benzamidine, 2 mg/ml aprotinin] and briefly sonicated. Insoluble material was removed by centrifugation at 13,000 rpm for 5 min. Protein concentration was determined by the Bio-Rad Dc Protein Assay (Bio-Rad Laboratories, Milan, Italy). Twenty micrograms of proteins were loaded and separated on 8.5% SDS-polyacrylamide gels and transferred onto polyvinyl difluoride membranes (GE Healthcare Europe, Milan, Italy) according to the instruction manual. Membranes were blocked in a blocking solution [10 mM Tris-HCl pH 7.5, 0.1 M NaCl, 0.1% Tween-20, 5% (w/v) nonfat dry milk] overnight at 4°C, and incubated with the following mouse monoclonal primary antibodies: anti-p53 (clone DO-1, Santa Cruz, Calif., USA; diluted 1:1,000) and antiactin (clone C4, Chemicon, Temecula, Calif., USA; diluted 1:5,000). Appropriate secondary antimouse antibodies, conjugated with horseradish peroxidase, were used (GE Healthcare Europe; diluted 1:4,000) and the reactions were visualized by enhanced chemiluminescence (ECL Western Blotting Detection Reagents; GE Healthcare, Little Chalfont, Buckinghamshire, UK), according to the manufacturer's instructions.

Two-Dimensional Gel Electrophoresis and Image Analysis

Cell pellets were dissolved in lysis buffer containing 7 M urea, 2 M thiourea, 40 mM Tris base, 50 mM DTT, 4% CHAPS and 0.5% Pharmalyte 3–10 (GE Healthcare). After sonication and incubation at 37°C for 1 h the protein concentration in the lysates was measured using a densitometric method (adapted TCA procedure) [18]. The proteins were precipitated with 80% acetone overnight at –20°C and then washed extensively with cold 80% acetone. For the first dimension, 100 μg of total proteins were loaded onto pH 3–10 nonlinear 18-cm gel strips (IPG; GE Healthcare) with a rehydration solution (6 M urea, 2 M thiourea, 4% CHAPS, 15 mM DTT, 0.5% IPG buffer 3–10 and trace amounts of bromophenol blue). After rehydration for 12 h, isoelectric focusing was performed at 20°C using an Ettan IPGphor II IEF System (Amersham Bioscience, Uppsala, Sweden). The second-dimension separation was performed 12 gels at a time on gradient (9–14%) SDS-PAGE gels (20 × 26 cm) using an EttanDALTtwelve System (Amersham Bioscience). After fixing, the protein spots in analytical

gels were visualized by Vorum silver staining (modified for MS) [19] and the stained gels were scanned with a UMAX Image Scanner (Amersham Bioscience). The image analysis was done using the PDQuest software (version 7.1.1; Bio-Rad).

For further protein identification larger amounts (1 mg) of total proteins, after treatment with DNAseI (GE Healthcare), RNAse A (USB, Cleveland, Ohio, USA) and 2-D Clean-Up Kit (GE Healthcare), were loaded on preparatory gels and subjected to isoelectric focusing to a total of 160 kVh. Following two-dimensional gel electrophoresis (2-DE), gels were stained using colloidal Coomassie brilliant blue staining. For the image analysis we chose the three highest quality gels, based on protein spot resolution and number, to allow experimental statistical comparison between the A549 cell line and A549 infected with the HPV constructs. A reference gel was selected from one of the experimental gels and unmatched protein spots of the remaining gels were manually added to this reference gel to create the master gel, a virtual image comprehensive of all matched spots derived from all analyzed samples. Subsequent to automatic spot detection and spot filtering, the matching of spots between gels was manually reviewed and adjusted as necessary.

Protein Identification

Protein spots of interest were excised from 2-DE gels using a robotic instrument (Proteineer SpotPicker; Bruker Daltonik, Bremen, Germany). In-gel digestion for peptide mass fingerprinting was carried out manually with porcine trypsin (Promega, Madison, Wisc., USA) in 50 mM ammonium bicarbonate at 37°C for 16–18 h. The reaction was stopped by adding a final concentration of 0.1% TFA. For the mass-spectrometric analysis of tryptic digests the samples were prepared by reverse phase extraction using ZipTip C18 (Millipore, Bedford, Mass., USA). Elution and spotting on 600-μm AnchorChip target plates were obtained as described [20]. Each spectrometric analysis was performed using a Reflex IV MALDI time-of-flight (MALDI-TOF; Bruker Daltonik) operating in a positive ion mode with a reflectron setup. Database searches with the measured monoisotopic peptide masses were performed against the National Center for Biotechnology Information (NCBInr) human database using the peptide search routine MASCOT (Matrix Science, London, UK, at http://www.matrixscience.com). Carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionine was allowed as variable modification of the peptides. The query was performed with the maximal tolerance of 100 ppm. For positive identification of the peptide mass fingerprinting, protein scores greater than 65 were considered significant ($p < 0.05$), as calculated by the MASCOT scoring algorithm.

Tryptic digests of some gel spots selected for validation were subjected to analysis by nanoliquid chromatography-electrospray ionization-tandem MS (nLC-ESI-MS/MS; Waters, Milford, Mass., USA). The generated data were screened in NCBInr database with the MASCOT search engine using a mass tolerance ≤ 50 ppm for parent and 0.2 Da for fragments. An MS/MS ion score >35 indicates identity or extensive homology ($p < 0.05$).

Pathway Analysis

Relationships between differentially expressed genes were assessed using Ingenuity Pathways Analysis (IPA; IPA 5.0; Ingenuity® Systems, www.ingenuity.com) that enables mining, visualization and exploration of relevant functional associations significant to the experimental results. We employed a data set containing gene identifiers corresponding to MS and MS/MS-identified proteins and their corresponding relative abundance derived from 2D-PAGE experiments. Only IPA interconnections with statistical significance lower than $p < 0.05$ were used to create the functional clusters employed to design the network graph. The network graphically displays genes/gene products as nodes and the biological relationships between the nodes as edges.

Results

RT-PCR

In order to investigate the expression level of proteins regulated by HPV16E6 and E7 oncogenes singularly and/or combined, we stably infected the A549 cell line with the retroviral vector pLXSN carrying HPV16E6, HPV16E7 and HPV16E6/E7 ORFs. Mock-infected cells were included as control. The expression of the HPV16E6 and E7 oncogenes, singularly or combined, is shown in figure 1.

Western Blot

P53 protein expression was detected by Western blotting. While p53 was detected in the A549 cell line, mock cells, and pLXSN-HPVE7-infected cells, it was undetectable in the A549 cells infected by pXLSN-HPVE6 and/or pXLSN-HPVE6/E7, respectively (fig. 2). The A549 cell line contains a wild-type p53 [21].

Proteomic Differential Analysis

2-DE was used to examine differences in protein profiles of A549 cell lines stably infected with HPV16E6, E7, and E6/E7 oncogenes compared to control (A549 cells and A549 empty vector/VEC). Representative images of silver-stained 2-DE are shown in figure 3.

After background subtraction, normalization and matching, the abundance of each single protein spot in gels from different cell lines was estimated by spot intensity and the results were evaluated in terms of optical density (OD) expressed as ppm. In our image analysis we considered only spots with an average relative intensity above 0.15%. For each 2-DE analysis, the ratio of the mean normalized spot intensity of treated versus control gels, and a Student t test were calculated for spots identified in the three replicates of each cell lines. Three hundred and eleven spots were compliant with these criteria (reference spots) and were employed to perform a comprehensive statistic comparison. In order to minimize the effects of the empty vector on the protein profile expression, we considered as significant only the spots with similar OD

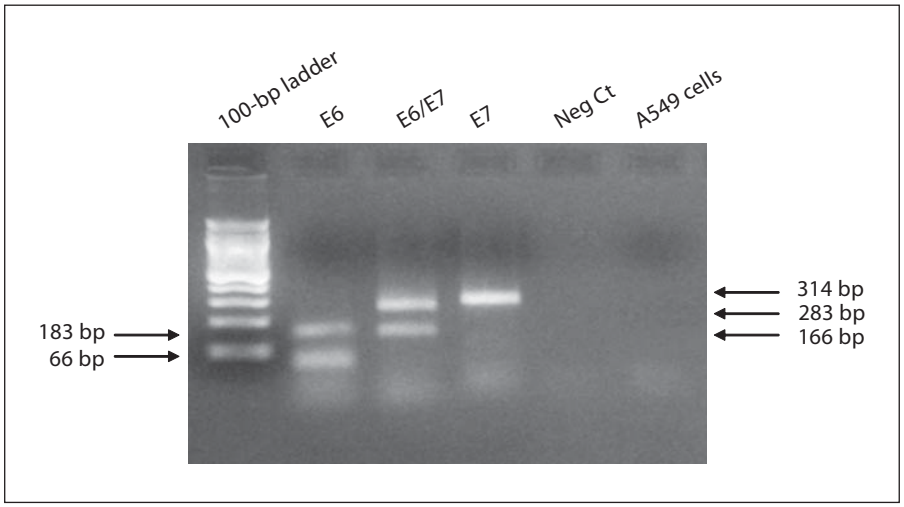


Fig. 1. Detection of HPV16E6, E7 and E6/E7 transcripts. Lane 1: 100-bp ladder; lanes 2–4: E6*I and II-spliced transcripts, E6/E7-spliced transcripts, and E7 transcript, respectively; lanes 5 and 6: PCR Neg Ct and mock cells, respectively.

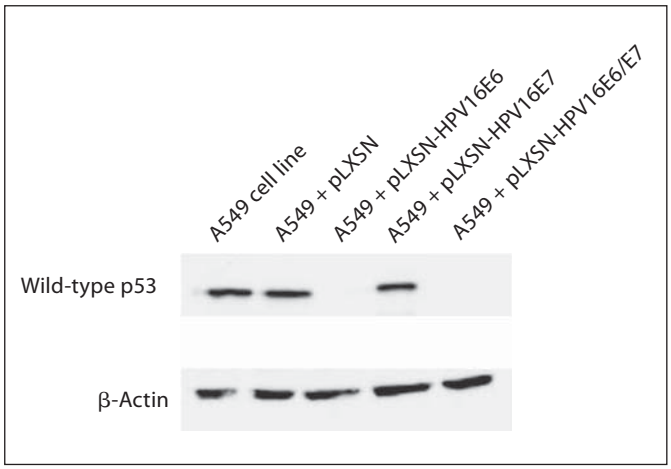


Fig. 2. Wild-type p53 protein expression. Lane 1: A549 cell line; lanes 2–5: A549 cells infected by pLXSN vector only, pLXSN-HPV16E6, pLXSN-HPV16E7, pLXSN-HPV16E6/E7, respectively. β-Actin was used as internal control.

between the A549 uninfected cell line (control) and the A549 cell line stably infected with the empty vector (VEC). Among them we analyzed proteins present in the E6-, E7- and E6/E7-infected cell lines showing at least a 2-fold OD up- or downregulation and whose t test statistic was <0.05. We found 17 differentially expressed spots (fig. 4): 7 spots upregulated in A549 infected with HPV16E6 (E6; fig. 4a), 2 spots upregulated in A549-HPV16E7 (E7; fig. 4b) and 8 spots upregulated in A549-HPV16E6/E7 (E6/E7; fig. 4c). Interestingly, one spot (No. 3105) was found to be upregulated both in the E7- and in the E6/E7-infected cell lines. None of them was significantly downregulated (more than 2-fold).

Identification of Differentially Expressed Polypeptides by Mass Spectrometry

The proteins selected after image analysis were in-gel digested with trypsin and the extracted peptides were analyzed using either MALDI-TOF-MS mass fingerprint or nLC-ESI-Q-TOF-MS/MS peptide sequencing. For both approaches, the acquired spectral data were compared to the theoretical molecular weight and fragmentation pattern of peptide sequence in a database to identify the proteins by means of probabilistic assignments ($p < 0.05$).

The identified proteins are shown in table 1 and the detailed results of the LC-ESI-MS/MS experiments are reported in table 2.

It is notable that two protein spots (No. 4605 and 4703) upregulated in the HPV16E6-A549 cell line were identified as the same polypeptide, Mitofilin p87/89, while spots No. 4202 (E6), 3203 and 3209 (E6/E7) turned out to be the same protein, transaldolase 1.

Computer Analysis of Functional Associations between Differentially Expressed Genes

Biological associations of the differentially expressed proteins in the HPV16-infected A549 cell lines were modeled using IPA. In the construction of the interaction network we used only functional associations with $p < 0.05$.

Two networks describe the proteins regulated by the HPV16E6 oncogene. The first with the highest fit is related to infectious disease, cell to cell signaling, interac-

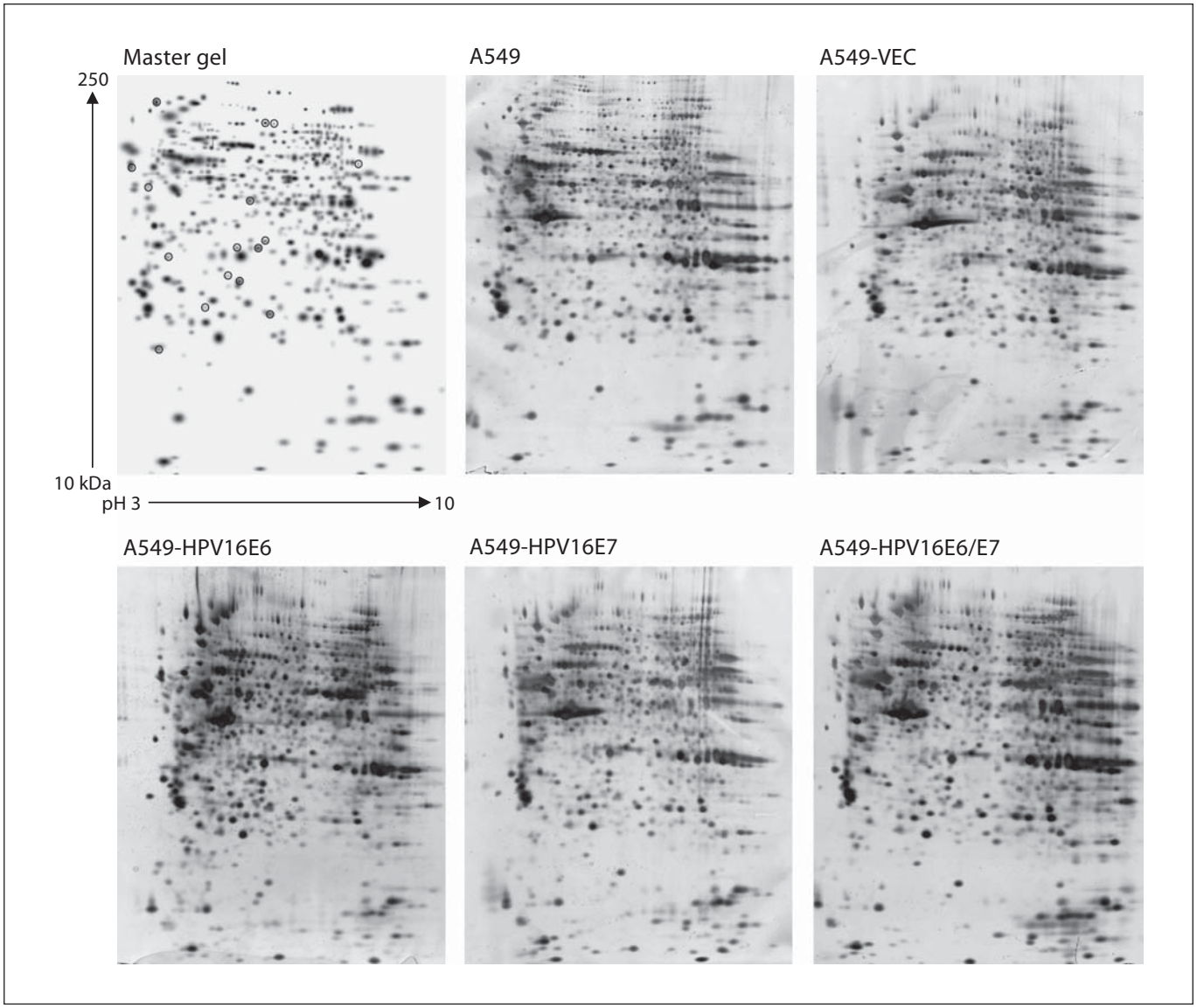


Fig. 3. Representative 2-DE gels used for protein profiling. 100 μg of each cell lysate were loaded on immobilized nonlinear pH gradients from pH 3–10 in the horizontal dimension followed by SDS-PAGE (9–14% gradient gels) in the vertical dimension. The gels were visualized by silver staining. The synthetic master gel was obtained from the image analysis (PDQuest). Protein spots in the master gel were differentially expressed in the infected cell lines compared to controls.

tion and immune response and merges all the proteins identified with the exception of mitofilin. This protein is also involved in a second network that refers to gene expression without the involvement of other identified proteins. Such a second network has not been considered further.

Two networks were also obtained from proteins identified as differentially regulated in the HPV16E7-infected A549 cell line. These proteins showed a relationship with cell cycle, cell morphology, gene expression and cell death.

Interestingly, only a single network was built by proteins identified as upregulated in the HPV16E6/E7-infected cell line. These proteins are involved in cellular growth, proliferation and death. These functional associations have a central position in a merge analysis of all the net-

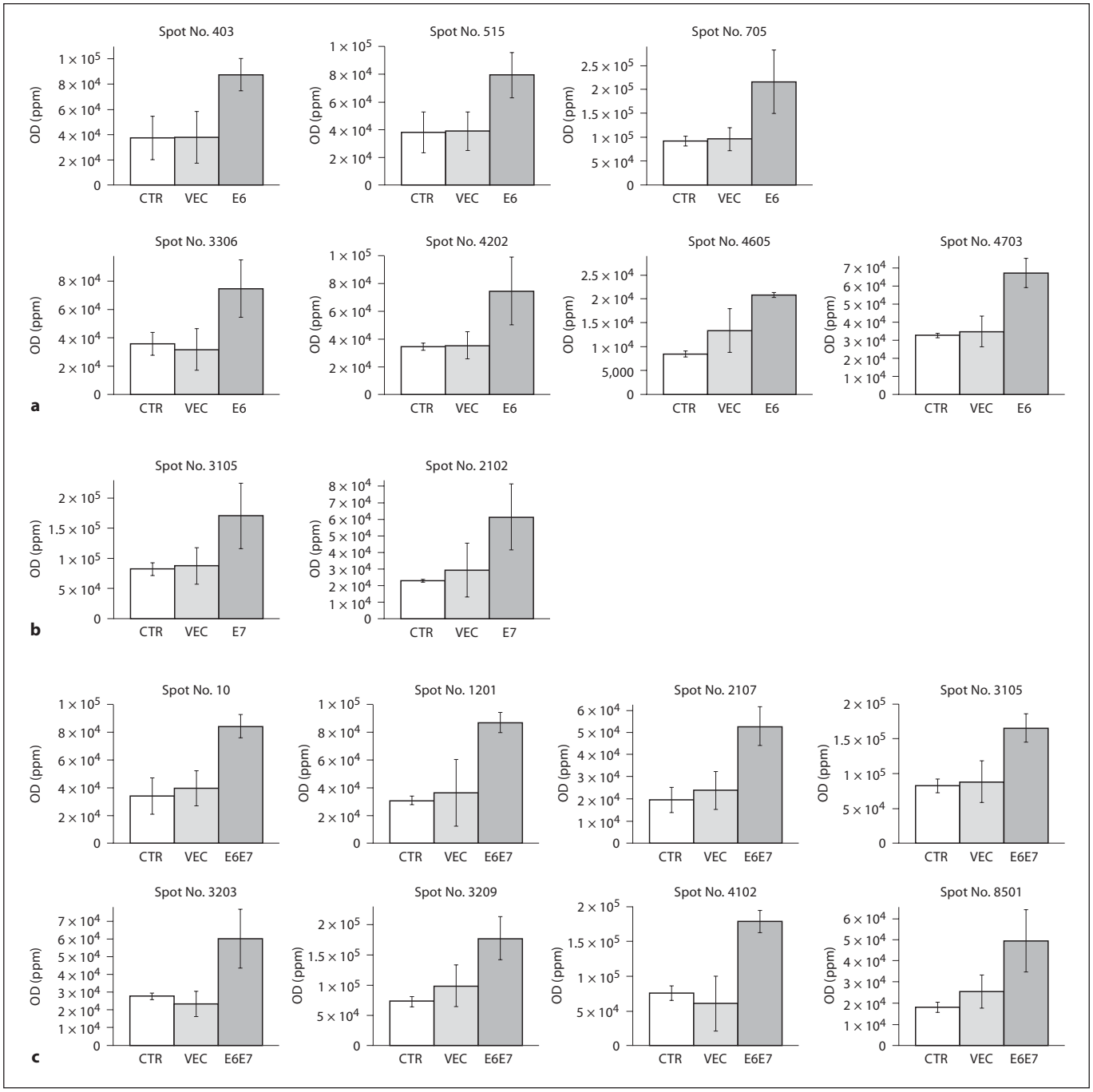


Fig. 4. Histogram plot of statistical protein spot differences between the HPV16-infected and uninfected A549 cell lines. Polypeptide quantities were calculated in parts per million (ppm) of the total integrated OD. Average and standard deviation (as error bars) of each differentially regulated protein spot are shown. **a** 7 spots were upregulated in HPV16E6-infected cell line compared to controls [A549 cell line (control, CTR) and empty vector-infected A549 (VEC)]. **b** 2 spots upregulated in HPV16E7-infected A549. **c** 8 spots upregulated in HPV16E6/E7-infected A549.

Table 1. Related information of proteins differentially expressed in the HPV16E6 (E6)-, HPV16E7 (E7)- and HPV16E6/E7 (E6/E7)-infected A549 cell lines and identified on the 2-DE gels by MALDI-TOF-MS and/or by nLC-ESI-Q-TOF-MS/MS (QTOF)

	Spot No.	Accession No.[a]	Protein identified	MASCOT score	Cov.[b] %	Number of peptides matched	pI[c]	M_r[d]	FC[e]
E6	403	gi|5292161	Proteasome 26S non-ATPase regulatory subunit 4	409 (QTOF)	21	6	4.72	41,024	2.34
	515	gi|4758756	Nucleosome assembly protein 1-like 1	73 (QTOF) 62	6 19	2 8	4.36	45,631	2.09
	705	gi|62088648	Tumor rejection antigen (gp96) 1	132	22	13	4.73	92,567	2.35
	3306	gi|4506209	Proteasome 26S ATPase subunit 2	172	31	14	5.71	49,002	2.09
	4202	gi|5803187	Transaldolase 1	521 (QTOF) 199	28 37	9 15	6.36	37,688	2.18
	4605	gi|29427676	Inner membrane mitochondrial protein (Mitofilin p87/89)	99	15	9	6.08	83,626	2.47
	4703	gi|29427676	Mitofilin p87/89	134	17	10	6.08	83,626	2.06
E7	2102	gi|5453790	Nicotinamide N-methyltransferase	416 (QTOF) 148	29 56	7 10	5.56	30,011	2.69
	3105[f]	gi|189617	Annexin IV	210	42	14	5.84	36,290	2.07
E6/E7	10	gi|4507669	Tumor protein, translationally-controlled 1	478 (QTOF) 107	48 47	6 7	4.84	19,697	2.48
	1201	gi|38522	Eucariotic translation elongation factor 1 delta	418 (QTOF) 120	24 37	5 9	4.95	31,316	2.83
	2107	gi|1421662	Annexin III	863 (QTOF) 129	37 33	12 10	5.63	36,480	2.71
	3105[f]	gi|189617	Annexin IV	210	42	14	5.84	36,290	2.01
	3203	gi|5803187	Transaldolase 1 (TALDO1)	540 (QTOF) 105	27 34	9 11	6.36	37,688	2.16
	3209	gi|5803187 + gi|4506209	Transaldolase 1 + protein phosphatase 1, catalytic subunit, beta isoform 1	736 (QTOF) 103 341 (QTOF)	33 21 23	11 8 5	6.36 5.84	37,688 37,961	2.45
	4102	gi|662841	Heat shock protein 27	152	41	10	5.98	22,826	2.36
	8501	gi|133274	Heterogeneous nuclear ribonucleoprotein L (hnRNP L)	772 (QTOF) 149	25 28	11 10	6.65	60,719	2.72

[a] Accession No. according to NCBInr database. [b] Percentage of amino acid sequence coverage by measured peptides. [c] Theoretical isoelectric point (pI) calculated by the MASCOT software. [d] Molecular weight (M_r) calculated by the MASCOT software. [e] Fold change calculated by taking the averaged ratio of spot intensity between infected cell lines and uninfected A549. [f] Spot upregulated both in HPV16E7- and HPV16E6/E7-infected cell lines; only one result (with the higher identification score) is reported for both samples.

works regulated by the three different infection conditions: E6, E7 and E6/E7 (fig. 5; table 3). Notably, of the identified proteins Hsp27, annexin IV, Gp96 and tumor protein, translationally-controlled 1 turned out to be the key genes involved in this top gene network. Moreover, the β-estradiol and the ubiquitin proteolysis functional group are included in the derived functional network.

Discussion

Lung cancer is the most common cancer in the world and today almost half of the cases occur in the developing countries [22]. Although cigarette smoking, environmental pollution and asbestos are recognized as the main risk factors for lung cancer development, about 15% of the cases remain unresolved [2]. The interest in the possible role of HPV in lung carcinogenesis has increased in the last years. Many papers have been published on the subject, with conflicting results as we recently reviewed [23]. However, the finding of E6 and E7 transcripts in a series of non-small lung cancer cases prompted us to further investigate the role of oncogenic HPVs in lung cancer development [7, 8]. We used the lung cancer cell line A549 stably infected with HPV16E6 and E7 constructs to identify proteins modulated by the expression of these two oncogenes. Although experiments performed on nonimmortalized or malignantly transformed cells would be

Table 2. Related information of proteins differentially expressed in the HPV16E6-, HPV16E7- and HPV16E6/E7-infected A549 cell lines and identified on the 2-DE gels by LC-ESI-QTOF-MS/MS

Spot No.		Accession No.	Protein identified	MASCOT score	MS/MS sequence	Ion score
E6	403	gi|5292161	Proteasome 26S non-ATPase regulatory subunit 4	409	LQAQQDAVNIVCHSK; ITFCTGIR; IIAFVGSPVEDNEKDLVK; VNVDIINFGEEEVNTEK; MTISQQEFGR; NAMGSLASQATK	93 37 68 81 61 70
	515	gi|4758756	Nucleosome assembly protein 1-like 1	73	LDGLVETPTGYIESLPR; FYEEVHDLER	35 38
	4202	gi|5803187	Transaldolase 1	521	MESALDQLK; LGGSQEDQIK; LSSTWEGIQAGK; ILDWHVANTDKK; SYEPLEDPGVK; TIVMGASFR; ALAGCDFLTISPK; LLGELLQDNAK; AAQASDLEK	53 76 111 26 42 13 69 74 58
E7	2102	gi|5453790	Nicotinamide N-methyltransferase	416	DTYLSHFNPR; HSAESQILK; IFCLDGVK; GDLLIDIGSGPTIYQLLSACESFK; SSYYMIGEQK; FSSLPLGR; EAVEAAVK	68 43 67 111 52 28 47
E6/E7	10	gi|4507669	Tumor protein, translationally-controlled 1	478	DLISHDEMFSDIYK; EIADGLCLEVEGK; GKLEEQRPER; VKPFMTGAAEQIK; NYQFFIGENMNPDGMVALLDYR; EDGVTPYMIFFK	112 118 18 63 106 61
	1201	gi|38522	Eucariotic translation elongation factor 1 delta	418	SLAGSSGPGASSGTSGDHGELVVR; IASLEVENQSLR; GVVQELQQAISK; LVPVGYGIR; VGTDLLEEEITK	132 60 90 31 88
	2107	gi|1421662	Annexin III	863	MLISILTER; EYQAAYGK; GAGTNEDALIEILTTR; DISQAYYTVYK; SLGDDISSETSGDFR; DESLKVDEHLAK; VDEHLAK; QDAQILYK; WGTDEDK; LTFDEYR; GIGTDEFTLNR; SEIDLLDIR	46 33 107 78 119 65 15 50 46 51 71 50

Table 2 (continued)

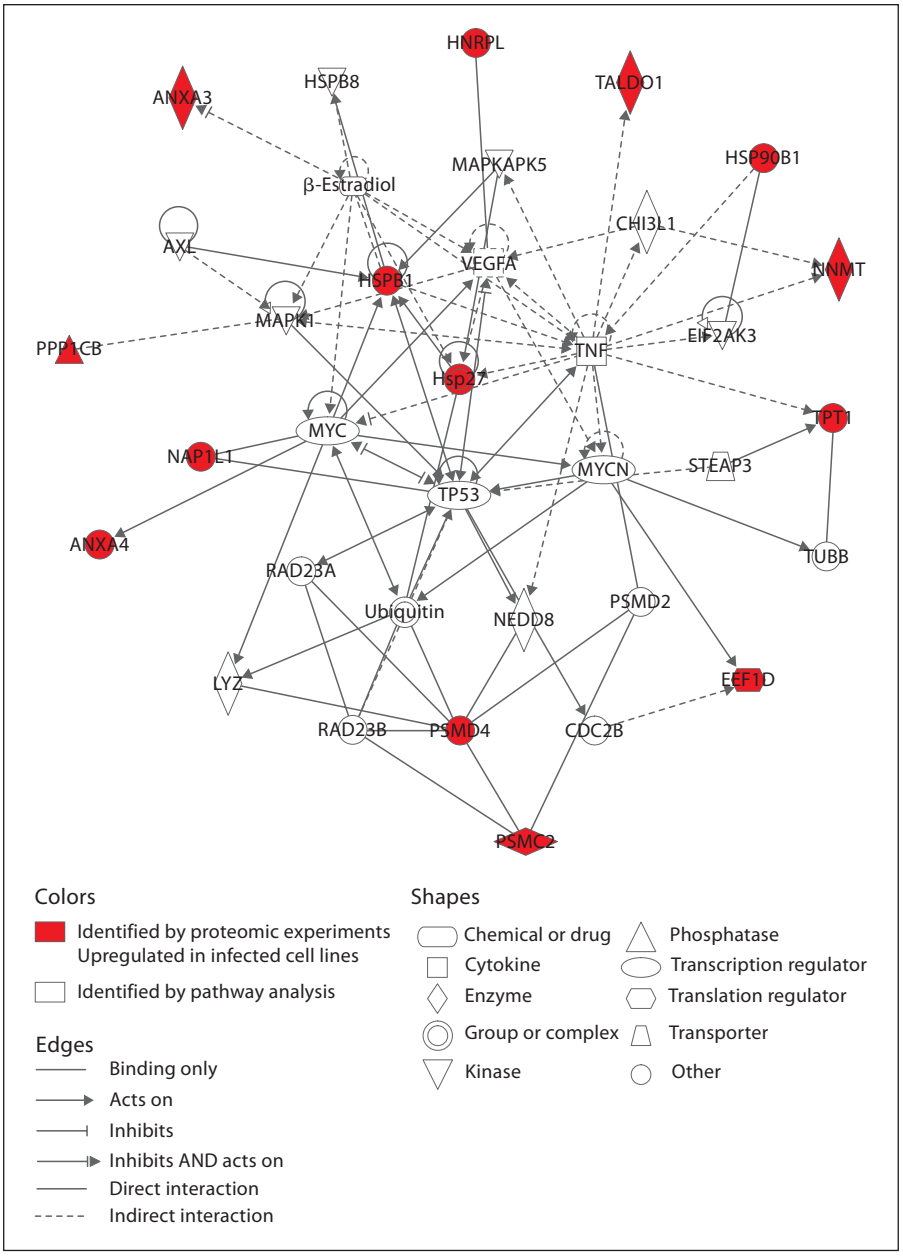
Spot No.		Accession No.	Protein identified	MASCOT score	MS/MS sequence	Ion score
E6/E7	3203	gi|5803187	Transaldolase 1	540	LGGSQEDQIK;	79
					LFVLFGAEILK;	28
					VSTEVDAR;	58
					LSSTWEGIQAGK;	85
					SYEPLEDPGVK;	52
					IYNYYK;	8
					ALAGCDFLTISPK;	100
					LLGELLQDNAK;	74
					AAQASDLEK	55
	3209	gi|5803187	Transaldolase 1	736	LGGSQEDQIK;	77
					LFVLFGAEILK;	53
					VSTEVDAR;	63
					LSSTWEGIQAGK;	118
					ILDWHVANTDKK;	72
					SYEPLEDPGVK;	43
					TIVMGASFR;	45
					ALAGCDFLTISPK;	106
					LLGELLQDNAK;	78
					LVPVLSAK;	37
					AAQASDLEK	44
		gi|4506209	Protein phosphatase 1, catalytic subunit, beta isoform 1	341	IVQMTEAEVR;	79
					EIFLSQPILLELEAPLK;	94
					LFEYGGFPPEANYLFLGDYVDR;	35
					YPENFFLLR;	50
					TFTDCFNCLPIAAIVDEK	84
	8501	gi|133274	Heterogeneous nuclear ribonucleoprotein L	772	YYGGGSEGGR;	62
					ISRPGDSDDSR;	60
					IEYAKPTR;	36
					NDQDTWDYTNPNLSGQGDPGSNPNKR;	116
					VFNVFCLYGNVEK;	80
					SKPGAAMVEMADGYAVDR;	88
					LNVCVSK;	40
					FSTPEQAAK;	50
					SSSGLLEWESK;	90
					SDALETLGFLNHYQMK;	95
					NPNGPYPYTLK	54

more informative, this cell line is commonly used in proteomic and mRNA studies [24, 25] instead of primary cultures because of technical problems in isolating, characterizing, growing and transfecting primary cultures.

To analyze the target molecules modulated by HPV16E6 and E7 oncogenes, singularly or combined, we used a proteomic approach based on 2-DE, peptide mass fingerprinting by MALDI-TOF-MS and MS/MS sequencing by nLC-ESI-Q-TOF-MS/MS.

The global protein content in the A549 cell lines expressing the oncogenes was compared with that of a control A549 cell line and A549 mock cells, respectively, to generate a differential protein expression profile. After 2-DE experiments (fig. 3), the differential proteomic analysis of A549 infected with HPV16 oncogenes revealed the variation of 17 proteins identified by MS and MS/MS (tables 1, 2).

IPA was used to investigate the protein pathways involved in the mechanism of HPV16 infection. This bioinformatic tool, using online database (Ingenuity Pathways Knowledge Base), provides a probabilistic model of the specific biological pathways activated under the experimental conditions selected. Results, derived from the protein networks obtained with the three different infec-



Color version available online

Fig. 5. IPA top gene network generated uploading all the differentially expressed proteins in the A549 cell line infected by HPV16E6, HPV16E7 and HPV16E6/E7 constructs. The network graphically displays gene products as nodes and the biological relationship between the nodes as edges. Proteins (focus genes) which were identified as upregulated in the infected cells by proteomics experiments are shown in red color and gene products which are not user specified but incorporated into the network through relationships with other proteins are represented as white nodes. The functional class of the gene products is displayed with different geometric shapes. Interaction between proteins is represented as edges (lines): solid lines for a direct interaction and dashed lines for an indirect interaction. Each edge between gene products is created using scientific information contained in the IPA database (Ingenuity Pathways Knowledge Base, IPKB). (For the relationship between the nodes refer to the legend at the bottom of the image.)

tion conditions, suggested the functional involvement of a cell death inhibition pathway with central nodes including MYC, TNF, vascular endothelial growth factor (VEGF) and annexin IV, Gp96, Hsp27 and tumor protein, translationally controlled 1 as major key proteins for cell viability and inhibition of apoptosis pathway (fig. 5; table 3).

VEGF is a major regulator of angiogenesis and it was found overexpressed in non-small cell lung and cervical cancers [26, 27]. The loss of the wild-type p53 upregulates the VEGF expression [28], supporting the hypothesis that HPV16E6 oncoprotein might be actively involved [29]. Actually, it has been established that HPV16E6 upregulated VEGF via a promoter region that contains four Sp-1 sites, and, importantly, that this occurred in a p53-independent manner.

Annexins constitute a large family of Ca^{2+} and lipid-binding proteins with the common biochemical hallmark of binding with negatively charged phospholipids in a Ca^{2+}-dependent and reversible manner [30]. They

have been implicated in a wide range of biological processes. Annexin IV has been directly identified in our 2-DE maps and is recognized as an early marker of apoptosis [31]. Annexin IV also plays a role in drug resistance as reported in the lung cancer cell line H460. In this model, annexin IV is one of the earliest proteins induced in H460 cells in response to cytotoxic stress due to antimitotic drug treatment [32]. These and our results suggest a link between annexin IV and cell proliferation. Annexin III has also been directly profiled in this study. This protein is a potential angiogenic factor; it induces migration and tube formation of vascular endothelial cells by stimulating the hypoxia-inducible factor-1 (HIF-1), which in turn, may induce increased secretion of VEGF [33], one of the nodal points of our protein function clustering analysis (fig. 5). We speculate that upregulation of annexin III encourages tumor progression through an increased vascularization of the tumoral mass.

The glucose-regulated proteins (GRPs) constitute a family of highly conserved stress response proteins that operate as molecular chaperones with both translocase and foldase activities. GRP94 (= gp96), identified in our analysis, is a Ca^{2+}-binding protein localized in the endoplasmic reticulum. It has previously been identified as tumor-specific antigen and, more recently, as regulator of antigen-presenting cell activation and so represents a potentially ideal candidate for cancer immunotherapy [34]. It can be considered an early marker of lung carcinogenesis [35].

The heat shock protein Hsp27 is an additional chaperone molecule found in our study, which is expressed in response to a wide variety of physiological and environmental insults including anticancer therapy [36]. The molecular mechanisms involving Hsp27 in resistance to cancer therapies can be explained in the following ways: (1) as molecular chaperone it can confer cytoprotection by repairing more efficiently the damaged proteins resulting from cytotoxic drug administration, (2) protecting cancer cells from apoptosis [37], (3) protecting the microvasculature inside tumors, because Hsp27 is found in endothelial cells [38], and (4) enhancing DNA repair [39, 40]. In rodent models, overexpression of Hsp27 increases tumor growth and metastatic potential, while its inhibition reduces tumor size [41]. Therefore, Hsp27 has become a new target for anticancer therapy [42].

Translationally controlled tumor protein (TPT1, also known as p23, fortilin and human recombinant histamine releasing factor) has been described as binding partner of MCL1 (Bcl-2 homologue) [43], Bcl-xL [44] and identified as an antiapoptotic protein. The most convinc-

Table 3. Ingenuity pathway system gene product designations of the top gene network identified in the Ingenuity Pathways Analysis

Top function	Score	Focus genes
Cell death p value: 6.92×10^{-6}	36	13

Proteins in the network	
Gene symbol	Protein name
ANXA3	Annexin III
ANXA4	Annexin IV
AXL	AXL receptor tyrosine kinase
CDC2B	Cell division cycle 2 homolog B *(S. pombe)*
CHI3L1	Chitinase 3-like 1
EEF1D	Eukaryotic translation elongation factor 1 delta
EIF2AK3	Eukaryotic translation initiation factor 2-alpha kinase 3
HNRPL	Heterogeneous nuclear ribonucleoprotein L
Hsp27	Protein family of heat shock protein 27
HSP90B1	Tumor rejection antigen (gp96) 1
HSPB1	Heat shock 27-kDa protein 1
HSPB8	Heat shock 22-kDa protein 8
LYZ	Lysozyme
MAPK1	Mitogen-activated protein kinase 5
MAPKAPK5	Mitogen-activated protein kinase-activated protein kinase 5
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
MYCN	v-myc myelocytomatosis viral-related oncogene, neuroblastoma derived (avian)
NAP1L1	Nucleosome assembly protein 1-like 1
NEDD8	Neural precursor cell expressed, developmentally downregulated 8
NNMT	Nicotinamide N-methyltransferase
PPP1CB	Protein phosphatase 1, catalytic subunit, beta isoform
PSMC2	Proteasome 26S, ATPase subunit 2
PSMD2	Proteasome 26S, non-ATPase subunit 2
PSMD4	Proteasome 26S, non-ATPase regulatory subunit 4
RAD23A	RAD23 homolog A *(S. cerevisiae)*
RAD23B	RAD23 homolog B *(S. cerevisiae)*
STEAP3	STEAP family member 3
TALDO1	Transaldolase 1
TNF	Tumor necrosis factor
TP53	Tumor protein p53
TPT1	Tumor protein, translationally controlled 1
TUBB	Beta tubulin
VEGFA	Vascular endothelial growth factor A

The score based on the number of the focus genes and network size is used for ranking the created network, the given p value represents the lower calculated threshold value for the involved proteins.

ing point in favor of a link between TPT1 and cancer was provided by the demonstration that during reversion of cells from the malignant phenotype, TPT1 levels are considerably reduced. Such an inhibition of TPT1 expression results in suppression of the malignant phenotype with TPT1 levels downregulated through activation of the tumor suppressor protein p53 [45, 46]. Notably, TPT1 specifically antagonizes the eEF-1δ-mediated GDP/GTP exchange reaction in the elongation reaction of protein synthesis in higher cells [47, 48]. In our study the HPV16E6/E7-infected A549 cell line shows an upregulation of both TPT1 and EEF1D. Elongation factors of translation have been implicated in cell transformation [49] and overexpression of EEF1D resulted in anchorage-independent growth and in the formation of tumors in nude mice [50]. A lot of evidence indicates that participation of EF-1 subunits in the control of virus expression can be part of a complex strategy for infected-cell protein synthesis optimization. In fact, EEF1D has been shown to interact with HIV Tat protein and this interaction reduces the translational efficiency of cellular but not viral mRNAs [51].

Finally, our results on the upregulation of the proteins annexin III, gp96, transaldolase 1, elongation factor δ1, proteasome 26 subunit have been confirmed at the transcriptional level [52, 53].

In conclusion, although careful molecular epidemiological studies are needed to establish a causal link between HPV infection and lung carcinogenesis, proteomic analysis could be a valuable tool for identifying the molecular pathways involved in the transformation process induced by HPV and for understanding its possible role as cocarcinogen in lung carcinogenesis.

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